Cyclin-dependent Kinase 9 Induces Regional and Global Genomic DNA Methylation Via Influencing DNMT Gene Expression in Mouse Myoblast C2C12 Cells During Differentiation

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Abstract

Objectives: Cyclin-dependent kinases (CDKs) including Cdk9 have been associated with cardiac differentiation. The increasing evidence has proposed that Cdk9 overexpression can regulate the epigenome. However, the current research is the first report of the Cdk9 affection on the regional and global DNA methylation during differentiation.

Materials and Methods: This study examined the effects of Cdk9 overexpression on the regional methylation patterns of cardiac miRNAs (miR-1, -133, -206) and myogenic regulatory factors (i.e., MyoD and Myogenin) and promoter DNA methylation in mouse myoblast C2C12 cells during differentiation by the methylation-specific polymerase chain reaction (MSP-PCR) method. Moreover, the mRNA expression levels of DNMT1, DNMT3A, DNMT3B, and global 5-methyl cytosine (5-mC) levels in mouse myoblast C2C12 cells were quantified during differentiation by RT-qPCR and ELISA methods, respectively.

Results: The results demonstrated that Cdk9 overexpression results in DNA methylation changes in mouse myoblast C2C12 cells. It was found that the average expression levels of DNMTs in line with global DNA methylation significantly increased in Cdk9 transfected cells upon Cdk9 overexpression (P<0.05). In addition, the results showed that the regional promoter methylation of miR-1 and miR-133 genes increased in transfected cells during differentiation. An interesting possibility raised by our study is that further active global DNA methylation observed in Cdk9-transfected C2C12 cells can be clarified through the increased DNMT expression by Cdk9 in these cells.

Conclusions: In general, our study provides a comprehensive mechanism that Cdk9 can promote epigenetic changes and modulate global and regional DNA methylation profiling of myoblast cells during differentiation.

Keywords: MicroRNA, Cdk9, DNA methylation, Gene expression, Myoblast cell differentiation

Introduction

Epigenetics is a set of multiple processes containing DNA methylation, alterations in histone structures, and the affection of various non-coding RNAs in the expression of genes and chromatin remodeling in the cell (1,2). After the egg cell formation, its development depends on the type of epigenetic changes that occur on the inherited material of the cell (1). DNA methylation is an epigenetic modification that has a critical role in different molecular pathways including chromatin remodeling, gene silencing, inactivation of X-chromosome, and genomic imprinting (3). DNA methylation occurs within CpG dinucleotide pairs on the 5’ position of cytosine bases, followed by 5-methylcytosine (5-mC) formation (4). Approximately 70% of annotated mammalian promoters belong to CpG islands so that its methylation is essential for the expression and development of genes, as well as genome stability (5,6). DNMT1, DNMT3A, and DNMT3B are activated forms that are responsible for DNA methylation. The main role of DNMT1 is the methylation of DNA in stable forms for the transmission of methylation patterns to daughter cells during cell divisions. DNMT3A and DNMT3B function as de novo DNMT enzymes and establish the patterns of genomic methylation in the post-fertilization stage (7). Additionally, all three DNMTs are needed for fetal development, and any dysfunction in this enzymatic system has profound effects on the developmental process and disease (1). According to (8-10), muscle differentiation as a process with multiple steps is controlled by myogenic regulatory factors (MRFs)
and microRNAs (miRNAs). For appropriate cardiac development, the expression of these MRFs including Myogenin and MyoD (myoblast determination protein) is critical for various stages of myogenesis and myogenic processes (11). Although MyoD, Myf5, and Myf6 are responsible for muscle formation, the functional role of Myogenin is differentiation and accompanied by MyoD, and Myf6 strongly applies a controlling role for the differentiation of myoblasts into adult muscle fibers (12). The modulation of MyoD phosphorylation by Cyclin-dependent kinase 9 (Cdk9) is a triggering factor for the induction of cardiac differentiation in the primary stage of cardiogenesis (13, 14). In our previous study, we have identified Cdk9 functions on myogenic transcriptional factor and myomiRs in myoblast C2C12 cells (15). Concerning Cdk9 regulatory roles, it was shown that the constant overexpression of Cdk9 can affect mouse myoblast C2C12 cell line differentiation. Accordingly, Cdk9 was over-expressed in mouse myoblast C2C12 cells and the expression level of myogenic transcription factors was determined by qPCR and western blotting. In addition, the expression profile of three main myomiRs including miR-1, miR-133, and miR-206 was investigated during cardiac differentiation. Although it induces cardiac differentiation in the primary stage of cardiomyogenesis, persistent Cdk9 overexpression represses differentiation by the alteration of myomiRs and myogenic gene expression. Based on our funding, the transient induction of Cdk9 in the primary steps of differentiation is crucial for myogenesis (16). However, the precise mechanism by which Cdk9 upregulates these miRNAs in myoblast cells is not understood thoroughly. There is no significant report on whether Cdk9 overexpression can affect DNMTs gene expression and the promoter methylation of miRNAs and MRFs. Thus, it was hypothesized that Cdk9 can affect miRNAs and MRF gene expression by enhancing their DNA methylation. For this purpose, the study focused on evaluating the potential regulatory functions of Cdk9 on the promoter DNA methylation status of miR-1, miR-206, miR-133, myoD, myogenin, and the gene expression level of DNMTs, as well as genomic DNA methylation in myoblast C2C12 stem cells.

