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Original Article

5-azacytidine inhibits Sox2 promoter methylation during the induction of Thy-1^{+}Lin^{-} cells into hepatocytes

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Abstract: Objective: To investigate the changes and functions of Sox2 gene expression and promoter methylation during induced differentiation of bone marrow mesenchymal stem cells (BMSCs) into hepatocytes (HCs). Methods: Rat bone marrow Thy-1^{+}Lin^{-} cells were prepared and divided into control group (directed induction of differentiation into HCs) and experimental group (5-azacytidine intervention induced differentiation). The mRNA expression levels of ALB and Sox2 were detected by fluorescence quantitative polymerase chain reaction (PCR), and the Sox2 gene promoter methylation level was determined by Bisulfite sequencing PCR (BSP). Results: Sox mRNA expression level was significantly increased in experimental group compared to the control group at 0, 7, and 14 days, respectively (all $P<0.05$). The Sox2 promoter methylation level was gradually increased after 0, 7 and 14 days induction in both groups, accompanied by an increase in methylated loci (all $P<0.05$). Statistical significance was present in CpG methylated loci between groups (all $P<0.05$). Conclusions: The expression of Sox2 gene increased first and then decreased in the process of inducing rat BMSCs into stem cells, and the methylation level of CpG loci in the promoter region changed dynamically, with an increased overall methylation level. After 5-aza treatment, the Sox2 promoter was in a non-methylated state, and its mRNA expression increased, which hindered the cell differentiation.

Keywords: Bone marrow mesenchymal stem cells, induced differentiation, Sox2, promoter methylation, bisulfite sequencing PCR

Introduction

Bone marrow mesenchymal stem cells (BMSCs), due to their high acquisition rate and good survival rate, have always been the focus of stem cell research and application [1]. A number of studies have proved that the process of stem cell differentiation involves the expression regulation of a large number of genes, and the change of promoter methylation status of various genes is an important direction of epigenetic regulation [2, 3]. The application of stem cells is the hope for the treatment of many diseases, but more experimental and clinical evidence is needed for their clinical application. Self-renewal and differentiation of stem cells have been the focus of academic research for many years [4]. The coordination of epigenetics and metabolism is known to be crucial for the correct differentiation of stem cells [5, 6].

Differentiation of stem cells is accompanied by precise dynamic changes in DNA and histone epigenetics [7]. Epigenetic networks play an important role in stem cell totipotency and somatic cell reprogramming, but the mechanisms of these networks are only partially understood [8]. Methylation and demethylation of gene promoters regulate gene expression [9]. Our research team has observed the promoter methylation and gene expression changes in pluripotent genes such as Oct4, Nanog, and Klf4 during the induced differentiation of BMSCs into HCs *in vitro*. Oct4, Klf4, C-myc, and Sox2 are the initial factors for inducing pluripotent stem cells [5, 6]. Among them, Oct4, Sox2, Nanog, Klf4, c-Myc, and Rex1 are well-known pluripotent factors, but their expression regulation needs to be further explored [10, 11]. MASUI et al. believed that the main role of Sox2 is to maintain the expression of Oct4, thus maintaining the pluripotency of embryonic stem cells. It has also been reported that the differentiation potential of mouse stem cells was not significantly affected when Sox2 was knocked out in mouse stem cells, but Oct4 expression was maintained [12-15]. Recently,

the regulation of Sox2 expression has been found to be highly sensitive, with small changes triggering the differentiation of embryonic cells into multiple cell types [16]. Sox2 expression is low in adult tissues and organs except brain tissue, and its abnormal expression is associated with many tumors such as lung [17], liver [18], gastric [19], colorectal [20], and breast cancers [21].

The purpose of this study is to understand the dynamic changes of methylated loci and gene expression of the Sox2 promoter at the single-molecule level during *in vitro* induction of BMSCs into hepatocytes (HCs).

Materials and methods

Experimental cells

Rat Thy-1^{+}Lin^{-} BMSCs preserved in liquid nitrogen were resuscitated and routinely cultured in a basic culture medium (DMEM-F12 medium + 10% fetal bovine serum [FBS] + penicillin-streptomycin) in a 37°C and 5% CO_2 incubator, with fluid change once every 3 days. When the cells were 90% confluent, they were digested with 0.25 trypsin-EDTA and inoculated in a T25 culture flask at 1:3. This study was approved by the ethical committee of Shanxi Cancer Hospital.

