Distinct patterns of histone modifications at cardiac-specific gene promoters between cardiac stem cells and mesenchymal stem cells

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Distinct patterns of histone modifications at cardiac-specific gene promoters between cardiac stem cells and mesenchymal stem cells. Am J Physiol Cell Physiol 304: C1080–C1090, 2013. First published April 3, 2013; doi:10.1152/ajpcell.00359.2012.—Mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs) possess different potential to develop into cardiomyocytes. The mechanism underlying cardiomyogenic capacity of MSCs and CSCs remains elusive. It is well established that histone modifications correlate with gene expression and contribute to cell fate commitment. Here we hypothesize that specific histone modifications accompany cardi-specfic gene expression, thus determining the differentiation capacity of MSCs and CSCs toward heart cells. Our results indicate that, at the promoter regions of cardiac-specific genes (Mylh6, Myl2, Actc1, Tnni3, and Tmnt2), the levels of histone acetylation of H3 (acH3) and H4 (acH4), as a mark indicative of gene activation, were higher in CSCs (Sca-1−/CD29+) than MSCs. Additionally, lower binding levels of histone deacetylase (HDAC) 1 and HDAC2 at promoter regions of cardiac-specific genes were noticed in CSCs than MSCs. Treatment with trichostatin A, an HDAC inhibitor, upregulated cardiac-specific gene expression in MSCs. Suppression of HDAC1 or HDAC2 expression by small interfering RNAs led to increased cardiac gene expression and was accompanied by enhanced acH3 and acH4 levels at gene loci. We conclude that greater levels of histone acetylation at cardiac-specific gene loci in CSCs than MSCs reflect a stronger potential for CSCs to develop into cardiomyocytes. These lineage-differential histone modifications are likely due to less HDAC recruitment at cardiac-specific gene promoters in CSCs than MSCs.

STEM CELL-BASED THERAPY holds tremendous promise for myocardial regeneration in the treatment of heart diseases, given that cardiomyocytes have very limited capacity to repair themselves. Among these, bone marrow-derived mesenchymal stem cells (MSCs), due to their unique properties (ease of isolation and expansion in vitro, multipotency, and immunological tolerance), have been widely used in many studies and clinical trials (19, 42, 44, 56). Implantation of MSCs after myocardial ischemia has been shown to improve cardiac function, promote neoangiogenesis, reduce cardiomyocyte death, and correct myocardial remodeling through their paracrine action and differentiation capacity (44, 56, 57). However, the insufficient ability of MSCs toward cardiomyocytes hinders their therapeutic efficacy for cardiac repair (16, 21, 38, 51).

Cardiac stem cells (CSCs) are residential stem cells in hearts that possess strong potential to replace damaged cardiomyocytes and restore heart function. However, the very small number of CSCs in each heart is not sufficient to repair heart damage when myocardial infarction occurs. MSCs and CSCs share some features in terms of cell morphology and expression of stem cell surface markers (11, 15, 28), but MSCs exhibit lower cardiac differentiation capacity than do CSCs (1, 8, 24). A recent study reported that CSCs exhibit greater cardiogenesis than bone marrow-derived MSCs, adipose tissue-derived MSCs, and bone marrow mononuclear cells (24). Interestingly, a portion of CSCs may be derived from bone marrow stem cells that home to the heart via the circulation and reside in the myocardium long enough to adopt CSC properties (1, 9), providing a developmental link between MSCs and CSCs. To improve the therapeutic application of MSCs and CSCs to heart disease treatment, it is important to investigate the mechanisms underlying the differentiation potential of CSCs and MSCs toward cardiac lineage.

Emerging evidence has demonstrated that gene regulation at multiple levels plays crucial roles in stem cell self-renewal, reprogramming, and lineage commitment. Epigenetic regulation, as a major form of transcriptional regulation, has been used extensively to investigate stem cell fate determination (13, 31, 32). Chromatin variation in the form of histone modifications has been considered critical to the basic regulatory events associated with stem cell differentiation. Histone modifications, such as histone acetylation at gene promoters, helps in establishing a transcriptionally permissive or nonpermissive status of local chromatin and in predicting transcriptional consequence in embryonic stem cell (ESC) fate commitment (13, 31, 32). Normally, acetylation of histone tails loosens chromatin structure and enhances activation of gene expression. It is evident that acetylation of histone tails (H3 and H4) increases the accessibility of Gata4 to the atrial natriuretic factor (ANF) promoter, facilitates activity of ANF gene expression, and, eventually, favors ESC differentiation into cardiomyocytes (18).

