Dnmt1 regulates adipogenesis by Cdkn1a methylation

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Abstract: Obesity has recently become a major healthy concern in developed countries. This leads to intensive interest in the mechanism study of adipogenesis, in which epigenetic mechanisms are speculated to play an essential role. To explore the function of Dnmt1, its expression was first profiled during the course of adipocyte differentiation of 3T3-L1 cells. The results revealed a dynamic regulation of its expression at the initiation stage. Knockdown of Dnmt1 compromised the differentiation process and decreased lipid production within the cells. To the aspect of epigenetic regulation, promoter methylation of Cdkn1a was significantly increased at the initiation stage of the differentiation, accompanied by decreased Cdkn1a expression. Furthermore, knockdown of Dnmt1 led to an increased Cdkn1a expression, indicating that Dnmt1 inhibits Cdkn1a expression by promoter methylation. Furthermore, we found that knockdown of Cdkn1a up-regulated the expression of PPARγ and resulted in enhanced adipocyte differentiation. In summary, our results demonstrated that Dnmt1 regulated the process of adipogenesis by methylation of Cdkn1a promoter, suggesting that Cdkn1a played a fundamental role in the prevention of adipocyte hyperplasia.

Keywords: Adipogenesis; Cdkn1a; Dnmt1; DNA methylation

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1. Introduction

As a common chronic disease, obesity has an increasing prevalence in developed countries. Excessive accumulation of adipose tissue serves as a major contributing factor in severe human diseases, including cardiovascular disease, type 2 diabetes, and several types of cancer[1–3]. By 2014, the incidence of obesity in United States is increased up to 35.0% in adult men and 40.4% in women[4–6]. This may be derived from the increased volume or numbers of adipocytes for a given individual. To this aspect, published studies have found that increased adipocyte number is a major contributor to adult fat mass[7]. However, our understanding of adipocyte hyperplasia is still limited.

In the studies of adipogenesis, mouse pre-adipocyte cell line 3T3-L1is the most frequently used cell model, established in 1974. Using a standard protocol, these cells are readily induced to differentiate into mature adipocyte[8–11]. During the course of the differentiation, the cells experience three distinct stages, cell-contact inhibition (growth arrest) stage, mitotic clonal expansion stage, and terminal differentiation stage. Briefly, the pre-adipocytes are cultured to confluence, maintained in a growth-arrest status for 48 h, and then induced to adipocyte differentiation by a hormone cocktail consisting of 3-isobutyl-1-methylxanthine, dexamethasone and insulin (MDI). After induction, these poised cells re-enter into cell cycling for several rounds and finally move into terminal differentiation to mature adipocytes[12–14].

The growth arrest stage is crucial for adipocyte differentiation. In the absence of this period, the normal differentiation process will be blocked. Studies have also revealed extensive epigenetic modifications, including DNA and histone modifications[15–17]. Disturbing epigenetic modification by methylation inhibitors or RNAi will

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lead to decreased differentiation potential of the cells[13]. For example, inhibiting DNA methylation by 5-aza-2′-deoxycytidine (5-aza-dC) markedly suppresses adipogenesis[17]. Transfection of Dnmt3a-targeting siRNA also compromises the adipocyte differentiation process[13]. Together, these data suggest that the cells are licensed to adipocyte differentiation within this stage via an epigenetic regulation.

Although DNA methylation has been well studied in cancer and mammalian development[18,19], its roles in adipocyte differentiation still remain elusive. In the present study, we aimed to explore the roles of Dnmt1 in adipocyte differentiation.

2. Materials and methods

2.1. Cell culture and MDI treatment.

3T3-L1 pre-adipocytes were cultured in DMEM supplemented with 10% bovine serum. Adipogenesis was induced as previously described. Briefly, 2 d after the cells reached confluence on day (0), cells were induced to differentiate by changing the culture medium to DMEM supplemented with 0.5 mM 3-isobutyl-1-methyxanthine (Sigma), 1 μM dexamethasone (Sigma) and 167 nM insulin (Sigma). At the end of day (2), culture medium was replaced by DMEM supplemented with only 167 nM insulin. At the end of day (4), insulin was withdrawn and the cells were allowed to grow in DMEM throughout the rest of the experiment. At each time point, the cells were collected, washed three times with ice-cold PBS and then stored at –80 °C prior to further analysis.