Materials and Methods

Cell Line and Cell Culture

Mouse myoblasts C2C12 cells were purchased from the Cell Bank of Iran (NCBI, Pasteur Institute, Tehran). All chemicals were purchased from Sigma Aldrich (Munich, Germany) and all reagents were obtained from Gibco Life Technologies (UK). The cell line was grown in the DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, streptomycin (and 0.1 mg/mL), and penicillin (100 U/mL) in a humidified 5% CO₂ atmosphere at 37°C. For inducing muscle differentiation, the mentioned medium was changed with a high-glucose (4.5 g/L) DMEM medium containing 2% horse serum (17).

Transfection of C2C12 Myoblast With pCEP4/cdk9 Plasmid

The pCEP4/Cdk9 plasmid has been constructed and transfected for Cdk9 overexpression as described in our previous study (16). Briefly, the exponential method was used for electroporation by the Gene Pulser Xcell Systems (Hercules, California) in the DMEM medium. Then, the cells were immediately re-cultured in the DMEM medium with 20% FBS. The medium was changed with the fresh DMEM medium including 10% FBS with 300 mg/mL of hygromycin after 24 hours for the separation of stably transfected cells. The differentiation of the cells toward myocells was carried out by the inclusion of 2% horse serum in the medium.

Extraction of RNA and RT-qPCR

Once the stably transfected cells were obtained and proliferated, total RNA was extracted from both non-transfected and transfected cells on days 0, 1, 2, 3, 4, and 5 after the induction of differentiation using the TriPure isolation reagent (Roche Applied Science, Germany) based on the protocol of the manufacturer. cDNAs were synthesized in 20 μL reaction volumes from 2 μg of total RNA based on the manufacturer’s instruction.

The transcript levels of DNMTs were analyzed by RT-qPCR assay and performed three times by using Rotor gene 6000. The sequences of primers are listed in Table 1. The PCR amplification was performed in 25 μL final reaction volume according to the method described by Tarhriz et al (16). The cDNA amplification reactions were carried out as preincubation at 95°C (5 minutes), followed by 35 amplification cycles consisting of denaturation at 95°C for 30 seconds, annealing, and extension at 60°C for 1 minute. The mRNA level of GAPDH was utilized as the internal control, and the relative quantification of the mRNAs of the samples was calculated using the 2⁻ΔΔCt standard method (18).

Genomic DNA Preparation and Methylation Specific Polymerase Chain Reaction

Genomic DNA was extracted from non-transfected (control) and transfected C2C12 cells according to the standard method by phenol-chloroform extraction, ethanol precipitation, and digestion with proteinase...
Global DNA Methylation Analysis

Genomic DNA methylation in the isolated DNA from non-transfected and transfected C2C12 cells on days 0, 1, 2, 3, 4, and 5 after differentiation induction was tested by the measurement of 5-methylated cytosines (5-mC) using the ELISA assay kit (Zymo Research, Germany) according to the protocol of the manufacturer. The amount of 5-mC was proportional to the optical density intensity measured in an ELISA plate reader at 450 nm. All measurements were carried out thrice. More details were found in our previously published study (21).