Selection of drug concentration

Cells grown to 70-80% confluent were seeded into the wells of a 96-well plate at 5.0×10^3 cells/well for culture. When 60-70% confluence was observed, they were cultivated in a basic culture medium containing 5-azacytidine (5-aza; Macklin Biochemical Co., Ltd.) with a concentration of 0, 2.5, 5, 10, 20, 30, 40, 60, 80, 100, and 120 μm, respectively. The cell activity and proliferation capacity were measured by Methyl Thiazolyl Tetrazolium (MTT) assay after 24 h with 6 duplicate wells per concentration.

Induced differentiation

Experimental group: The culture medium was renewed when the cells were fused to 70%, and 20 μm 5-aza was added. The culture medium was discarded 24 h later, after which the cells were treated with two PBS rinses and subsequently cultured in an induction medium (DMEM-F12 medium + 10% FBS + penicillin-streptomycin + 0.025 μg/L recombinant human hepatocyte growth factor (HGF) + 0.1 nmol/L dexamethasone), with the medium changed once every 3 days for 14 days. Control group: Normal cultured cells without treatment.

Identification of differentiated HC-like cells

Cell morphology was observed under the inverted microscope at 0, 7, and 14 days after induction. Total RNA was extracted from cells using TRIzol reagent (Takara) according to the manufacturer's instruction. cDNA was synthesized by reverse transcription using HiScript®Q Select RT SuperMix for qPCR (Vazyme, Nanjing, China). The SOX2 and ALB mRNA expression levels were detected using AceQ®Universal SYBR®qPCR Master Mix (Vazyme) on a LightCycler®480 (Roche, Basel, Switzerland). SGAPDH was used as an internal reference. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were: SOX2, forward: 5'-ATGATGGAGACGGAGCTGAA-3', reverse: 5'-CATCTTGGGGTTCTCCTGG-3'; 195 bp; ALB, forward: 5'-TGCCGTGAAAGAGAAAGCAC-3', reverse: 5'-GTCATCCGCGCATTCCAACA-3'; GAPDH, forward: 5'-AAGGTCGGAGTCAACGGATTTG-3', reverse: 5'-CCATGGGTGGAATCATATTGGAA-3'. The primers were synthesized by Shanghai Sangon Biotech.

Determination of methylation level in the promoter region of the SOX2 gene by BSP

(1) The rat Sox2 gene sequence was queried in Genbank, and the 2000 bp sequence upstream of the exon start site was selected. Four CpG islands were predicted by Methprimer software, and the optimal sequencing product combined with BSP results was about 300 bp, which contained many CpG loci. The target sequence was finally determined as the third CpG island (738-997 bp), and BSP-PCR primers were designed based on upstream and downstream sequences.

The below are target sequences: CCTGGGCGGGCAAGATTCTTGAGCCCTACCTCCGCCCCCAGCCCATTCTCCCACAGCCTGGGCTTGCTCGGTCCCGGCTCCTCGGGTTCGGCCGGCCTTCCCTCTGCGCACGCCGCCTGCACCTGCACCTCTGGACCCCGCGCGTTTCCCAGGCCCCGGCGCCTCTCGGCCGGGCCTTCGCGACTACCACTGTGCCAATCAG-

CAGGGGCAATGCGGGCTGCGCTGCCCCATCCTGGGAACGCTGCGTGGGAGGGAGTTTGTGAGCGCAGCCTAAATGCCACCTCCGTGCCTCGCGGTGGGATGCCAGGAAGTTGCGTG. The two framed sequences indicate the primer positions, and the middle part is the sequence to be tested. The fragment contained 26 CpG loci, which are located in the sequence as follows: 34, 70, 76, 84, 90, 94, 108, 112, 115, 140, 142, 144, 158, 161, 168, 172, 180, 182, 215, 222, 241, 246, 266, 286, 293, and 295. Shanghai Sangon Biotech was commissioned to synthesize methylation-specific primers: F: 5' TTTGGGYGGGTAAGATTTTTGAG; R: 5' CACRCAACTTCCTAACATCCCAC; 318 bp.