2.2. Oligonucleotide

DNA oligonucleotides were purchased from Invitrogen (Beijing, China). RNA oligonucleotides were obtained from RiboBio (Guangzhou, China).

2.3. qRT-PCR

Total RNA was extracted by TRI Reagent (Sigma) according to the manufacturers' instructions. cDNA was synthesized using SuperScript II (Invitrogen), and qPCR was performed using SYBR Green PCR mix. Relative expression values were calculated using ΔΔCT method, and β-actin was selected as the housekeeping gene. Table 1 lists the primers used in the assay.

Table 1. qRT-PCR primers.

Primer name	Sequence
β-Actin-F	5′-GAAGAGCTATGAGCTGCCTGA
β-Actin-R	5′-CTCATCGTACTCC TGCTTGCT
Dnmt1-F1	5′-ACCCAAGGAAGAGTCGGAAG
Dnmt1-R1	5′-TCCTCCTTTGATTTCCGCCT
Dnmt1-F2	5′-ATGGTGAAGTTTGGTGGCAC
Dnmt1-R2	5′-TCTCCTCATCGATGCTCACC
Cdkn1a-F1	5′-TTAAGGACGTCCCACTTTGCC
Cdkn1a-R1	5′-AAAGTTCCACCGTTCTCGGG
Cdkn1a-F2	5′-GTACTTCCTCTGCCCTGCTG
Cdkn1a-R2	5′-CACAGAGTGAGGGCTAAGGC
PPARγ-F	5′-AAGAGCTGACCCAATGGTTG
PPARγ-R	5′-ACCCTTGCATCCTTCACAAG

2.4. Gene silencing assay

siRNAs were individually transfected with the following steps. Cells were seeded at a density of 10 000 cells/well in 1 mL of growth medium 1 d before transfection. A targeting siRNA or a scrambled control siRNA was transfected using Lipofectamine 2000 (Invitrogen). Lipofectamine/siRNA complexes were formed in 0.2 mL of serum-free Opti-MEM reduced serum medium (GIBCO) for 10 min at room temperature and then added to each well. Cells were cultured for another 2 d until they reached confluence and subjected to MDI treatment on day (0). At the same day, a second transfection was performed. The sequences of Dnmt1 siRNA were as follows: sense 5′-GCUAUCAUUGA-GGCGGAAATT-3′ and antisense 5′-UUUCCGCCUC-AAUGAUAGCTT-3′.

2.5. Bodipy staining assay high content assay

Lipid accumulation was evaluated using Bodipy 493/503 staining (Life Technologies). Cells at different differentiation stages were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.5% Triton X-100

Triton X-100 for 15 min at room temperature. After washing with PBS, the cells were stained with Bodipy 493/503 solution at 1:1000 and then Hochest 350/461 buffer at 1:10 000 for 45 min.

Cell observation and documentation were performed with an Operetta High Content Screening and Analysis Reader (Perkin Elmer) using a 20× objective. Fluorescence of Hoechst (the nuclei) and Bodipy 493 (Lipid) was measured using Hoechst 33342 and Alexa 488 channels. Bright field images were documented as cell number control. Fluorescence images were analyzed using Columbus Image Data Storage and Analysis System (Perkin Elmer).

2.6. MeDIP-CHIP array and mRNA microarray

Methylated DNA immunoprecipitation CHIP array (MeDIP-CHIP) was performed by KangChen Bio-tech (Shanghai, China). NimbleGen mouse DNA methylation 3 × 720 K CpG Island Plus RefSeq promoter Array (Roche-NimbleGen) was used to identify methylated DNA regions by a high throughput multiplex array platform. mRNA microarray was performed by KangChen Bio-Tech (Shanghai, China).

