RA-Induced Transcriptional Silencing of Checkpoint Kinase-2 through Promoter Methylation by Dnmt3b Is Required for Neuronal Differentiation of P19 Cells

Muhammad Abid Sheikh[1,2,†], **Yousra Saeed Malik**[1,†] **and Xiaojuan Zhu**[1]

*1 - **Institute of Cytology and Genetics,** Northeast Normal University, Changchun, China*
*2 - **Department of Microbiology and Molecular Genetics,** University of the Punjab, Lahore, Pakistan*

***Correspondence to Xiaojuan Zhu:** Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China. zhuxj720@nenu.edu.cn*
http://dx.doi.org/10.1016/j.jmb.2017.07.005
Edited by M. Yaniv

Abstract

In a previous study, we identified several novel targets of Dnmt3b using a chromatin library from retinoic acid (RA)-treated P19 cells. The present study describes the regulation of expression and function of checkpoint kinase (Chk2), which was one of the target genes of Dnmt3b. Chromatin immunoprecipitation followed by quantitative PCR analysis showed that recruitment of Dnmt3b on Chk2 promoter is induced following RA treatment of P19 cells. Both bisulfite genomic sequence and COBRA analyses showed that the methylation level of Chk2 promoter is progressively increased during RA-induced neuronal differentiation of P19 cells. Concomitantly, both mRNA and protein expression of Chk2 are reduced as determined by real-time PCR and Western blot analysis, respectively. Suppression of Dnmt3b expression by lentiviral-mediated shRNA resulted in increased expression and reduced methylation of Chk2, which clearly showed that Dnmt3b is responsible for transcriptional silencing of Chk2 gene in RA-treated P19 cells. Neuronal differentiation of P19 cells was inhibited upon enforced Chk2 expression in P19 cells, which showed that the decrease in endogenous expression of Chk2 is essential for normal differentiation. Ectopic Chk2 expression also negatively regulated cell cycle arrest and apoptosis following RA treatment, which could also contribute to impaired neuronal differentiation. Together, this study described the regulation of Chk2 expression through promoter methylation and also presented a novel role of Chk2 during neuronal differentiation, which is independent of its previously known function in DNA damage response.

Introduction

DNA methylation is an epigenetic mechanism of gene silencing whereby a DNA methyltransferase (Dnmt) enzyme catalyzes the methylation of 5′ carbon position of a cytosine residue at the CpG dinucleotide [1,2]. CpG-rich areas (500–2000 bp long), designated as CpG islands, are found in the promoter regions of almost 50% of mammalian genes [3]. Gene silencing through DNA methylation is carried out by either of the following two mechanisms. First, transcription factors are unable to bind with promoters when CpG islands are heavily methylated, thus inhibiting transcription [4,5]. Second, methyl-CpG-binding domain proteins can bind with the methylated DNA and in turn recruit histone deacetylases that result in the formation of a large repressor complex at the promoter of the gene [6,7]. Dnmts primarily consist of three functional family members, namely, Dnmt1, Dnmt3a, and Dnmt3b [8]. Dnmt1 prefers hemimethylated substrates, and thus, it is the major maintenance methylation enzyme that copies methylation pattern during replication [9]. Dnmt3a and Dnmt3b are main *de novo* Dnmts that establish new methylation patterns during development [10]. These Dnmts and their isoforms are expressed in a cell- or tissue-specific manner suggesting their involvement in distinct pathways underlying development and differentiation [11].

A previous study by our group showed an increase in Dnmt3b expression, while the levels of Dnmt1 and Dnmt3a were reduced during retinoic acid (RA)-induced neuronal differentiation of P19 cells. Next, Dnmt3b-enriched chromatin library identified several novel targets of Dnmt3b during RA-induced neuronal

differentiation of P19 cells that have already been reported in our previous study [12]. The present study is focused on checkpoint kinase 2 (Chk2), which was one of the target genes in our Dnmt3b-enriched chromatin library. We selected Chk2 for further study based on its important role in ensuring genomic integrity, cell cycle progression, differentiation, and apoptosis [13,14].