Statistical Analysis

The obtained data were statistically analyzed using GraphPad Prism and (SPSS, Inc., Chicago). In addition, the mean (± SD) was calculated by one-way ANOVA, followed by Tukey’s multiple comparison tests. Pearson correlation test was utilized for the analysis of the correlation between DNMT expression and global DNA methylation. P<0.05 was considered as the level of statistical significance.

Results

DNMT1, DNMT3A, and DNMT3B Gene Expression in C2C12 Cells

This study evaluated the expression level of the DNMTs in both non-transfected and transfected cells at days 0, 1, 2, 3, 4, and 5 of the C2C12 cell line after treatment with horse serum for the investigation of the relationship between DNMT expression profiles with DNA methylation changes during the differentiation of C2C12 cells. The relative expression levels of the DNMTs in both non-transfected and transfected cells (with PCEP4/Cdk9 plasmid) are shown in Figures 1 and 2. The average expression levels of DNMT mRNA were normalized with the expression levels of GAPDH mRNA in both non-transfected and transfected cells at days 0, 1, 2, 3, 4, and 5 of the C2C12 cell line. Non-transfected and Cdk9-transfected C2C12 cells at days 0 were utilized as a reference, and the expression levels of DNMTs in these cells were adjusted to 1.0, and the expressions in all other cells at days 1-5 were expressed.

<p>| Table 1. Applied Primer Sequences for qRT-PCR |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing T (°C)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>5′-ACCGGAAAACCAAGAGAAGTC-3′</td>
<td>5′-ACCGACACTCCTCTGTGCTA-3′</td>
<td>60</td>
<td>107</td>
</tr>
<tr>
<td>DNMT3A</td>
<td>5′-ACCGGAAATGACGGAAGTCA-3′</td>
<td>5′-ACGCGTCTGTAGGTGAGCCG-3′</td>
<td>60</td>
<td>170</td>
</tr>
<tr>
<td>DNMT3B</td>
<td>5′-GGGAGCTTCTGAGGCCTTGA-3′</td>
<td>5′-GGCAGTTCCTCAGCTGAGC-3′</td>
<td>60</td>
<td>87</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-TCCTGGAGCCATGAGGCGAT-3′</td>
<td>5′-TCGGGAGTCACTCCTCAATG-3′</td>
<td>60</td>
<td>240</td>
</tr>
</tbody>
</table>

Note: U: Unmethylated; M: Methylated; PCR: Real-time polymerase chain reaction.

<p>| Table 2. Applied Primer Sequences and Annealing Temperature for Methylation-specific PCR |</p>
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Annealing T (°C)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myo D</td>
<td>U: 5′-GGGGATTTTACGGGCGGTGGTG-3′</td>
<td>5′-ACCTTTGAGAA ACTACCTAT TCAAGT-3′</td>
<td>54</td>
<td>186</td>
</tr>
<tr>
<td>Myo G</td>
<td>U: 5′-GATTTGGGGCTTTATT TATTACCC-3′</td>
<td>5′-GGGCGGCGGAG GTTCTGTC-3′</td>
<td>54</td>
<td>186</td>
</tr>
<tr>
<td>miR-1</td>
<td>U: 5′-GCTATGTTTGTGCTGCTGCTGCT-3′</td>
<td>5′-CCTTTGAGAACCT CAAGTCTTTC-3′</td>
<td>54</td>
<td>186</td>
</tr>
<tr>
<td>miR-133</td>
<td>U: 5′-GAAATCGGAGCTT TTAATGCTTTC-3′</td>
<td>5′-CACTTTGAGAACCT CAAGTCTTTC-3′</td>
<td>54</td>
<td>186</td>
</tr>
<tr>
<td>miR-206</td>
<td>U: 5′-TAGGAGAAACCATTTTTTCTAAGG-3′</td>
<td>5′-CTCTAACCTTGTATATG TATGTT-3′</td>
<td>54</td>
<td>186</td>
</tr>
</tbody>
</table>