3. Results

3.1. The involvement of Dnmt1 in adipocyte differentiation

Adipocyte differentiation of 3T3-L1 cells was induced by MDI in the present study. During the differentiation, the expression of Dnmt1 was determined at several critical time points by qRT-PCR. Figure 1 shows that the expression of Dnmt1 was steadily increased at the initiation stage of the differentiation, followed by a dynamic decline, and this finding was consistent with the published data. On day (+1), the expression of Dnmt1reached a peak about 207% compared with the levels of day (–2). The dynamic regulation of Dnmt1 suggested that it was involved in adipocyte differentiation of 3T3-L1 cells.

To evaluate the effects of Dnmt1 on adipocyte differentiation, loss-of-function assays were performed.

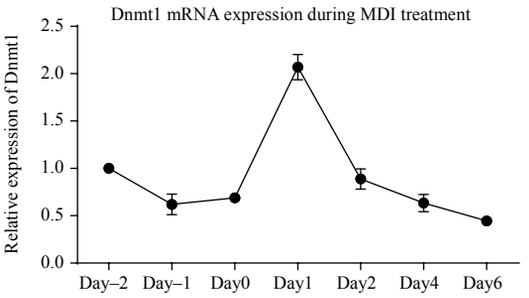

Figure 1. Regulation profile of Dnmt1 in adipocyte differentiation of 3T3-L1 cells. Expression levels are plotted as fold-change relative to that of day–2. Data are presented as Mean±SD.

Using Dnmt1-targeting siRNAs, the expression of DNMT1 was significantly repressed in 3T3-L1 cells. When these cells were subjected to differentiation, decreased lipid accumulation was observed on day (+4), evidenced by Bodipy staining. Compared with scramble siRNA, treatment with a Dnmt1-targeting siRNA caused a 25% reduction in lipid production (Fig. 2). These results demonstrated that Dnmt1 was a major contributing factor in adipocyte differentiation.

3.2. MeDIP-CHIP array and mRNA microarray

Dnmt1 is critical for the maintenance of DNA methylation, and it mediates transcriptional silencing by methylating CpG island[20]. To understand the epigenetic regulation of adipocyte differentiation, we simultaneously performed methylated DNA immuno-precipitation (MeDIP) assays (GEO accession: GSE81739) and mRNA microarray at several critical time points of the differentiation. The result showed that promoter methylation of 582 genes was increased during the differentiation. As earlier study has reported that when the growth-arrested 3T3-L1 preadipocytes are treated with hormone induction, they are induced into mitotic clonal expansion (MCE) before the expression of differentiation-related genes[21]. Therefore, we focused on the proliferation- or differentiation-related genes in further investigations. Taking these data together with gene ontology and pathway analyses, 58 proliferation genes were found with increased levels of promoter methylation. Among them, seven genes were down-regulated during the differentiation (Fig. 3). It was

interesting to note that Cdkn1a (*p21*), a cyclin-dependent kinase inhibitor, was within this gene set. Consistent with its increasing promoter methylation levels (peak score = 2.60), the expression of Cdkn1a was constantly down-regulated during the course of adipocyte differentiation (Fig. 4).