(2) Determination of methylation level of each locus in the promoter region of the SOX2 gene by BSP: Genomic DNA was extracted by column centrifugation and incubated with 2 mg/ml Proteinase K in a water bath at 50°C for 3 hours, followed by a bisulfite denaturation reaction (A 140-μL mixture, comprising 13 μL of DNA, 7 μL of hydroquinone, and 120 μL of denaturation buffer, was incubated in the dark in a PCR tube in the dark, with the incubation process as follows: 95°C for 30 s; 58°C for 20 min; three cycles of 95°C for 10 s and 58°C for 20 min; storage at 4°C). The denatured DNA was desulphurized, and the DNA was extracted by column centrifugation. The concentration of single-stranded DNA (ssDNA) was calculated by spectrophotometer. After that, methylation-specific PCR reaction was performed (50 μL system: 0.25 μL of 5 U/μL TaKaRa EpiTaq HS, 5 μL of 10× EpiTaq PCR Buffer, 5 μL of 25 mM $MgCl_2$, 6 μL each of 2.5 mM dNTP Mixture, Template <100 ng, 2 μL each of 10 μM F and R primers, and sterilized distilled water to a final volume of 50 μL. PCR reaction conditions: 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s, for 40 cycles in total). The PCR reaction products were collected for 3% gel electrophoresis, observed, isolated and purified, and then cloned and sequenced. The sequences were analyzed by QUMA Quantification tool for methylation analysis.

Statistical methods

SPSS 20.0 statistical software was used for data analysis. Measurement data were presented as the mean ± standard deviation (SD). Statistical significance between groups was analyzed by using repeated measures one-way analysis of variance (ANOVA) with Dunnett multiple comparisons test for post-hoc comparison. Counting data such as methylation were expressed as percentage, tested using the Fisher's exact test to identify the differences at each point and the Wilcoxon-Mann-Whitney rank sum test for overall differences between groups. The significance level of $P<0.05$ was adopted.

Results

Morphology and growth of Thy-1$^+$Lin$^-$ BMSCs

As shown in **Figure 1**, the cells in the control group grew stably and continuously for more than 20 generations after resuscitation, and showed typical long shuttle-shaped growth, in which a few cells were polygonal with long protrusions, and the cell colonies were arranged in a vortex. After 7 days of induction, the cell morphology was shuttle or polygonal, and the vortex of cell colonies gradually disappeared. After 14 days of induction, the cells were predominantly polygonal. In the experimental group, after 24 h of 20 μm 5-aza treatment, a few cells died and most of them grew adherently. In addition, the cells lost their typical spindle shape and presented mainly in long columns or rods, with chromatin deposition in the nucleus and relative growth inhibition (**Figure 2**). Cells resumed growth 7 days after induction, and cells showed complete polygonal growth 14 days after induction.

Proliferation of rat Thy-1$^+$Lin$^-$ BMSCs cultured with different concentrations of 5-aza determined by Methyl Thiazolyl Tetrazolium (MTT) assay

The optical density (OD) reading (590 nm) 24 h after treatment showed that the cell proliferation activity decreased statistically when the concentration of 5-aza was ≥ 30 μm, as shown in **Figure 3**.

Comparison of relative expression levels of target genes in BMSCs between the experimental and control groups on day 0, day 7, and day 14 of induction

As shown in **Table 1**, the relative mRNA expression levels of ALB on day 0, 7, and 14 were 1.00±0.13, 4.49±0.02 and 28.77±0.09 in the

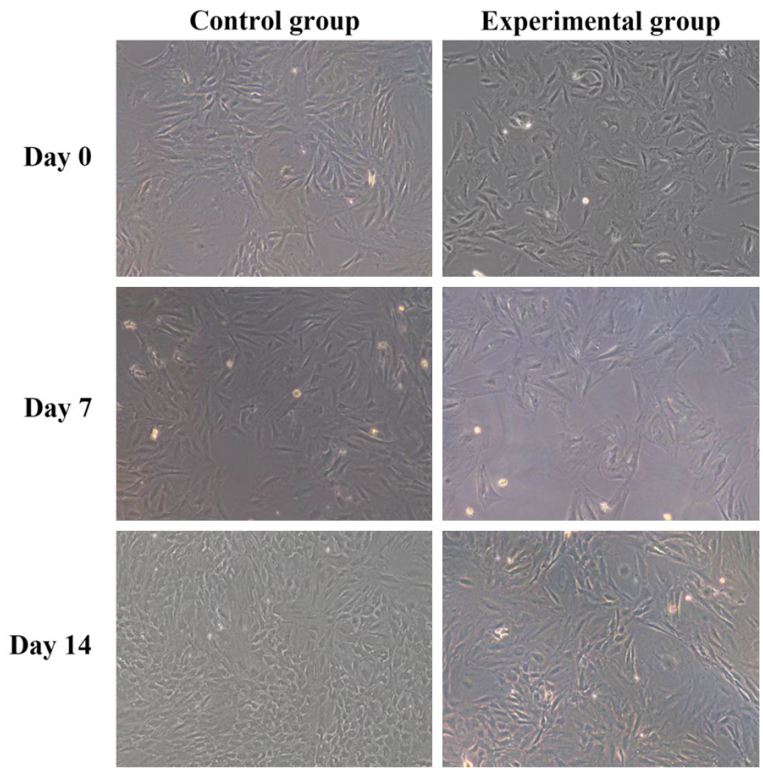

Figure 1. Rat Thy-1^{+}Lin^{-} BMSCs (×100) at 0, 7, and 14 days after induction in the experimental group and control group. BMSCs, bone marrow mesenchymal stem cells.