The balance between histone acetylation and deacetylation is controlled by histone acetyl transferases and histone deacetylases (HDACs). Among these, the class I HDACs, HDAC1 and HDAC2, have been implicated in the regulation of cardiac development. Activity of HDAC1 and HDAC2 is directly related to cardiac-specific gene expression (26, 49). Cardiac-specific deletion of HDAC1 and HDAC2 leads to neonatal lethality, with abnormalities in the heart (34). However, the role of gene-specific histone acetylation in determining the cardiomyogenic potential of MSCs and CSCs and whether HDACs are implicated in this process remain unknown. Our results from the current study show that distinct gene-specific histone modifications contribute to the cardiac differential potential of CSCs (Sca-1−/CD29+) vs. MSCs and that HDACs...
may be responsible for these lineage-specific histone modification changes.

MATERIALS AND METHODS

All animal studies conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The protocols were reviewed and approved by the Animal Care and Use Committee of Indiana University.

Preparation of cells. MSCs and CSCs were isolated from bone marrow (femur/tibia) and heart of C57BL mice (Jackson Laboratories, Bar Harbor, ME), respectively, according to our established methods (15, 53, 54). MSCs and CSCs were expanded in vitro and used for measurement of expression of cell surface markers at passage 3, as previously described (15, 53, 54). MSCs and CSCs show an extensive capacity for expansion in vitro without losing their characteristics and differentiation potential (1, 10, 28, 33, 37, 53, 54). Cell morphology was detected by contrast microscopy.

Cell proliferation. Cell proliferation was assessed by cell counting and the CellTiter 96 AQueous nonradioactive cell proliferation [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)]-2H-tetrazolium (MTS)] assay (Promega, Madison, WI). For comparison of cell growth between MSCs and CSCs, cells were seeded into 12-well plates on day 1. Between days 2 and 5, the numbers of living cells were counted using Trypan blue. In addition, MSCs in 12-well plates were treated with or without trichostatin A (TSA, 3, 10, and 30 ng/ml). For 24 h, the cells were collected for cell counting. For the MTS assay, MSCs were cultured in 96-well plates and treated with or without TSA (10 ng/ml). After 24 h, MTS (final concentration 333 μg/ml) and phenazine methosulfate, an electron coupling reagent (final concentration 25 μM) were added to the cell culture. MTS was converted to soluble formazan in metabolically active cells. After 3 h of incubation at 37°C, the amount of formazan product was measured by the amount of 490-nm absorbance, which indicates the number of live cells in culture. Furthermore, MSCs in 12-well plates were treated with or without TSA (10 ng/ml). After 1, 6, or 24 h of treatment, cells were collected for determination of Gata4 and sarcomeric (SM)-actinin by qPCR.

In vitro differentiation capacity. The differentiation ability of MSCs and CSCs toward adipocytes, osteocytes, and cardiomyocyte-like cells was assayed. For adipogenesis, MSCs or CSCs at passage 4 were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies, Grand Island, NY) containing 50 μg/ml indomethacin (Sigma-Aldrich), ST, and 100 nM dexamethasone (Sigma-Aldrich). After 3 wk of cultivation, the cells were fixed with 10% formaldehyde for 10 min and stained with 0.5% oil red O (Sigma-Aldrich) for 15 min. Adipocytes were detected under a fluorescence microscope.

For osteogenesis, MSCs or CSCs were cultured in IMDM supplemented with 50 μg/ml ascorbic acid 2-phosphate (Sigma-Aldrich), 10 nM dexamethasone, and 10 mM β-glycerol phosphate (Sigma-Aldrich). After 3 wk of culture, the cells were fixed and stained with alizarin red (Sigma-Aldrich) for 15 min. Under fluorescence microscopy, the mineralized matrix of the bone was examined.

Differentiation potential of MSCs and CSCs toward cardiomyocyte-like cells was assessed in cells cultured in cardiac differentiation medium (EMD Millipore). Differentiated MSCs and CSCs were harvested on day 6 or 10. Samples were used for detection of cardiac-specific transcription factors by RT-qPCR and for determination of protein levels of Gata4 and sarcomeric (SM) α-actinin by Western blotting.

Flow cytometric analysis. FITC-conjugated anti-CD45, phycoerythrin-conjugated anti-Sca-1, CD29, CD31, CD34, CD44, and CD45, and allophycocyanin-conjugated CD11b and CD117 (e-Kit) were purchased from eBioscience (San Diego, CA) and BD Biosciences (San Jose, CA). The standard immunofluorescence staining method was used. Cell surface antigen was analyzed using a FACSCalibur flow cytometer.