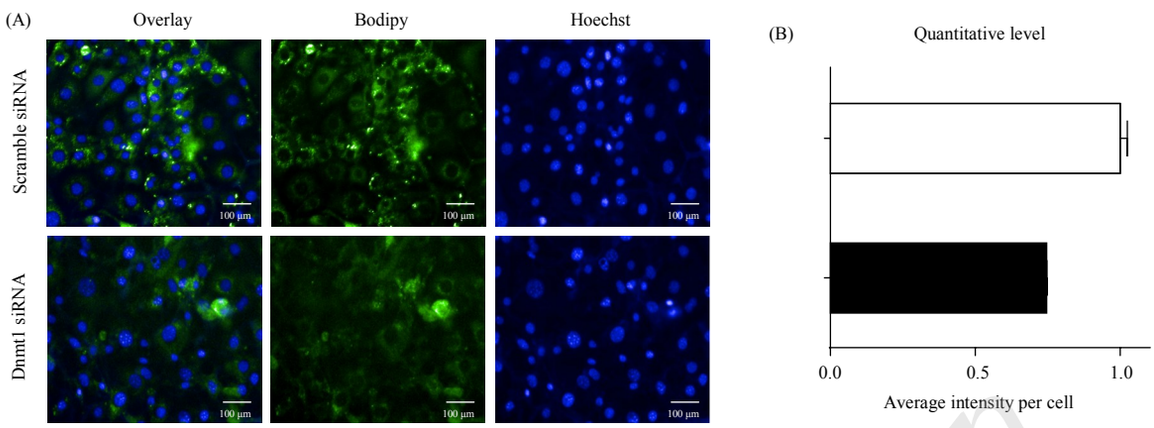

Figure 2. Knockdown of Dnmt1 led to decreased lipid production. (A) Bodipy staining of the nucleus (blue) and lipid droplet (green). (B) Quantitative measurement of lipid production per cell.

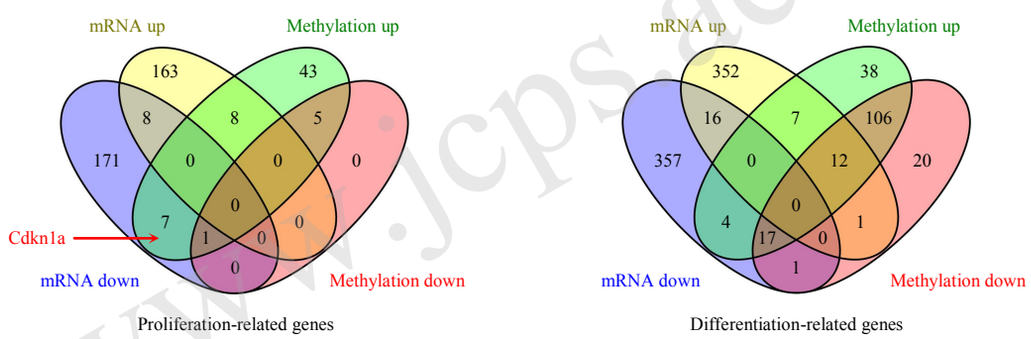

Figure 3. Venn diagram analysis of microarray data.

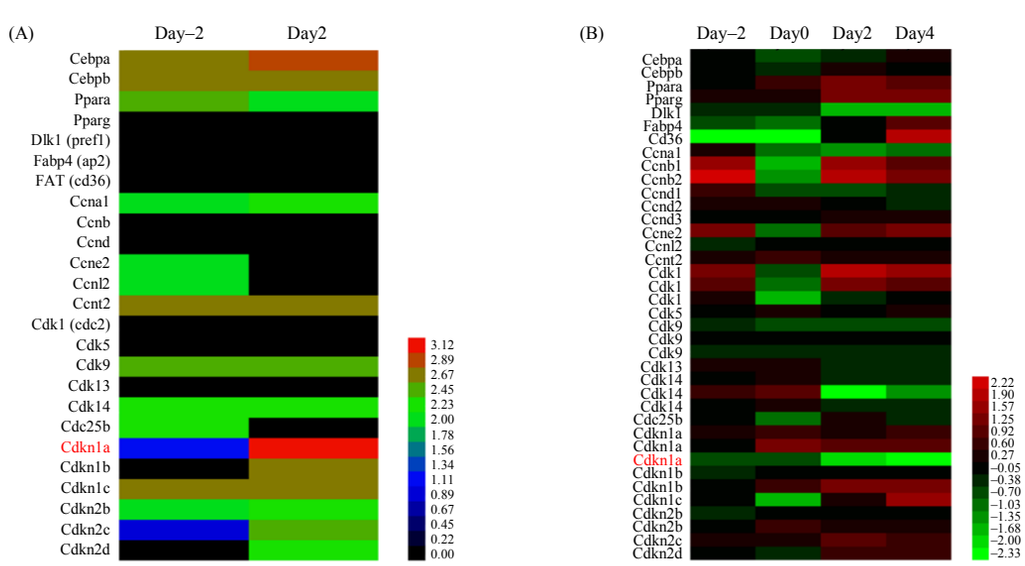

Figure 4. Heat map of microarray results. (A) Heat map of MeDIP-CHIP data about proliferation genes. (B) Heat map of mRNA microarray data about proliferation genes.