Note: U: Unmethylated; M: Methylated; PCR: Real-time polymerase chain reaction.
as an n-fold. Our results indicated that $DNMT1$ gradually increased for 2 days, and then leveled down on days 3, 4, and 5 compared to day 0 in non-transfected C2C12 cells. As shown in Figure 1A, $DNMT1$ started decreasing on day 2 and had the lowest level on day 3 and then its level elevated on days 4 and 5. However, its level on days 2-5 was lower than that of the non-transfected cells. $DNMT3A$ demonstrated nearly the same pattern as $DNMT1$, where the lowest level belonged to day 3. $DNMT3A$ gradually increased for 2 days and then decreased on days 3, 4, and 5 as compared to day 0 (Figure 1A). $DNMT3B$ increased on the first day and decreased on days 3, 4, and 5 of differentiation as compared to day 0 (Figure 1A). Moreover, these findings demonstrated that the average mRNA level of all three $DNMTs$ ($T1/3A/3B$) in the non-transfected C2C12 cells of the third day of differentiation were at the lowest expression compared to those of the cells on day 0 and then started leveling up on days 4 and 5. For $Cdk9$-transfected cells, the lowest level of $DNMT1$ was observed on day 5 in spite of non-transfected cells and its level started decreasing from day 2 until day 5 (Figure 2A). With a fluctuation, the overall level of $DNMT3A$ increased all days compared to day 0 (Figure 2A). Based on the results (Figure 2B), there was a trend for the higher expression of the average levels of all three $DNMTs$ ($T1/3A/3B$) in transfected C2C12 cells during differentiation as compared to day 0 although differences were not significant ($P > 0.05$). Overall, the results (Figure 3) demonstrated that the average expression levels of $DNMTs$ noticeably increased (~2-fold) in transfected cells as compared to non-transfected control cells (non-transfected: 0.8-fold, transfected: 1.61-fold) ($P < 0.01$).

Global Methylation Analysis

The total 5-mC content of the genomic DNA of non-transfected and transfected C2C12 cells were quantified to examine if DNA methylation changes take place during C2C12 differentiation. First, the levels of global DNA methylation in non-transfected C2C12 cells were investigated in this regard. Our finding indicated that nearly, 1.143%, 1.07%, 1.06%, 1.05%, 1.14%, and 1.08% of the cytosine residues were methylated at 0, 1, 2, 3, 4, and 5 day(s) in the genome of non-transfected cells, respectively (Figure 4A). Approximately 1.2%, 1.17%, 1.07%, 1.11%, 1.14%, and 1.13% of the cytosine residues were methylated at 0, 1, 2, 3, 4, and 5 day(s) in the genome of the transfected cells, respectively (Figure 4B). Interestingly, the findings (Figure 4A-B) demonstrated that global DNA methylation levels remarkably reduced on the 2nd day in both non-transfected and transfected C2C12 cells ($P < 0.05$). Based on data in Figure 4C, the levels of global methylation significantly increased in transfected cells in comparison with their respective non-transfected cells (increased: 1.044-fold, $P < 0.05$).

Association of $Cdk9$ Overexpression With Cardiac Specific miRNAs and MRFs Promoter DNA Methylation

The MSP on the specific sites was conducted to study the impact of $Cdk9$ overexpression on the promoter DNA methylation status of $MyoD$ and $Myogenin (MyoG)$, and

![Figure 1. A. The Relative Expression of $DNMT1$, $DNMT3A$, and $DNMT3B$ in C2C12 Non-transfected Cells During Differentiation Measured by RT-PCR and B. A Summary of the Change in the Average Expression of All 3 $DNMTs$ ($T1/3A/3B$) in Non-transfected (Control) C2C12 Cells During Differentiation. Note. The expression of each gene was normalized to GAPDH. C2C12 cells at day 0 were considered as controls and the rate of the expression of the genes of interest in other groups was calculated as well. The data were presented as the mean ± SD and each experiment was conducted in triplicate. The results were analyzed by one-way ANOVA and Tukey’s post hoc tests. The significance level is considered as $P < 0.05$. The bars marked with different letters are significantly different from other samples. C: Control (non-transfected); T: Transfect; RT-PCR: Real-time polymerase chain reaction; SD: Standard deviation.]

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miR-1, miR-206, and miR-133 on days 0, 1, 2, 3, 4, and 5 in C2C12 cells. The representative MSP of MyoD and Myogenin promoter methylation are shown in Figure 5. The MSP analysis indicated that the MyoD promoter was unmethylated in both non-transfected (control) and transfected C2C12 cells during differentiation although it was methylated only in control cells at day 5 (Figure 5). The hypermethylation of the Myogenin gene promoter was also found in both non-transfected and transfected cells at days 1-5 of differentiation (Figure 5). For miR-1, promoter methylation was only observed in transfected cells on day 4 (Figure 5). Moreover, the results confirmed that the promoter methylation of the miR-133 gene increased at days 4 and 5 in transfected cells as compared to control cells (Figure 5). Eventually, the results revealed a trend for miR-206 hypermethylation in both non-transfected and transfected cells, especially at days 4 and 5 of differentiation (Figure 5).