Cell cycle analysis. MSCs with or without 24 h of TSA treatment (10 ng/ml) were harvested, washed twice with PBS, fixed in cold 70% ethanol, and stored at −20°C. On the next day, the fixed cells were washed with PBS and resuspended in staining solution (25 μg/ml propidium iodide, 0.2 mg/ml RNase, and 0.05% Triton X-100 in PBS) for 1 h at 37°C in darkness. The stained cells were analyzed using a FACSCalibur flow cytometer. The percentages of cells in the G1, S, and G2 phases of the cell cycle were determined using ModFit LT software.

RT-qPCR. Total RNA was extracted using TRIzol (Life Technologies). cDNA was synthesized using the cloned avian myeloblastosis virus first-strand cDNA synthesis kit (Life Technologies). SYBR green-based qPCR was used to determine the gene expression levels. The primer sequences, except 5'-ACT CCT TTG TCA CCA CTG AAC GTG-3' (forward) and 5'-ATG CAG CTG TGG CCA TCT CGT TCT C-3' (reverse) for Actc1 and 5'-GTA TCT CCG AAT TCT GCT CGT-3' (forward) and 5'-TCT GCT CCA CAG TGC CAG CTG TCG-3' (reverse) for Cdkn1b, are described elsewhere (15).

Small interfering RNA transfection. HDAC1 small interfering RNA (siRNA), HDAC2 siRNA, and scramble control siRNA were purchased from Dharmacon (Laayette, CO). Lipofectamine 2000 (Life Technologies) was used to deliver these siRNAs into MSCs. MSCs were plated in six-well plates at 0.6 × 10^5 cells/well or 60-mm dishes at 1.2 × 10^5 cells/dish on day 1. On day 2, cells were transfected with siRNAs (50 nM). After 6 h, siRNA complexes containing siRNA were washed out, and normal IMDM was added. On day 4, MSC nuclear lysates and total RNA were collected from the cells in the six-well plates. The cells in the 60-mm dishes were harvested and fixed in 1% formaldehyde for chromatin immunoprecipitation (ChIP)-qPCR analysis.

Western blotting. The whole cell lysates were harvested using RIPA buffer (Sigma-Aldrich), and nuclear extracts were isolated using NE-PER Nucleolar and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL). The protein extracts (10–20 μg) were subjected to electrophoresis on a 4–12% precise protein gel (Life Technologies) and transferred to a nitrocellulose membrane. The membranes with the transferred whole cell extracts were incubated with the antibodies of Gata4 (Santa Cruz Biotechnology, Santa Cruz, CA), SM α-actinin (Sigma-Aldrich), and G2P (Biodesign International, Saco, ME), whereas the membranes with the transferred nuclear extracts were incubated with HDAC1 and HDAC2 (Santa Cruz Biotechnology), acetylated histone H3 (acH3) and acetylated histone H4 (acH4) (EMD Millipore), and anti-tATA-binding protein (Abcam, Cambridge, MA). The horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibody was utilized. The presence of proteins was visualized using SuperSignal West Pico stable peroxide solution (Pierce).

ChIP. ChIP assay was performed as we previously described (58, 59). Briefly, cultured MSCs and CSCs were fixed in 1% formaldehyde at room temperature for 20 min. The same number of cells was used for each cell to facilitate comparisons among samples. The cells were lysed and sonicated to generate 150- to 300-bp chromosomal DNA fragments. After preclearance with salmon sperm DNA, BSA, and protein A-agarose slurry (EMD Millipore), the supernatants were incubated overnight with the antibody against acH3, acH4, HDAC1, HDAC2, or control IgG (all antibodies were purchased from EMD Millipore, except HDAC1 and HDAC2, which were obtained from Santa Cruz Biotechnology). Protein A-agarose beads were used to precipitate the antibody-protein-DNA complexes. The precipitates were washed, and DNA was eluted. DNA cross-links were reversed at 65°C, and DNA was purified. The amount of DNA was quantified by SYBR green-based PCR using site-specific primer sets (Fig. 1). Results are expressed as percent input.
Characterization of MSCs and CSCs. MSCs and CSCs grew as adherent monolayers and had the same morphological features as spindle-shaped cells (Fig. 2A). Similar proliferation ability was also observed in MSCs and CSCs (Fig. 2B). After in vitro expansion, flow cytometry data revealed that both cell types stained negative for the hematopoietic stem cell markers CD34 and CD45 and the endothelial cell markers CD31 and CD117. MSCs and CSCs expressed similar levels of Sca-1, CD29, and CD44 (Fig. 2C), which was consistent with the previous observation from our group and others (15, 28, 29).