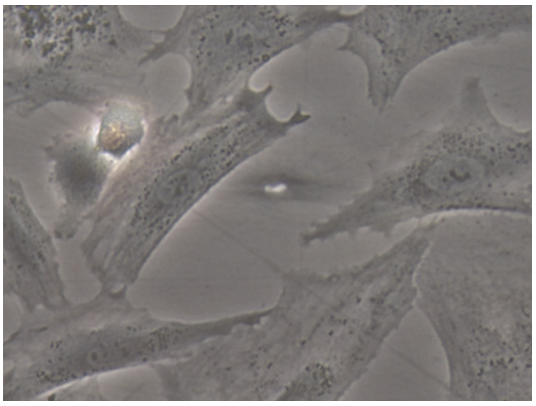

Figure 2. Rat Thy-1^{+}Lin^{-} BMSCs after 20 μm 5-aza treatment for 24 h (×200). BMSCs, bone marrow mesenchymal stem cells.

control group, and 3.00±0.22, 17.84±1.21 and 0.50±0.08 in the experimental group, respectively. Significant differences were found in the intra-group comparisons at different time points and the inter-group comparisons at the same time point (all P<0.05). The relative Sox2 mRNA expression levels in the control group were 1.00±0.000, 1.29±0.000 and 0.97±0.001 at day 0, 7, and 14 after induction, respectively, with statistical significance between day 0 and day 7 and between day 0 and day 14 (all P<0.05). In the experimental group, the relative Sox2 mRNA levels were 1.85±0.000, 0.90±0.000, and 1.25±0.000, respectively. Statistical significance was also identified between the two groups in Sox2 mRNA at day 7 and 14 after induction (P<0.05).

Methylation levels of various loci of the promoter region in the control and experimental groups on day 0 of induction

Cells in both the experimental and control group were hypomethylated, while a higher methylation level was found at the 17th locus in the control group, which was statistically significant compared with the experimental group (**Table 2** and **Figure 4**).

Methylation levels of various loci of the promoter region in the control and experimental groups at 7 days after induction

The overall methylation level was statistically lower in the control group than in the experimental group. The experimental group exhibited significantly higher methylation levels at the 10th, 14th, 16th, and 25th loci than the control group. While the methylation level was higher in the control group than in the experimental group at the 23rd locus, and the difference was statistically significant (**Table 3** and **Figure 5**).

Methylation levels of various loci of the promoter region in the control and experimental groups at 14 days after induction

No significant difference was identified between groups in the methylation level on the 14th day of induction; while the control group had a higher methylation level at the 15th locus than the experimental group, with statistical significance (**Table 4** and **Figure 6**).

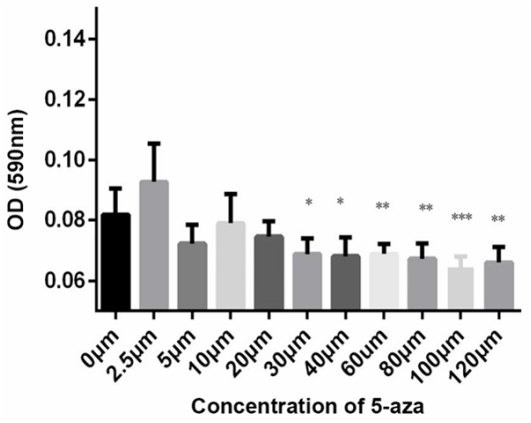

Figure 3. Proliferation of Thy-1^{+}Lin^{-} BMSCs cultured with 5-aza in 96-well plates. *, **, and *** indicate P<0.05, P<0.01, and P<0.001 compared with 0 μm 5-aza treatment, respectively. BMSCs, bone marrow mesenchymal stem cells.

Table 1. Relative mRNA expression of the gene

		ALB	Sox2
0 d	Control	1.00±0.13	1.00±0.00
	Experimental	3.00±0.22*	1.85±0.00*
7 d	Control	4.49±0.02$^{\#}$	1.29±0.00$^{\#}$
	Experimental	17.84±1.21$^{*,\#}$	0.90±0.00$^{*,\#}$
14 d	Control	28.77±0.09$^{\#,\&}$	0.97±0.001$^{\#,\&}$
	Experimental	0.50±0.08$^{*,\#,\&}$	1.25±0.00$^{*,\#,\&}$

Note: *P<0.05 vs Control group; $^{\#}$P<0.05 vs 0 d in the same group; $^{\&}$P<0.05 vs 7 d in the same group; ALB: Albumin; Sox2: SRY-box transcription factor 2.