Checkpoints are intricate kinase pathways that regulate DNA repair and cell cycle progression. Checkpoints regulate transitions of cell cycle at crucial points before or during DNA replication (G1/S) and before cell division (G2/M) in response to DNA damage [15]. Mammalian cells contain two checkpoint kinases, that is, Chk1 and Chk2. Chk1 senses single-strand DNA breaks [16]; however, Chk2 is the effector kinase in ATM–CHK2–CDC25 pathway that is responsible for sensing double-strand DNA breaks [17]. The cumulative effect of these pathways is to ensure genomic integrity during cellular differentiation and development and deregulated expression of these kinases can lead to neoplastic transformations [18]. For example, increased expression of Chk2 has been reported in human gastric carcinomas [19]. In contrast, a reduced expression of Chk2 is observed in non-small cell lung cancer due to hypermethylation of Chk2 promoter region [20]. The present study also deals with the regulation of Chk2 expression through promoter methylation during RA-induced neuronal differentiation of P19 cells. We also presented a novel role of Chk2 during neuronal differentiation which is independent of its previously known function in DNA damage response.

Results and Discussion

Recruitment of Dnmt3b on Chk2 promoter is induced following RA treatment of P19 cells

In a previous study [12], we reported the identification of several novel targets of Dnmt3b by using a Dnmt3b-enriched chromatin library from RA-treated P19 cells. We selected Chk2 for further study based on its important role in ensuring genomic integrity, cell cycle progression, differentiation, and apoptosis [13,14]. In order to validate the results of chromatin library as well as to study the recruitment of Dnmt1, Dnmt3a, and Dnmt3b on Chk2 promoter, we again performed ChIP followed by real-time quantitative PCR analysis at different time points during differentiation of P19 cells. Chk2 promoter enrichment above background (IgG-negative control) was not observed with either Dnmt3a or Dnmt1 pulled-down DNA (Fig. 1). In untreated proliferating P19 cells, Dnmt3b is not recruited on Chk2 promoter as the fold enrichment values obtained are similar to the values of IgG, which served as a negative control in this assay. We also observed that recruitment of Dnmt3b on Chk2 promoter was induced by RA treatment. As compared to IgG, more than 50-fold enrichment of Chk2 promoter was observed with Dnmt3b pulled-down DNA from cells differentiated for 6 days. We also observed a dramatic increase in the recruitment of Dnmt3b on Chk2 promoter region from day 2 to day 4, which is a critical time period during neuronal differentiation of P19 cells (Fig. 1).

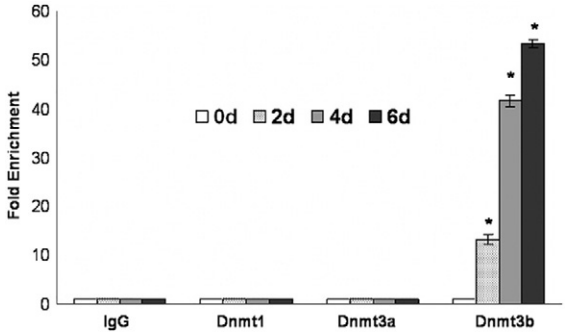

Fig. 1. Recruitment of Dnmt1, Dnmt3a, and Dnmt3b on Chk2 promoter at different days during RA-induced differentiation of P19 cells by using quantitative ChIP analysis. Data were normalized to input fraction. The value of IgG (negative control) was set as 1, and the results were presented as relative fold enrichment of IgG. Error bars represent ±SEM ($n = 3$). *$P < 0.05$ *versus* untreated P19 cells.

Methylation level of Chk2 promoter CpG island is increased after RA treatment of P19 cells

Association of Dnmt3b with the promoter of Chk2 led us to study the methylation pattern of Chk2 promoter region in RA-treated and -untreated P19 cells. For this purpose, we screened the promoter region of Chk2 for the presence of any CpG island using CpG island searcher online analysis tool.[‡] The screening showed that Chk2 promoter region indeed contained a CpG island that was used in this study. The primers were designed by using MethPrimer online software to amplify a region of 289 bp within the CpG island of Chk2 promoter region, which contained 23 CpG dinucleotides (Fig. 2a). In order to determine the methylation pattern of these individual CpG sites within Chk2 promoter, bisulfite genomic sequence (BGS) and COBRA analyses were performed at different days during differentiation of P19 cells (Fig. 2). For BGS, the 289-bp PCR product was amplified from bisulfite-converted DNA and cloned into pGEM-T vector. BGS analysis was performed by using at least 15 individual clones with the help of BISMA online analysis tool. The results of BGS analysis were in agreement with the ChIP results. The promoter of Chk2 is unmethylated in RA-untreated P19 cells as less than 1% of the scored CpG sites are methylated in uninduced cells. During RA-induced