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Epigenetic regulation of neonatal cardiomyocytes differentiation

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ABSTRACT

The relationship between DNA methylation, histone modifications and terminal differentiation in cardiomyocytes was investigated in this study. The upregulation of methylation-related proteins, including DNA methyltransferase (DNMT) 1, methyl-CpG binding domain proteins 1, 2 and 3, and the increase in global methylation during rat neonatal heart development were observed. Moreover, an increase in DNA synthesis and a delay in differentiation were found in 5-azacytidine (5-azaC)-treated cardiomyocytes. Increase in acetylation of H3-K9, H4-K5, H4-K8 and methylation of H3-K4 suggested a more accessible chromatin structure in 5-azaC-treated cells. Furthermore, methyl-CpG-binding protein 2 was found to be upregulated in differentiated cardiomyocytes. Overexpression of this protein resulted in an increase of global methylation levels. Therefore, we suggest that a hypermethylated genome and a more compact chromatin structure are formed during terminal differentiation of cardiomyocytes.

1. Introduction

Neonatal cardiomyocytes in humans, mice and rats undergo a transition from hyperplastic to hypertrophic growth, such that further increase in myocardial mass is not accompanied by cardiomyocyte proliferation [1]. Unlike adult skeletal muscle which contains satellite cells that can be stimulated to proliferate and develop into mature adult myocytes, mammalian adult cardiomyocytes lack progenitors and thus they cannot regenerate. Consequently, myocardial loss due to injuries or diseases during adulthood is irreversible.

Most cardiomyocytes in rats gradually cease to undergo DNA replication within the first 2 weeks after birth [2]. They still undergo hyperplastic growth in day 1 postnatal, whereas hypertrophic growth is observed in day 7 postnatal [3]. Normally, postnatal heart at day 14 is regarded as an adult myocardium. For decades, the underlying mechanism of terminal differentiation in adult myocardium has remained obscure. The elucidation of cellular and molecular events behind this hyperplastic to hypertrophic switch has been one of the major focuses of cardiovascular research.

The involvement of DNA methylation in the differentiation process has been reviewed [4]. DNA methylation in mammals is catalyzed by at least three DNA methyltransferases (DNMTs). DNMT3a and 3b are believed to be the primary *de novo* DNMTs that establish new methylation patterns [5] while DNMT1 ensures the DNA methylation pattern is faithfully copied to the newly synthesized DNA strand after DNA replication [6]. DNA methylation within gene promoters can directly regulate gene expression by altering the binding of transcription factors to DNA or by recruiting methyl-CpG-binding proteins, e.g., methyl-CpG-binding protein 2 (MeCP2) and methyl-CpG binding domain (MBD) protein 2 that recognize methylated CpG dinucleotides, leading to a change in chromatin conformation and thus turning off transcription [7–9].

We previously identified differentially expressed genes in rat neonatal hearts [10,11]. Many of these genes are involved in DNA methylation and chromatin-remodeling processes. In the current study, the possible roles of DNA methylation and histone modifications in the terminal differentiation of rat neonatal cardiomyocytes were investigated.

2. Materials and methods

2.1. Primary culture of rat ventricular cardiomyocytes

Apical 1/3 of the ventricles from Sprague–Dawley (SD) rats were dissected, minced and trypsinized at 4 °C for 16–20 h. Afterwards, tissues were digested with collagenase type II (1 mg/ml) in HEPES-Buffered Saline at 37 °C for 15 min, followed by washing, centrifugation and then cultured in DMEM/F-12 with 0.1% insulin–transferrin–selenium-G supplement (Invitrogen), 5% horse serum, 0.1 mM ascorbic acid and 0.1 mM bromodeoxyuridine for 48 h. The medium was then replaced with DMEM supplemented with

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0.5% HS, 10% M199 media and 0.1% ITSG for 24 h. The purity of cardiomyocytes was ⩾90%, as determined by immunohistochemistry with anti-sarcomeric actin and FITC-conjugated anti-mouse antibodies.

2.2. Cytosine-extension assay

The global methylation assessment was accomplished by the cytosine-extension assay [12]. Briefly, 2 μg of genomic DNA was digested overnight with 10-fold excess of HpaII, BstUII, HhaI and MspI endonucleases according to the manufacturer's protocol (New England Biolabs). Each 20 μl-reaction mix was composed of 10 U restriction endonuclease, 2 μg genomic DNA and 1× nuclear extract buffer (New England Biolabs). The reaction mix was incubated at 60 °C (for BstUII only) or at 37 °C (for HpaII, HhaI and MspI) overnight. DNA incubated in the absence of restriction enzyme was served as a negative control. The single nucleotide extension reaction was performed in a 25 μl-reaction mix containing 0.25 μg genomic DNA, 1× PCR buffer, 0.25 U AmpliTaq DNA polymerase and 0.3 μl [^{3}H]-dCTP (GE Healthcare). The reaction mixture was incubated at 95 °C for 7 min and then DNA was added. Afterwards, the mixture was incubated at 56 °C for 1 h and then placed on ice. Duplicated 10 μl aliquots from each reaction were applied on DE-81 ion-exchange filters (Whatman) and washed three times with Na-phosphate (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. Background radiolabel incorporation was subtracted from enzyme-treated samples and the results were expressed as relative [^{3}H]-dCTP incorporation/0.5 μg DNA. As a result, the extent of radiolabeled [^{3}H]-dCTP incorporation would be proportional to the number of unmethylated CpG cleavage sites.