Discussion

DNA methylation is a major epigenetic modification that regulates cell maintenance, differentiation, and development through the modulation of gene expression [22]. DNA methylation patterns are usually established and maintained by DNA methyltransferases [23]. Abnormal cell proliferation caused by aberrant methylation level of the Sox2 gene promoter can be seen in tumorigenesis, and regulating SOX2 expression through its methylation level has become one of the new targets for tumor therapy. This process occurs primarily at the cytosine-phosphate-guanine (CpG) site, where one methyl group is covalently bound to cytosine [24]. Most gene promoters are located at the CpG site, and DNA methylation of CpG-enriched promoters leads to transcriptional silencing of pluripotent genes by altering the binding affinity of proteins or transcription factors to DNA [25]. The BSP method has long been the gold standard for DNA methylation level determination [26]. After the genomic DNA is extracted and treated with bisulfite, the unmethylated cytosine is converted to uracil, while the methylated cytosine is not. All uracil is converted to thymine during gene fragment cloning, allowing final sequencing to determine the CpG island methylation frequency.

In this study, the changes of promoter methylation level of the Sox2 gene during the induced differentiation of rat Thy-1^{+}Lin^{-} BMSCs into HCs was determined by the BSP method. According to the experimental results, the Sox2 gene expression in the control group increased first and then decreased during the induction, and the Sox2 promoter methylation level gradually increased, accompanied by the gradual increase of CpG methylated loci. Studies have shown that SOX2 is a transcription factor that promotes stem cell proliferation but hinders cell type differentiation. It is expressed even after stem cell differentiation [27-30]. The Sox2 promoter of Thy-1^{+}Lin^{-} BMSCs treated with 20 μm 5-aza (a DNA methyltransferase inhibitor) was unmethylated, and the expression of Sox2 increased obviously. On the 7th day of induction, the methylation level of the Sox2 promoter of the experimental group was higher than that on the day 0, with increased methylated loci, together with lower Sox2 and higher ALB levels than the control group. On induction day 14, the overall methylation level of the Sox2 promoter was not significantly different between the two groups; however, a difference in the methylated loci was determined, with higher Sox2 gene expression and lower ALB expression in the experimental group as compared to the control group. Interestingly, it has been shown that silenced DNA can be demethylated by epigenetic modification factors such as 5-azacytidine (5-AzaCR), a well-characterized inhibitor of DNA methyltransferases, cells pretreated with 5-AzaCR can remove these epigenetic marks as a whole [31, 32]. In this study, the methylation level of the Sox2 promoter was found to be inversely correlated with Sox2 gene expression, and that CpG methylated loci were more prominent than the overall methylation level for Sox2 expression. During the process of induced differentiation, the methylation level of the Sox2 promoter

Table 2. Methylation levels of CpG loci on day 0 of induction (%)

Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Control group	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	100	0	0	0	0	0	0	0	0	0
Experimental group	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

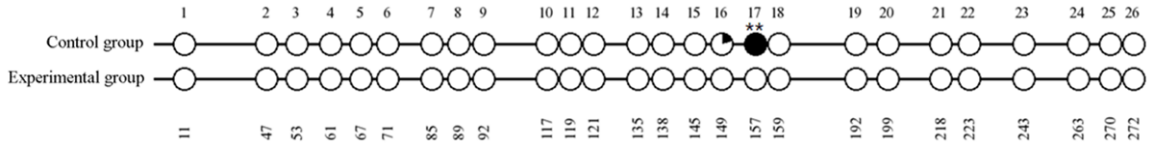

Figure 4. Circos plot of methylation levels at various CpG loci at day 0 of induction. ** indicate P<0.01 compared with experimental group. CpG, cytosine-phosphate-guanine.