In vitro differentiation potential. MSC and CSC differentiation capacity into mesodermal lineages was assessed in cells cultured in adipogenic or osteogenic medium. MSCs exhibited strong adipogenesis and osteogenesis, whereas CSCs indicated low levels of adipogenic and osteogenic potential (Fig. 3).

Cardiomyogenic ability of MSCs and CSCs was determined in cells cultured in cardiomyocyte differentiation medium. RT-qPCR data revealed that, after 6 days of cardiac induction, the levels of cardiac-specific transcription factors (Nkx2.5, Gata4, and Tbx5) were increased in MSCs and CSCs and were much higher in CSCs (Fig. 4, A and B). Protein levels of Gata4 and SM α-actinin were also elevated in CSCs after 10 days of cardiac induction (Fig. 4C). Collectively, these results indicate stronger cardiac development ability of CSCs than MSCs.

Gene-specific histone acetylation. To investigate whether histone modifications play a role in establishing cardiomyogenic capacity of MSCs and CSCs, we examined the local histone acetylation levels at the conserved promoter regions of cardiac-specific genes. In Fig. 5, the Vista alignment plots indicate the conserved noncoding sequences of cardiac-specific genes: Myh6 (α-myosin heavy chain), Myl2 (myosin light chain 2v), Actc1 (cardiac α-actin), Tnni3 (cardiac troponin I), and Tnt2 (cardiac troponin T) in human and mouse. It is believed that important regulatory DNA regions overlap with the conserved noncoding sequences (shown as pink peaks in the plots) and the proximal gene promoters are generally located upstream to the transcriptional start sites of genes. To quantify the enrichment of acH3 and acH4 at the promoter regions of these genes, we utilized ChiP-qPCR assay and the primers located at the pink areas highlighted by the blue rectangles.

Our results indicate that more acH3 and acH4 was enriched at the promoter regions of Myh6, Myl2, Actc1, Tnni3, and Tnt2 in CSCs than MSCs, as shown in Fig. 6. Cardiomyocytes (terminally differentiated heart cells) from adult mouse hearts served as controls and exhibited the highest levels of acH3 and acH4 at the promoters among these three cell types. Taken together, these results indicate that histone acetylations correlate with a subset of cardiac gene expression in MSCs, CSCs, and cardiomyocytes.

Level of HDAC1 and HDAC2 binding to cardiac-specific gene promoters. Histone deacetylation is mainly regulated by HDACs. To determine whether different degrees of histone acetylation at cardiac-specific gene promoters in MSCs and CSCs are attributable to activity of HDAC1 or/and HDAC2, we used ChiP-qPCR assay to examine the local enrichment of HDAC1 and HDAC2 at these gene promoters in MSCs and CSCs. Our data indicate more enrichment of HDAC1 and HDAC2 at the promoter regions in MSCs than CSCs (Fig. 7, A and C), suggesting that histone deacetylation occurs at a greater level at these gene promoters in MSCs than CSCs. Meanwhile, we examined HDAC1 and HDAC2 protein levels.
in MSCs and CSCs. As shown in Fig. 7, B and D, the nuclear levels of HDAC1, HDAC2, and TATA-binding protein (a nuclear loading control) were similar in MSCs and CSCs, implying that protein expression of HDAC1 and HDAC2 does not account for the different levels of HDAC1 and HDAC2 binding at cardiac-specific gene promoters in MSCs and CSCs. We did not detect HDAC3 binding at the tested gene promoters.

HDAC inhibitor increases cardiac-specific gene expression and histone acetylation in MSCs. We have demonstrated that lower levels of histone acetylation in MSCs were likely due to higher levels of HDAC1 and HDAC2 binding at the promoters of cardiac-specific genes. Next, we tested whether TSA, a HDAC inhibitor, would affect MSC cardiac differentiation capacity through alteration of cardiac-specific gene expression. We treated MSCs with or without 10 ng/ml TSA for 24 h. The

Fig. 3. Adipogenic and osteogenic differentiation abilities of MSCs and CSCs. In vitro expanded MSCs and CSCs were cultured in adipogenic or osteogenic medium for 3 wk. Oil red O or alizarin red stain was utilized to detect adipocytes or mineralized matrix of the bone, respectively. Magnification ×200.