2.3. Tritiated-thymidine incorporation assay

Cells were pulsed with 1 μCi/ml [^{3}H]-thymidine (GE Healthcare) in DMEM supplemented with 5% FBS for 8 h. After labeling, cells were washed with PBS and then fixed with 15% trichloroacetic acid (TCA) at 4 °C for 1 h. After aspiration of TCA, the cells were washed twice with distilled water. The precipitated DNA was solubilized in 1 M NaOH for 20 min and then neutralized by 1 M HCl. Finally, lysates were subjected to scintillation counting.

2.4. 5-Azacytidine treatment

Primary rat neonatal cardiomyocytes (about 1.6×10^5) were seeded overnight in 24-well plates, 5-azaC was added and was refreshed every 24 h until 48 h treatment finished. The medium containing PBS only was regarded as a control.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from rat hearts of different ages using TRIZOL reagents (Invitrogen). The first-strand cDNA was synthesized from total RNA using Thermoscript RT-PCR System (Invitrogen) according to the manufacturer's instructions. RT-PCR was carried out under standard protocol using the following primers: β-actin (forward: 5′-TGAGCTGCGTGTGGCCCCTGAG-3′; reverse: 5′-GGGGCATCGGAACCGCTCATTG-3′), MBD1 (forward: 5′-CAAGAGGGTACCAGCAGTCTCATGCA-3′; reverse: 5′-GTTTATGTAGCCT TGGCAACCAGGC-3′), MBD2 (forward: 5′-TGAAATGGACAG TGGAGATGA-3′; reverse: 5′-CTTATTAAAACTGTGGGGGAAA-3′), MBD3 (forward: 5′-ACTTGCCCAAGGGCCTGCAGG-3′; reverse: 5′-CTCC TCCTCAGCACAGGCCTTGTCC-3′), DNMT1 (forward: 5′-GGAAAGG AGGAGACTACTAC-3′; reverse: 5′-TCTCACTTGCCACCCACACA-3′), DNMT3a (forward: 5′-AGGGACATGGGGGCAAACT-3′; reverse: 5′-GCAAGAAACAAAAACCCAAAC-3′), DNMT3b (forward: 5′-GGACT ACTTTGCATGTGAATA-3′; reverse: 5′-GCTGTTGTTTTGGTATCTTAG-3′), ANF (forward: 5′-CTTCGGGGGTAGGATTGACAGGATTG-3′; reverse: 5′-AGGGGTGAGGATCTACCTTAATATGCAGA-3′), β-MHC (forward: 5′-GCATTGCATCCTGTTGGATACAGAGTTG-3′; reverse: 5′-CTCCCTGCTTGGCCTCACTCCCCTA-3′), α-MHC (forward: 5′-CTCC TAAACCAAAAGCAAACTCAGACCC-3′; reverse: 5′-ACAGTTAAACGTT GCGCCTTCATAGTG-3′). PCR was performed at 94 °C for 2 min, followed by 26–33 cycles of amplification at 94 °C for 36 s, 52 °C for 36 s and 72 °C for 1 min by using PTC-100 Programmable Thermal Controller (MJ Research). The band intensities were measured by a densitometer and the results were normalized with β-actin. The results were repeated by at least three times independently from three different pools of templates, while each pool of template was extracted from at least eight ventricles.

2.6. Transient transfection

The MeCP2-pcDNA4 construct was created by cloning the coding region of rat *MeCP2* into pcDNA4 vector (Invitrogen). The *MeCP2* coding region was amplified from a SD rat ventricular cDNA template by PCR with the following primers (forward: 5′-ATGGTAGCTGGGATGTTAGGGCTCAG-3′; reverse: 5′-ACCGCTTTGC AATCCGCTCTATGTAA-3′) and the reaction profile was as follows: denaturation at 95 °C for 3 min, followed by 32 cycles of denaturation at 94 °C for 36 s, annealing at 55 °C for 36 s and extension at 68 °C for 1.5 min. Overexpression of MeCP2 in 7-day-old cardiomyocytes was carried out by transient transfection of the MeCP2-pcDNA4 construct for 48 h using Lipofectamine with Plus Reagent (Invitrogen).

2.7. Western blotting

Cells were lysed with lysis buffer and samples containing 40 μg of total protein lysates were separated by SDS–PAGE and blotted onto a PVDF membrane. The primary antibodies against MeCP2 (Upstate, 1:1000 dilution), proliferating cell nuclear antigen (PCNA) (Novo castra, 1:750 dilution), p21 (BD Biosciences Pharmingen, 1:500 dilution), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (Trevigen, MD, 1:5000 dilution), acetylated histone H3 lysine-9 (Upstate, 1:500 dilution), acetylated histone H4 lysine-5 (Upstate, 1:750 dilution), acetylated histone H4 lysine-8 (Upstate, 1:1000 dilution) and dimethylated H3 lysine-4 (Upstate, 1:750) were applied. Horseradish peroxidase conjugated anti-goat, anti-mouse and anti-rabbit antibodies (GE Healthcare) were used as secondary antibodies correspondingly. Proteins were visualized with ECL-chemiluminescent kit (GE Healthcare).

2.8. Statistical analysis

All quantitative data are presented as mean ± S.E.M. Student's *t*-test was used for paired data. A value of $P \leqslant 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Differential expression of DNA methylation-related proteins and global methylation pattern during the development of rat neonatal hearts

In order to study the role of DNA methylation in heart development, the expression profiles of DNA methyltransferases and methly-CpG binding domain proteins during rat myocardial development were comprehensively investigated by using semi-quantitative RT-PCR. DNMT1 was found to be upregulated gradually from day 1 to day 90 postnatal rat hearts, while the changes in the