Table 3. Methylation levels of CpG loci on the 7th day of induction (%)

Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Control group	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	20	0	0	0	0	0	100	40	0	40
Experimental group	0	0	0	0	0	0	0	0	20	80	0	0	0	80	80	80	80	0	20	0	0	20	0	0	0	0

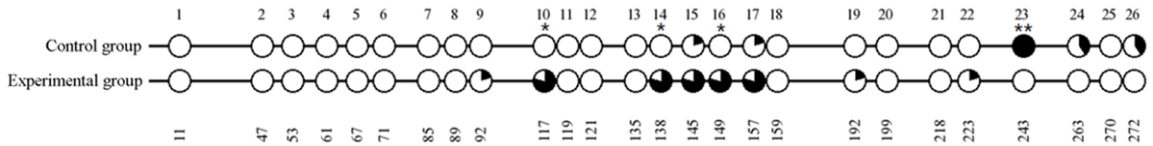

Figure 5. Circos plot of methylation levels at CpG loci on the 7th day of induction. *, ** indicate P<0.05 and P<0.01 compared with experimental group. CpG, cytosine-phosphate-guanine.

Table 4. Methylation levels at CpG loci on the 14th day of induction (%)

Locus	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Control group	0	0	0	0	20	20	20	0	0	0	20	20	0	0	80	0	20	0	20	0	60	20	0	0	0	20
Experimental group	0	20	20	0	20	40	0	0	0	0	0	0	0	0	0	0	0	0	0	0	40	80	40	40	0	20

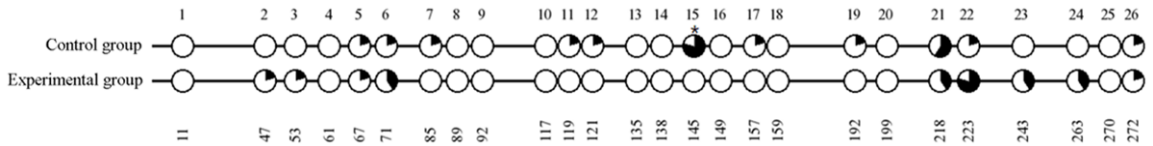

Figure 6. Circos plot of methylation levels at CpG loci on the 14th day of induction. * indicates P<0.05 compared with experimental group. CpG, cytosine-phosphate-guanine.

methylated loci changed dynamically. Methylation inhibitor 5-aza can reduce the methylation level of the Sox2 promoter and increase gene expression, interfering with the induced differentiation of Thy-1^{+}Lin^{-} BMSCs into HCs. Therefore, it is considered that Sox2 expression, promoter methylation locus changes, and methylation level alterations all play important roles in the induced differentiation of rat BMSCs into HCs. Same results were found in the study of Manzoni et al. that exposure to the epigenetic modifier 5-azacytidine caused the onset of pluripotency gene expression, such as OCT4, NANOG, REX1 and SOX2 [33].

However, this study still has some limitations. Although the use of BSP to determine methylation frequency at the nucleotide level is by far

the most intuitive method, the large DNA damage during sodium bisulfite treatment and the lack of measurement from the original DNA can lead to false negatives [34]. In recent years, single-molecule real-time sequencing (SMRT), also known as the third generation sequencing technology, can quickly and efficiently distinguish the dynamic changes of methyladenine, methylcystein and hydroxymethylcytosine in DNA molecules and template DNA damage [35]. It would be nice to be able to measure DNA methylation changes at the single-molecule level with high throughput, which is of great significance to reveal stem cell differentiation and provide therapeutic targets for tumors and other diseases more quickly. Moreover, despite the fact that we determined the dynamic changes in the expression of the Sox2 gene and the methylation level of the CpG site in the promoter region during the induction of rat bone marrow mesenchymal stem cells into HCs, as well as the inducing effect of 5-azacytidine, this only preliminarily establishes the possibility that the inhibition of the methylation of the Sox2 promoter by 5-azacytidine induces the entry of $Thy\text{-}1^{+}Lin^{-}$ cells into the hepatocytes, and more studies will be needed in the future to further validate the results.

In summary, the expression of Sox2 gene first increased and then decreased during the induction of rat bone marrow mesenchymal stem cells into HCs, and the methylation level of the CpG site in the promoter region changed dynamically, and the overall methylation level increased. In contrast, after 5-aza treatment, the Sox2 promoter was in a non-methylated state, and its mRNA expression increased, hindering cell differentiation.

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Disclosure of conflict of interest

None.

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References

[1] Dueñas F, Becerra V, Cortes Y, Vidal S, Sáenz L, Palomino J, De Los Reyes M and Peralta OA. Hepatogenic and neurogenic differentiation of bone marrow mesenchymal stem cells from abattoir-derived bovine fetuses. BMC Vet Res 2014; 10: 154.

[2] Atlasi Y and Stunnenberg HG. The interplay of epigenetic marks during stem cell differentiation and development. Nat Rev Genet 2017; 18: 643-658.