Fig. 4. Cardiac differentiation potential of MSCs and CSCs. A and B: RT-qPCR data showing transcription levels of cardiac-specific transcription factors (Nkx2.5, Gata4, and Tbx5) in MSCs (A) and CSCs (B) at 6 days with or without cardiac induction. D-MSC and D-CSC cultured in differentiation medium; ND, nondetectable. Values are means ± SE; n = 2–3 individual experiments. *P < 0.05, ***P < 0.001 vs. uninduced counterparts. C: Western blot analysis demonstrating expression of Gata4 and sarcomeric (SM) α-actinin in MSCs and CSCs at 10 days with or without cardiac induction.
Myc2, mRNA levels of A

dose of TSA was chosen on the basis of a previous study (18). RT-qPCR analysis was performed to determine gene expression changes. As shown in Fig. 8A, mRNA levels of Myh6, Myl2, Tnni3, and Tnnt2 were much higher in TSA-stimulated than untreated MSCs, indicating that inhibition of HDAC activity resulted in enhanced cardiac gene expression in MSCs. In addition, we also analyzed Gata4 expression in TSA-treated MSCs. MSCs treated with or without 10 ng/ml TSA for 24 h were cultured for a total of 10 days. TSA treatment upregulated expression of Gata4 in MSCs (Fig. 8B), further confirming that the HDAC inhibition promoted cardiomyogenesis of MSCs.

To determine the effects of HDAC inhibition by TSA on histone acetylation changes in MSCs, we assessed the acetylation status of H3 and H4. As shown in Fig. 9A, TSA treatment enhanced protein levels of acH3 and acH4 in MSCs. Consistent with this result, the ChIP-qPCR data demonstrated that TSA induced more enrichment of acH3 and acH4 at the promoter regions of Myh6, Myl2, Actc1, Tnni3, and Tnnt2 in MSCs (Fig. 9B), thus facilitating higher levels of gene expression.

Effects of TSA on MSC cycle progression and proliferation. The HDAC inhibitor TSA has been shown to induce cytostatic effects via regulation of cyclin-dependent kinase (Cdkn1b) expression. To determine whether TSA modulated MSC cycle progression and, consequently, affected gene expression, we investigated the roles of TSA in MSC growth and cell division, as well as Cdkn1b expression. The living cell numbers were similar in MSCs treated with TSA (10 ng/ml) and nontreated

MSCs (Fig. 10, A and B), indicating that treatment with 10 ng/ml TSA did not alter MSC proliferation. When TSA concentration was increased to 30 ng/ml (Fig. 10A), reduced cell growth was noticed in MSCs. A time-course experiment indicated that 10 ng/ml TSA did not affect Cdkn1b expression in MSCs (Fig. 10C). Furthermore, percentages of cells in the G1, S, and G2 phases of the cell cycle were similar in MSCs treated with 10 ng/ml TSA for 24 h and nontreated MSCs (Fig. 10D), indicating that TSA at this concentration did not arrest the MSC cycle progression. These results suggest that cardiac differentiation of MSCs induced by 10 ng/ml TSA is not a cytostatic effect.

Roles of HDAC1 and HDAC2 in regulation of cardiac gene expression and histone acetylation. We have shown that TSA treatment leads to increased cardiac gene expression and higher levels of acH3 and acH4 at the tested gene promoters in MSCs, but TSA is a nonspecific HDAC inhibitor. To determine the specificity of HDAC1 and HDAC2 activity on cardiomyogenesis of MSCs, siRNA technology was utilized to suppress the expression of HDAC1 or HDAC2. As shown in Fig. 11A, HDAC1 or HDAC2 expression was dramatically decreased in MSCs transfected with HDAC1 siRNA or HDAC2 siRNA, respectively, compared with cells treated with scramble siRNA or vehicle. Next we determined the influence of HDAC1 or HDAC2 knockdown on cardiac-specific gene expression. In Fig. 11, B and C, inhibition of HDAC1 or HDAC2 by siRNA resulted in increased Myh6 and Tnni3 expression, as well as enhanced acH3 and acH4 levels at their promoters in MSCs. Taken together, these findings suggest that HDAC1 and HDAC2 played inhibitory roles in cardiac gene expression in MSCs.

DISCUSSION

Although accumulating evidence has demonstrated that epigenetic regulation is implicated in stem cell fate decision, the current study provides the initial evidence showing the chromatin-based mechanism underlying cardiomyogenic capacity of MSCs and CSCs. Our results indicate more acetylated H3 and H4 enrichment at the promoters of cardiac-specific genes in Sca-1+/CD29+ CSCs than MSCs, reflecting stronger cardiomyogenic potential of CSCs. These higher levels of acH3 and acH4 at the tested gene loci in CSCs are likely attributable to lower levels of HDAC binding at these gene promoters than in MSCs. In addition, HDAC inhibition by TSA increased protein levels of acH3 and acH4 in MSCs and induced more enrichment of acH3 and acH4 at cardiac-specific gene promoters, which was related to upregulated expression of these cardiac-specific genes in MSCs. Furthermore, suppression of HDAC1 or HDAC2 expression by siRNAs increased cardiac gene expression and histone acetylation in MSCs.