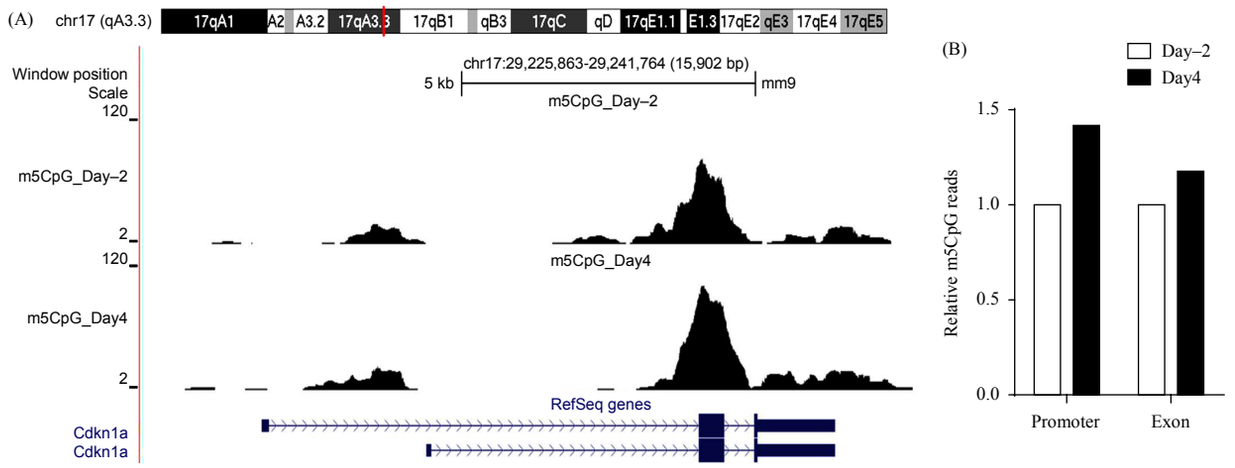

Figure 5. m5CpG ChIP-Seq data showed methylation level at Cdkn1a locus. (A) Genome browser presentation of m5CpG ChIP-Seq data (GEO Series GSE73434). (B) Quantitative measurements of m5CpG at Cdkn1a locus from m5CpG ChIP-Seq data.

In addition, the increased promoter methylation of Cdkn1a was also shown by m5CpG ChIP-Seq data from other studies (GEO accession GSE73434)[22]. Compared with preadipocytes, an increase of 142% in m5CpG methylation was found in differentiated adipocytes (Fig. 5). Taken all the data together, we proposed that the down-regulation of Cdkn1a was derived from its increased promoter methylation, which was mediated by Dnmt1.

3.3. Dnmt1 inhibits Cdkn1a expression by promoter methylation

To further characterize the regulation of Cdkn1a, a Dnmt1-targeting siRNA was transfected into cultured 3T3-L1 cells. Its effects on Cdkn1a expression were examined by qRT-PCR during the course of adipocyte differentiation. Compared with a scramble siRNA, the expression of Cdkn1a was up-regulated by 31% after Dnmt1 knockdown (Fig. 6). This finding suggested that Dnmt1 played a role in the inhibition of Cdkn1a expression during adipogenesis.