[3] Bhave VS, Paranjpe S, Bowen WC, Donthamsetty S, Bell AW, Khillan JS and Michalopoulos GK. Genes inducing iPS phenotype play a role in hepatocyte survival and proliferation in vitro and liver regeneration in vivo. Hepatology 2011; 54: 1360-1370.

[4] Zhang J, Ratanasirintrawoot S, Chandrasekaran S, Wu Z, Ficarro SB, Yu C, Ross CA, Cacchiarelli D, Xia Q, Seligson M, Shinoda G, Xie W, Cahan P, Wang L, Ng SC, Tintara S, Trapnell C, Onder T, Loh YH, Mikkelsen T, Sliz P, Teitell MA, Asara JM, Marto JA, Li H, Collins JJ and Daley GQ. LIN28 regulates stem cell metabolism and conversion to primed pluripotency. Cell Stem Cell 2016; 19: 66-80.

[5] Pan G and Thomson JA. Nanog and transcriptional networks in embryonic stem cell pluripotency. Cell Res 2007; 17: 42-49.

[6] Schmidt R and Plath K. The roles of the reprogramming factors Oct4, Sox2 and Klf4 in resetting the somatic cell epigenome during induced pluripotent stem cell generation. Genome Biol 2012; 13: 251.

[7] Agostinho de Sousa J, Wong CW, Dunkel I, Owens T, Voigt P, Hodgson A, Baker D, Schulz EG, Reik W, Smith A, Rostovskaya M and von Meyenn F. Epigenetic dynamics during capacitation of naïve human pluripotent stem cells. Sci Adv 2023; 9: eadg1936.

[8] Chen C, Gao Y, Liu W and Gao S. Epigenetic regulation of cell fate transition: learning from early embryo development and somatic cell reprogramming†. Biol Reprod 2022; 107: 183-195.

[9] Kouzmenko A, Ohtake F, Fujiki R and Kato S. Hormonal gene regulation through DNA methylation and demethylation. Epigenomics 2010; 2: 765-774.

[10] Qi H and Pei D. The magic of four: induction of pluripotent stem cells from somatic cells by Oct4, Sox2, Myc and Klf4. Cell Res 2007; 17: 578-580.

[11] Zhang C, Samanta D, Lu H, Bullen JW, Zhang H, Chen I, He X and Semenza GL. Hypoxia induces the breast cancer stem cell phenotype by HIF-dependent and ALKBH5-mediated