Attention has focused on MSCs because of their appreciated properties in regenerative medicine in the past several years. In addition to development into mesodermal lineages, MSCs have shown potential for differentiation into vascular endothelial cells, smooth muscle cells, neurons, and myocytes (19, 42, 44, 56). Although animal experiments and in vitro cell culture studies have indicated that MSCs are capable of differentiating into cardiomyocytes (22, 27, 57), a relatively low rate of MSC differentiation toward cardiomyocytes has been observed (16, 21, 38, 51). It is known that cardiac lineage commitment is a
well-controlled process requiring the gene expression-regulating activities of cardiac-specific transcription factors, including Nkx2.5, Gata4, MEF2c, and Tbx5 (3, 20, 25, 45). In the current study, after cardiac induction, MSCs demonstrated increased transcript levels of Nkx2.5 and Tbx5, implying their potential for development toward cardiac lineage. However, Gata4 and SM α-actinin proteins were at minimal levels in MSCs following cardiac induction, suggesting the low cardiomyogenic capacity in MSCs.

Compelling evidence has indicated that adult heart contains residential stem cells, i.e., CSCs, which possess strong cardiomyogenic potential. CSCs have shown greater cardiac differentiation ability than other types of stem cells (24). Similarly, our present study demonstrated much higher levels of...
Nkx2.5, Gata4, and Tbx5 in CSCs than MSCs. In addition, increased levels of Gata4 and SM α-actinin have been observed in cardiac-induced CSCs, but not MSCs. These results suggest stronger cardiomyogenic capacity in CSCs than MSCs. Notably, CSCs and MSCs share some features regarding cell morphology and expression of stem cell surface markers (11–15). The present data are also in agreement with these previous observations and imply that a connection between CSCs and MSCs is likely. Indeed, a notion that a portion of circulated bone marrow stem cells may home to the heart and grow CSC properties (1, 9, 35) brings a developmental link between MSCs and CSCs. Therefore, determination of the mechanisms underlying cardiac differentiation potential of tissue-specific CSCs and MSCs would improve their therapeutic application in heart disease treatment.

Activation of a cardiac-specific gene program is required for stem cell differentiation into cardiac lineage. It is established that epigenetic regulation is one of the major mechanisms involved in control of stem cell fate decision (13, 31, 32). Acetylation of histone H3 and H4 promotes ESC development into cardiomyocytes through increased accessibility of Gata4 to the promoter of the cardiac-specific gene ANF and, thereby, facilitates ANF gene expression (18). In addition, difluoromethylornithine helps cardiac commitment of MSCs, partly via enhancement of acH3 (36). In this study, we demonstrated, for the first time, higher levels of acH3 and acH4 enrichment at the promoter regions of cardiac-specific genes (Myh6, Myl2, Actc1, Tnni3, and Tnnt2) in CSCs than MSCs, indicating that gene-specific histone modifications reflect cardiac differentiation potential in MSCs and CSCs.

Deacetylation by histone-modifying enzymes (HDACs) condenses chromatin and represses gene transcription because of the removal of acetyl groups from histone tails (12, 30). Emerging evidence has suggested that HDAC1 and HDAC2 may play important roles during cardiac development. Inhibition of HDAC1 activity induced cardiac differentiation and upregulated the expression of cardiac-specific genes, whereas HDAC1 overexpression decreased cardiomyocyte formation, as well as expression of Gata4 and Nkx2.5 in ESCs (26). Reduced HDAC1 expression was associated with increased gene expression of Tnnt2, Tnnt3, SM α-actinin, and α-MHC in bone marrow progenitor cells (43). It has also been shown that HDAC2 was upregulated in CSCs to maintain their “stemness” (23), suggesting that HDAC2 was involved in the cardiomyogenesis. HDAC2 might also regulate gene expression of Myh7 (β-MHC), Acta1 (α-skeletal actin), Nppa (ANF), MLC2a, and Myh6 (34, 49). Trivedi et al. (50) demonstrated that HDAC2 affected cardiac gene expression through hypoacetylation of the cardiac-specific transcription factor Gata4. In the current study, we found more HDAC1 and HDAC2 binding to promoter regions of cardiac-specific genes in MSCs than CSCs, which contributed to the lower levels of H3 and H4 acetylation at gene promoters in MSCs than CSCs. Different DNA-binding levels of HDAC1 and HDAC2 were not due to disparity in expression of HDAC1 and HDAC2 between MSCs and CSCs. Inhibition of HDAC1 or HDAC2 by siRNA targeting increased cardiac-specific gene expression and local enrichment of acH3 and acH4 in MSCs, further verifying that HDAC1 and HDAC2 are involved in the cardiac differentiation. HDACs are usually recruited to their target genes primarily through their combination into transcriptional complexes or direct conjunction with transcriptional factors. Therefore, it is plausible to speculate that through the activity of unidentified transcriptional factors, HDAC1 and HDAC2 might be recruited to cardiac gene loci at a higher level in MSCs than CSCs, leading to differential histone acetylation levels in these two cell lineages.