3.4. Knockdown of Cdkn1a inhibits adipocyte differentiation

To explore the significance of Cdkn1a regulation, Cdkn1a-specific siRNAs were transfected into cultured cells (Fig. 7A). These cells were then subjected to MDI

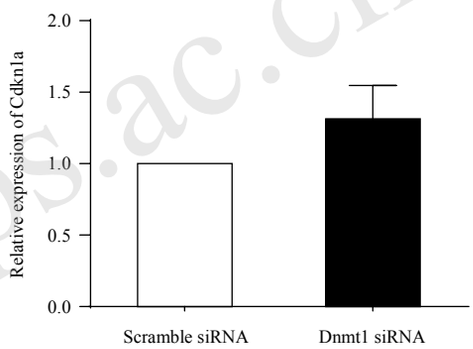

Figure 6. Knockdown of Dnmt1 promotes Cdkn1a expression. Data are shown as means±SD (n = 3).

induction. The effects on adipocyte differentiation were examined in terms of the expression of PPARγ, a key regulator of adipogenesis[23–25]. Compared with scramble siRNAs, the expression of PPARγ was up-regulated by 1032% in Cdkn1a siRNA treated cells (Fig. 7B). To evaluate the effects on lipid production, a 'High Content' assay using Bodipy staining was carried out. The results showed that down-regulation of Cdkn1a promoted lipid production in mature adipocytes (Fig. 8).

Compared with wild-type mice, previous studies have found that Cdkn1a knockout mice have a 90% increase in fat pad weight and 70% increase in fat cell numbers[26]. The obesity of Cdkn1a knockout mice is likely due to adipocyte hyperplasia. Taking together with its inhibitory role in cell proliferation, we proposed a

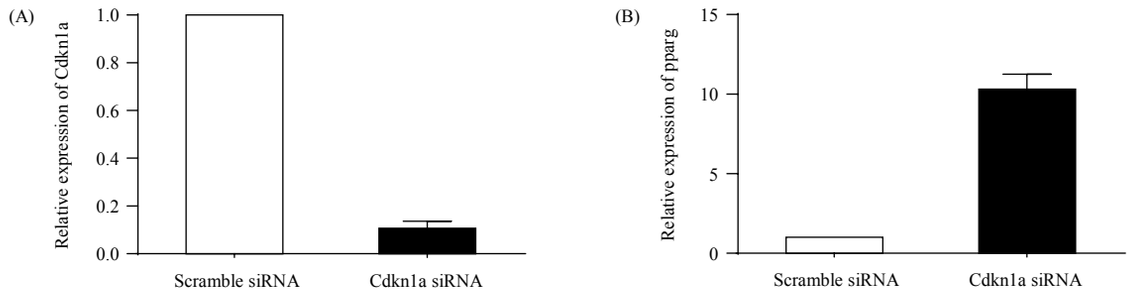

Figure 7. Knockdown of Cdkn1a inhibits adipocyte differentiation. (A) Gene silencing effects of Cdkn1a-targeting siRNA. (B) The effects on PPARγ expression. Data are shown as fold changes relative to β-actin (means ± SD, n = 3).

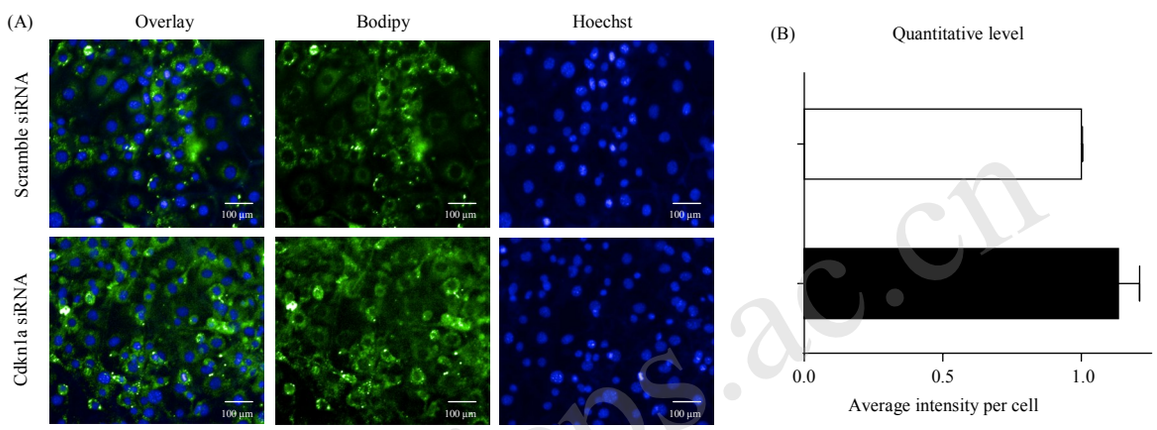

Figure 8. Knockdown of Cdkn1a led to increased lipid production. (A) Bodipy staining. Nuclei are in blue and lipid droplets are in green. (B) Quantitative measurement of lipid production per cell.