Discussion

This study highlights the significance of Cdk9 overexpression on regional and global DNA methylation in addition to DNMT gene expression changes in C2C12 cells during differentiation. Moreover, regional and global DNA methylation changes and gene expression patterns were observed in Cdk9-transfected myoblast cells during differentiation. It was further found that Cdk9 overexpression is able to increase the average expression level of DNMTs and global DNA methylation, as well as modulating the regional DNA methylation of MRFs and myoRNAs in C2C12 during differentiation. Muscle differentiation is a procedure with various steps organized by Cdk9, MRFs, and other epigenetic mechanisms including miRNAs and DNA methylation (22-28). Epigenetics define a regulatory mechanism of gene expression without changes in the underlying DNA sequences and play key roles in embryogenesis and differentiation (25). DNA methylation is a mechanism that donates a methyl group to the cytosine residue in CpG islands (5) by DNMT enzymes at the primary stage of embryogenesis (5,26). Additionally, experimental studies indicated that the upregulation of specific DNMT isoforms leads to manifesting cell activation and differentiation of myogenic stem cells (27,29,30).

A recent study has reported that Cdk9 is linked to gene silencing in mammal by chromatin remodeling and epigenetic modification (31). From the mechanistic viewpoint, it has been suggested that the Cdk9 induction pattern is roughly similar to DNMTs, and the synergy with DNMTs suggests Cdk9 as a novel epigenetic target for clinical developments (31,32). However, the precise mechanism by which Cdk9 regulates DNMT gene expression, DNA global methylation, and MRFs and miRNAs promoter methylation in myoblast cells is not
fully understood yet. Concerning Cdk9 regulatory roles, we examined the potential regulatory functions of Cdk9 overexpression on the entire genomic DNA methylation and regional promoter DNA methylation status of cardiac specific miRNA, MRFs as well as DNMT gene expression in myoblast C2C12 cells.

To the best of our knowledge, this is the first time that Cdk9 is linked to DNA methylation during C2C12 cell differentiation. This study is the first one to show that the Cdk9 overexpression is able to induce DNMT gene expression in Cdk9-transfected C2C12 cells in comparison to non-transfected (control) C2C12 cells during differentiation. Our results indicated that, in control C2C12 cells, only DNMT3B significantly increased in the first day of differentiation as compared to days 0, 3, 4, and 5 (Figure 1A). The results (Figure 1A) also represented the lowest level of DNMT1 and DNMT3A in non-transfected cells on day 3, as well as a significantly higher expression level of DNMT1 and DNMT3A in non-transfected C2C12 cells on the 0-2 days of differentiation as compared to day 3 (P<0.05). Some researchers speculated that DNMT1 has responsibility for DNA methylation maintenance although DNMT (3A & 3B) contributes to de novo methylation during cell differentiation (33,34). Overall, the highest and lowest levels of DNMTs (T1/3A/3B) were observed on the first and the third to fifth days of C2C12 differentiation in non-transfected cells (Figure 1B). Previous research also indicated that the expressions of DNMT1 and DNMT3A were diminished during the myogenic process (35). Moreover, the results regarding PCEP4/Cdk9 plasmid transfected cells demonstrated an increased expression level of DNMTs (T1/3A/3B) during differentiation (Figure 2B). Interestingly, our study showed that the expression...
levels of DNMTs decrease in non-transfected cells while DNMTs expression levels increase in Cdk9-transfected cells during the differentiation process. Overall, although the control and transfected C2C12 cells were cultured during the same conditions, the average transcription level of DNMT changes induced by Cdk9 was higher in transfected cells in comparison with non-transfected cells (wild: 0.8-fold, transfected: 1.61-fold, P<0.05, Figure 3). This clearly indicates a different epigenetic modification during the differentiation in control and transfected C2C12 cells. It has been suggested that Cdk9 is one of the main kinases required for epigenetic modifications and has a mechanistic function in epigenetic modifications for instance histone methylation (36). In addition, increasing evidence suggests that Cdk9 in combination with other transcription factors including CDK7 and RNA pol III regulates and increases gene body methylation, and therefore, increases the gene expression in cancer cells (37).