m6A-demethylation of NANOG mRNA. Proc Natl Acad Sci U S A 2016; 113: E2047-E2056.
[12] Masui S. Transcriptional network controlling pluripotency in embryonic stem cells. Seikagaku 2009; 81: 597-601.
[13] Miyagi S, Masui S, Niwa H, Saito T, Shimazaki T, Okano H, Nishimoto M, Muramatsu M, Iwama A and Okuda A. Consequence of the loss of Sox2 in the developing brain of the mouse. FEBS Lett 2008; 582: 2811-2815.
[14] Sharov AA, Masui S, Sharova LV, Piao Y, Aiba K, Matoba R, Xin L, Niwa H and Ko MS. Identification of Pou5f1, Sox2, and Nanog downstream target genes with statistical confidence by applying a novel algorithm to time course microarray and genome-wide chromatin immunoprecipitation data. BMC Genomics 2008; 9: 269.
[15] Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MS and Niwa H. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol 2007; 9: 625-635.
[16] Topalovic V, Krstic A, Schwirtlich M, Dolfini D, Mantovani R, Stevanovic M and Mojsin M. Epigenetic regulation of human SOX3 gene expression during early phases of neural differentiation of NT2/D1 cells. PLoS One 2017; 12: e0184099.
[17] Maehara R, Fujikura K, Takeuchi K, Akita M, Abe-Suzuki S, Karbanová J, Corbeil D, Itoh T, Kakeji Y and Zen Y. SOX2-silenced squamous cell carcinoma: a highly malignant form of esophageal cancer with SOX2 promoter hypermethylation. Mod Pathol 2018; 31: 83-92.
[18] Zhong F, Cheng X, Sun S and Zhou J. Transcriptional activation of PD-L1 by Sox2 contributes to the proliferation of hepatocellular carcinoma cells. Oncol Rep 2017; 37: 3061-3067.
[19] Niu H, Jia Y, Li T and Su B. SOX2 inhibition promotes promoter demethylation of CDX2 to facilitate gastric intestinal metaplasia. Dig Dis Sci 2017; 62: 124-132.
[20] Li J, Du L, Yang Y, Wang C, Liu H, Wang L, Zhang X, Li W, Zheng G and Dong Z. MiR-429 is an independent prognostic factor in colorectal cancer and exerts its anti-apoptotic function by targeting SOX2. Cancer Lett 2013; 329: 84-90.
[21] Sengupta S, Nagalingam A, Muniraj N, Bonner MY, Mistriotis P, Afthinos A, Kuppusamy P, Lanoue D, Cho S, Korangath P, Shriver M, Begum A, Merino VF, Huang CY, Arbiser JL, Matsui W, Győrffy B, Konstantopoulos K, Sukumar S, Marignani PA, Saxena NK and Sharma D. Activation of tumor suppressor LKB1 by honokiol abrogates cancer stem-like phenotype in breast cancer via inhibition of oncogenic Stat3. Oncogene 2017; 36: 5709-5721.
[22] Mattei AL, Bailly N and Meissner A. DNA methylation: a historical perspective. Trends Genet 2022; 38: 676-707.
[23] Loyfer N, Magenheim J, Peretz A, Cann G, Bredno J, Klochendler A, Fox-Fisher I, Shabi-Porat S, Hecht M, Pelet T, Moss J, Drawshy Z, Amini H, Moradi P, Nagaraju S, Bauman D, Shveiky D, Porat S, Dior U, Rivkin G, Or O, Hirshoren N, Carmon E, Pikarsky A, Khalaileh A, Zamir G, Grinbaum R, Abu Gazala M, Mizrahi I, Shussman N, Korach A, Wald O, Izhar U, Erez E, Yutkin V, Samet Y, Rotnemer Golinkin D, Spalding KL, Druid H, Arner P, Shapiro AMJ, Grompe M, Aravanis A, Venn O, Jamshidi A, Shemer R, Dor Y, Glaser B and Kaplan T. A DNA methylation atlas of normal human cell types. Nature 2023; 613: 355-364.
[24] Kaur G, Rathod SSS, Ghoneim MM, Alshehri S, Ahmad J, Mishra A and Alhakamy NA. DNA methylation: a promising approach in management of Alzheimer's disease and other neurodegenerative disorders. Biology (Basel) 2022; 11: 90.
[25] Jones PA and Liang G. Rethinking how DNA methylation patterns are maintained. Nat Rev Genet 2009; 10: 805-811.
[26] Šestáková Š, Šálek C and Remešová H. DNA methylation validation methods: a coherent review with practical comparison. Biol Proced Online 2019; 21: 19.
[27] Berezovsky AD, Poisson LM, Cherba D, Webb CP, Transou AD, Lemke NW, Hong X, Hasselbach LA, Irtenkauf SM, Mikkelsen T and deCarvalho AC. Sox2 promotes malignancy in glioblastoma by regulating plasticity and astrocytic differentiation. Neoplasia 2014; 16: 193-206, 206.e119-125.
[28] Lathia JD, Mack SC, Mulkearns-Hubert EE, Valentim CL and Rich JN. Cancer stem cells in glioblastoma. Genes Dev 2015; 29: 1203-1217.
[29] Bradshaw A, Wickremsekera A, Tan ST, Peng L, Davis PF and Itinteang T. Cancer stem cell hierarchy in glioblastoma multiforme. Front Surg 2016; 3: 21.
[30] Ellis P, Fagan BM, Magness ST, Hutton S, Taranova O, Hayashi S, McMahon A, Rao M and Pevny L. SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. Dev Neurosci 2004; 26: 148-165.
[31] Kim M and Costello J. DNA methylation: an epigenetic mark of cellular memory. Exp Mol Med 2017; 49: e322.
[32] Tobiasson M, Abdulkadir H, Lennartsson A, Katayama S, Marabita F, De Paepe A, Karimi M, Krjutskov K, Einarsdottir E, Grövdal M, Jansson

M, Ben Azenkoud A, Corddedu L, Lehmann S, Ekwall K, Kere J, Hellström-Lindberg E and Ungerstedt J. Comprehensive mapping of the effects of azacitidine on DNA methylation, repressive/permissive histone marks and gene expression in primary cells from patients with MDS and MDS-related disease. Oncotarget 2017; 8: 28812-28825.

[33] Manzoni EF, Pennarossa G, deEguileor M, Tettamanti G, Gandolfi F and Brevini TA. 5-azacytidine affects TET2 and histone transcription and reshapes morphology of human skin fibroblasts. Sci Rep 2016; 6: 37017.

[34] Varley KE and Mitra RD. Bisulfite Patch PCR enables multiplexed sequencing of promoter methylation across cancer samples. Genome Res 2010; 20: 1279-1287.

[35] Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J and Turner SW. Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods 2010; 7: 461-465.