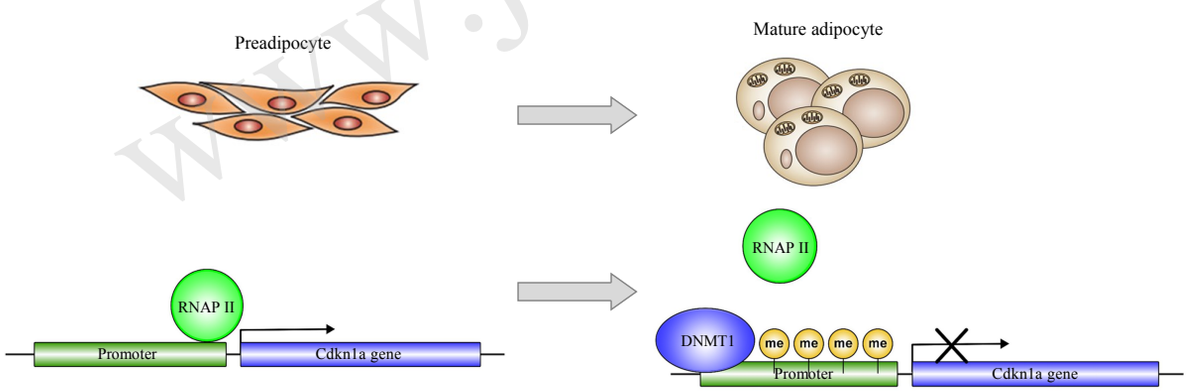

Figure 9. Functional Model of Dnmt1 regulated the process of adipogenesis by Cdkn1a promoter methylation.

hypothesis that Cdkn1a functioned in adipocyte differentiation by preventing over-proliferation of cells, therefore keeping the cells in growth arrest status.

4. Conclusions

Our results first showed that knockdown of Dnmt1 led to compromised adipocyte differentiation and decreased lipid production, suggesting that Dnmt1 played a key role in the process. Further study found that Dnmt1 inhibited Cdkn1a expression by promoter methylation, and knockdown of Cdkn1a promoted the process of adipocyte differentiation. Taken together with previous studies, these data indicated that Dnmt1 regulated adipocyte differentiation through Cdkn1a pathway via epigenetic regulation (Fig. 9). These findings provided a new potential drug target for obesity and adipocyte hyperplasia.

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Dnmt1通过Cdkn1a甲基化调控脂肪分化的过程

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摘要: 表观遗传调控机制在脂肪细胞分化中发挥了重要的作用。为了研究DNA甲基转移酶Dnmt1在脂肪细胞分化中的作用, 我们利用3T3-L1前脂肪细胞体外诱导分化模型, 首先分析了该基因在脂肪细胞分化中的表达变化, 发现Dnmt1的表达水平在分化起始阶段显著升高。如果在该过程中敲低Dnmt1的表达, 则会显著影响细胞分化进程, 抑制细胞脂质的生成。利用甲基化基因芯片分析, 我们发现在分化起始阶段, Cdkn1a基因的启动子甲基化水平显著升高, 并伴随基因表达水平的降低; 而敲低Dnmt1的表达则能抑制Cdkn1a启动子的甲基化, 提高基因的表达。这些研究结果表明, Dnmt1通过调控Cdkn1a启动子的甲基化, 影响该基因的表达。进一步的研究发现, Dnmt1通过Cdkn1a启动子的甲基化调节脂肪细胞分化, 表明Cdkn1a有可能在脂肪细胞分化、以及预防脂肪细胞增生中发挥关键的作用。

关键词: 脂肪分化; Cdkn1a; Dnmt1; DNA甲基化