To investigate the global methylation level for non-transfected and Cdk9-transfected C2C12 cells, the average level of the 5-mC content of the genomic DNA related to these cells was calculated during differentiation and it was found that the global DNA methylation level reduced in the same day (2d) in both non-transfected and transfected C2C12 cells (P<0.05, Figure 4A-B). Moreover, the findings (Figure 4C) generally revealed that the Cdk9 overexpression is able to increase global DNA methylation in transfected C2C12 cells in comparison to respective non-transfected (control) cells during differentiation (P<0.05). Hence, an interesting probability raised in our research is that further active global DNA methylation in C2C12 transfected cells can be explained by the increased DNMT expression by Cdk9 in the cells. DNA methylation is a key molecular mechanism required for the self-renewal and maintenance of stem cells during differentiation (38). Further, the Cdk9 regulatory role leads to increased efficiency of cardiac developments by the epigenetic programming of miRNA and MRFs including MyoD and Myogenin (8,16). In this study, it was reported that the Cdk9 overexpression is able to induce the promoter methylation of miR-1 and miR-133 in Cdk9- transfected C2C12 cells (Figure 5). It was also found the promoter methylation of miR-1 on day 4 and the promoter methylation of miR-133 increased on days 4 and 5 in transfected cells as compared to non-transfected cells (Figure 5). Some researchers speculated that the regulatory role of Cdk9 may be mediated by decreasing miR-133 gene expression and increasing miR1 and miR-206 transcription in C2C12 myoblast cells (15,17,39,40). Moreover, our results revealed that the MyoD promoter was unmethylated in both non-transfected and transfected C2C12 cells during differentiation although it was methylated only in control non-transfected cells on day 5 (Figure 5). MyoD is the main regulator of the cardiac myogenic program and its expression is regulated by promoter epigenetic modifications during differentiation (41,42). The increasing evidence suggests that the Cdk9 in combination with other key factors including Cyclin T2a plays a key role in the initiation of the myogenic procedure by stimulating MyoD family transcription (43, 44). Moreover, our previous study showed that Cdk9 overexpression increased MyoD gene expression in myoblast C2C12 cells (15). Based on these results, Cdk9 probably increased MyoD gene expression by reducing its promoter methylation. Furthermore, the hypermethylation of the Myogenin promoter was found in both cells on all days (Figure 5). The MRF transcripts including Myogenin are strongly critical for several stages of myogenesis (11). The precise mechanisms regulating DNA methylation throughout myogenesis remain unknown. However, extensive data indicate that the promoter demethylation of MyoD and the Myogenin is necessary for proceeding the differentiation program (12). Our previous study indicated that continuous Cdk9 overexpression in transfected cells reduces Myogenin gene expression during differentiation (16). Our finding is in line with that of other studies, demonstrating that the Myogenin gene is present in a transcriptionally inhibited form in proliferating myoblasts (45). Moreover, the current findings revealed that Myogenin gene expression is reduced by the de novo methylation of its promoter region in terminally differentiated muscle cells, and other myogenic genes requiring terminal differentiation may be regulated by promoter DNA methylation (46, 47). Overall, a positive correlation was found between genomic global DNA methylation, DNMT gene expression, and regional methylation for cardiac-specific miomiRs including miR-1 and miR-133 in Cdk9-transfected C2C12 cells.

Conclusions
In general, our results indicated that Cdk9 overexpression can promote the regional promoter DNA methylation of myomiRs including miR-1 and miR-133 in addition to increasing global DNA methylation and DNMT gene expression in myoblast C2C12 cells. The finding offers novel insights into the mechanisms of epigenetic by which Cdk9 promotes epigenetic changes and modulates the global and regional DNA methylation profiling of myoblast cells during differentiation.

Authors’ Contribution
MSH and MMS conducted this study. LA, HK and VT performed the experiments. MSH, HG and MMS supervised the experiments. LA, SM, MMS and VT wrote the manuscript. MMS and MSH revised the manuscript. All of the authors assisted in writing the manuscript, discussed the results and commented on the manuscript.

Conflict of Interests
None.

Ethical Issues
There is no involvement of humans or animals in this study.

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