Fig. 8. Role of the HDAC inhibitor trichostatin A (TSA) in cardiomyogenesis. A: TSA upregulated expression of cardiac-specific genes in MSCs. TSA (10 ng/ml) treatment for 24 h significantly increased transcription levels of Myh6, Myl2, Tnni3, and Tnnt2 in MSCs. Relative quantity of mRNAs was obtained by comparison with CSCs. Values are means ± SE; n = 3 individual experiments. *P < 0.05; **P < 0.01 vs. vehicle. B: increased Gata-4 expression after 9 days of culture in TSA-treated MSCs compared with untreated MSCs (vehicle).

Fig. 9. Effect of TSA on histone acetylation. A: Western blot showing higher levels of acH3 and acH4 in MSCs treated with TSA (10 ng/ml) for 24 h. Representative immunoblots from 1 of 3 individual experiments are shown. B: increased local enrichment of acH3 and acH4 at promoter regions of Myh6, Myl2, Actc1, Tnni3, and Tnnt2 in MSCs by TSA treatment. Values are means ± SE; n = 3 individual experiments.
Future research may focus on the interaction between specific transcription factors and histone modifiers in the development of CSCs and MSCs toward cardiac cell lineages. It has been reported that class II HDACs, especially HDAC5 and HDAC9, were involved in cardiac development (4, 46, 61). Our present study has been focused on determining effects of class I HDACs (HDAC1 and HDAC2) on the cardiac differentiation potential of MSCs. It is likely that class II HDACs may also

![Graph A](image1.png)

**Fig. 10.** Role of TSA in regulation of cell proliferation and cell cycle progression. **A:** cell growth determined by cell counting. MSCs are cultured with 0, 3, 10, and 30 ng/ml TSA for 24 h. **B:** 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay of MSC proliferation 24 h after treatment with 10 ng/ml TSA. **C:** role of TSA in mRNA level of cyclin-dependent kinase inhibitor 1B (Cdkn1b) in MSCs. Values are means ± SE; *n* = 3–4 individual experiments. *P* < 0.05 vs. 0 (vehicle).

![Graph D](image2.png)

**D:** flow cytometry of cell cycle progression in MSCs treated with TSA (10 ng/ml) for 24 h. Percentages of populations in G0-G1, S, and G2-M phases are listed. Histograms represent results from 4 individual experiments.

![Graph B](image3.png)

**Fig. 11.** Effect of HDAC1 and HDAC2 on cardiac gene expression and histone acetylation. **A:** dramatically decreased expression of HDAC1 and HDAC2 in MSCs transfected with specific small interfering RNA (siRNA) targeting of HDAC1 or HDAC2 compared with scramble siRNA-treated or vehicle control group. Immunoblots show results from 1 of 3 individual experiments. **B:** inhibition of HDAC1 or HDAC2 by specific siRNA of HDAC1 or HDAC2 in cardiac-specific gene expression (Myh6 and Tnni3). Values are means ± SE from experiments repeated 3 times. *P* < 0.05, **P** < 0.005 vs. scramble siRNA. **C:** local enrichment of acH3 and acH4 at promoter regions of Myh6 and Tnni3 in MSCs transfected with siRNAs of scramble control, HDAC1, and HDAC2. Values are means ± SE; *n* = 2–3 individual experiments.
contribute to gene regulation during cardiac differentiation of MSCs. To study the epigenetic implication on cardiac lineage commitment, a couple of pharmacological compounds have been investigated. TSA, an inhibitor of HDACs (40, 48), promotes cardiomyogenesis through increasing expression of Gata4, Nkx2.5, and MEF2c (5, 43). TSA has also been reported to enhance acetylation of histone H3 and H4 and, thus, to promote cardiac differentiation in monkey ESCs (14). Furthermore, TSA treatment is able to upregulate expression of cardiac-specific genes, including Actc1, Myh6, Tnni3, and Gja1 (connexin43) in human adipose-derived stem cells (6). Similarly, our present data demonstrate that TSA-treated MSCs express higher levels of cardiac-specific genes and Gata4, further confirming that a chromatin-modifying agent such as TSA could enhance the cardiomyogenic capacity of MSCs. Notably, although TSA was shown to induce cell cycle arrest and inhibit cell proliferation in a variety of cells, in this study, treatment with 10 ng/ml TSA for 24 h did not decrease cell growth and affect cell cycle progression in MSCs, indicating that TSA-enhanced cardiac gene expression was not due to its cytostatic effect. In fact, previous studies demonstrated that TSA at 12 ng/ml or 80 nM (24 ng/ml) did not significantly disrupt cell cycle progression (17, 55). TSA treatment increased global levels of acH3 and acH4 at the tested cardiac gene loci in MSCs, which was accompanied by elevated cardiac gene expression.

There is a lack of optimal cell surface markers for identifying CSCs. Many studies have utilized Sca-1 (28, 29, 39, 41, 47) or c-Kit (1, 60) for the isolation of murine CSCs. In the current study, we defined Sca-1+ cardiac cells as CSCs for subsequent experimental analysis. By means of the CSC isolation kit, ~50% of the freshly isolated cardiac cells were Sca-1+. After prolonged in vitro expansion, the cell culture reached a very high purity level in terms of Sca-1 expression (>98%), suggesting that the Sca-1+ cells in the freshly isolated cell population were removed during frequent subculturing. Notably, Sca-1+ cardiac cells may contain several cell types, including CSCs/cardiac progenitor cells (CPCs), endothelial cells, and hematopoietic cells. In our expanded CSC cultures, ~5% of the cells were CD34+ and 3% were CD31+, suggesting that a very small portion of Sca-1+ cells expressed hematopoietic or endothelial progenitor cell markers. On the basis of the relevant data from a previous publication (28), Sca-1+ CPCs purified by the clonogenic technique were not homogenous after in vitro expansion, and 20% of these Sca-1+ cells expressed CD34, suggesting that a subset of Sca-1+ cells might spontaneously differentiate into CD34+ cells in vitro. Moreover, CSCs purified by Sca-1 magnetic microbeads contained a small percentage of CD31+, CD34+, CD38+, or CD45+ cells (29, 39). Therefore, the CSCs used in our study exhibited very similar phenotypes to the CSCs/CPCs defined by other groups. Meanwhile, we have looked at the percentages of c-Kit+ cells in the freshly isolated cardiac cells, given recent clinical trials using c-Kit+ CSCs for treatment of patients with heart failure (2, 7). Our results show very little c-Kit+ expression in the freshly isolated cardiac cells (0.19 ± 0.04%, n = 4). Several studies from other groups also demonstrated very few (0.1–0.5%) c-Kit+ cells in adult mouse hearts (39, 47, 60). Nevertheless, differentiation of epigenetic regulation alterations between c-Kit+ CSCs and Sca-1+ CSCs would be an interesting area of study in the future.

It has been demonstrated that H3 lysine 27 trimethylation (H3K27me3) correlates with transcriptional repression, while H3 lysine 4 trimethylation (H3K4me3) is related to transcriptional activation. H3K27me3 and H3K4me3 may coexist on developmentally regulated genes to constitute the bivalent chromatin structure (52). We have observed the enrichment of H3K27me3 and H3K4me3 at the tested cardiac gene promoters (data not shown), suggesting that the coexistence of H3K27me3 and H3K4me3 at cardiac gene promoters may reflect the plasticity of both cell types. We will investigate the implications of histone methylation in cardiac gene regulation in our future research.

In summary, our study provides the first evidence of how a decision for cardiac commitment is programmed at the chromatin level. Gene-specific histone modifications affect cardiac differentiation potential in MSCs and CSCs. Our results reveal the role of an epigenetic mechanism underlying the cardiomyogenic capacity of adult stem cells toward cardiac lineage commitment and shed light on epigenetic modulation for therapeutic purpose in the future.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
M.W. and Q.Y. are responsible for conception and design of the research; M.W., Q.Y., L.W., and H.G. performed the experiments; M.W., L.W., and H.G. analyzed the data; M.W. and Q.Y. interpreted the results of the experiments; M.W. prepared the figures; M.W. and Q.Y. drafted the manuscript; M.W. and Q.Y. edited and revised the manuscript; M.W., Q.Y., L.W., and H.G. approved the final version of the manuscript.

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