Original Article
Changes in OCT4 promoter methylation during rat bone marrow mesenchymal stem cell differentiation into hepatocytes

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Abstract: In this study, we explored the specific mechanism associated with OCT4 gene promoter methylation variation in differentiation induction of rat bone marrow-derived liver stem cells (RBMLSCs) into hepatocytes in vitro. RBMLSCs were induced by 25 ng/mL recombinant human hepatocyte growth factor and 0.1 nmol/L dexamethasone. DNA and RNA were extracted from cells at different time points (day 0, 7 and 14) after induction. Real-time quantitative PCR (RT-qPCR) was performed to detect OCT4 and albumin (Alb) mRNA expression. Pyrosequencing was performed to detect methylation variation of 4 CpG sites in OCT4 promoter. During cell differentiation, Alb mRNA expression was increased significantly (P < 0.05), whereas OCT4 mRNA was decreased significantly (P < 0.005) at day 7 and day 14. Methylation frequency of the first 3 CpG sites in OCT4 promoter upstream was increased significantly (P < 0.001); however, no significant methylation changes were observed at 4th CpG site. The results showed that the hypermethylation of OCT4 promoter could result in the reduction of mRNA expression and disappearance of RBMLSCs pluripotency; the 4th CpG site, however, might not be involved in the regulating process.

Keywords: Rat bone marrow-derived liver stem cells (RBMLSCs), OCT4, promoter methylation, pyrosequencing

Introduction
End-stage liver disease in acute or chronic liver failure, cirrhosis or liver cancer is associated with poor prognosis. Currently, in the absence of effective surgical or medical treatment, orthotopic liver transplantation is the only effective and available treatment, but is greatly limited clinically [1]. Recent advancements in stem cell offer the exciting possibility of pluripotent differentiation into hepatocytes and other cell types [2]. A variety of mature protocols inducing rat bone marrow-derived liver stem cells (RBMLSCs) into hepatocytes in vitro have provided a theoretical basis for autologous transplantation [3, 4]. RBMLSCs-a type of adult stem cells-are derived from a variety of sources such as embryonic stem cells, which possess a high ratio of differentiation into hepatocytes, thereby serving as a bridge for liver transplantation [5]. However, the disadvantages of RBMLSCs include tumorigenicity and induction of carcinogenesis. Rubio [6] firstly reported that RBMLSCs showed a malignant potential after several generations. Li [7] reported that RBMLSCs promoted tumor growth in vivo.

Mammalian growth and development are controlled by multiple genes. Pluripotency gene, OCT4, which is expressed mainly in embryonic and germinal stem cells, is a member of POU (Pit-Oct-Unc) transcription factor family and plays an important role in differentiation [8]. However, OCT4 expression has been reported in a variety of tumors, such as bladder and breast cancers [9, 10]. Epigenetic studies have shown that gene expression level in cells and tissues is correlated with the level of promoter methylation [11]. Currently, most researches on gene methylation focus on tumorigenesis; however, the relationship between OCT4 expression level of adult stem cells and its promoter methylation status is still unknown.

Available techniques to identify methylation include methylation-specific PCR (MSP), DNA
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Figure 1. Nucleotide sequence of OCT4 promoter. 5'UTR means 5' untranslated region; ATG means transcription start site; red boxes present CpG sites; three sections of sequencing cover all CpG sites in promoter.

Table 1. Characteristics of pyrosequencing

<table>
<thead>
<tr>
<th>Sections</th>
<th>Primers</th>
<th>Biotin-labeled locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>01-F</td>
<td>5'TGGAGAAATGTGAAAGATAGGT3'</td>
<td>5'Biotin</td>
</tr>
<tr>
<td>01-R</td>
<td>5'CCAACCATCTCACTTAACCTA3'</td>
<td>5'Biotin</td>
</tr>
<tr>
<td>01-S</td>
<td>5'GAAGGGTATTTTGGTT3'</td>
<td>5'Biotin</td>
</tr>
<tr>
<td>02-F</td>
<td>5'GAGGAATGTGGAGAAGATAGGT3'</td>
<td>5'Biotin</td>
</tr>
<tr>
<td>02-R</td>
<td>5'CCAACCATCTCACTTAACCTA3'</td>
<td>5'Biotin</td>
</tr>
<tr>
<td>02-S</td>
<td>5'AGGTATTTTATTTAGTAAATT3'</td>
<td>5'Biotin</td>
</tr>
<tr>
<td>03-F</td>
<td>5'TGGAGAAATGTGAAAGATAGGT3'</td>
<td>5'Biotin</td>
</tr>
<tr>
<td>03-R</td>
<td>5'CCAACCATCTCACTTAACCTA3'</td>
<td>5'Biotin</td>
</tr>
<tr>
<td>03-S</td>
<td>5'CTTAACCTCTCAACCC3'</td>
<td>5'Biotin</td>
</tr>
</tbody>
</table>

direct sequencing (Sanger method), methylation-specific melting curve analysis (MS-MCA) and high-resolution melt (HRM). These methods can only be used for qualitative detection of gene methylation, not for quantitative determination of methylation frequency in certain CpG sites. They also require abundant DNA samples, and operate inconveniently. Pyrosequencing offers accurate, quantitative analysis and sensitive detection of CpG methylation, and thus is the gold standard of methylation detection. Pyrosequencing is a next-generation sequencing method, which is based on Frommer's theory [12]. Because of the principle of unmethylated cytosine conversion to uracil by sodium bisulfite in denatured DNA, methylation frequency could be calculated according to the ratio of the level of cytosine methylated to the total levels of methylated cytosine and thymine [13].

Pyrosequencing had been used to detect methylation frequency of all CpG sites in OCT4 promoter. In this study, we explored the methylation variation of OCT4 promoter in the process of RBMLSCs differentiation to further reveal epigenetic regulation mechanism of bone marrow mesenchymal stem cells and strategies for stem cell transplantation.

Materials and methods

Cell culture

We cultured the CD90+Lin−RBMLSCs, which were the primary cells isolated from rat bone marrow and conserved by our research group, in basal medium containing high sugar DMEM F12 medium supplemented with 10% premium Australian fetal bovine serum (Biolind, Israel), 100 U/mL penicillin G & 100 μg/mL streptomycin solution (Gibco). After recovering, the cells were incubated at 37°C in the presence of 5% CO₂. The medium was replaced every three days, and the cells were subcultured using 0.25% trypsin-EDTA (Gibco) digestion after reaching 80% confluence.

Third generation of well growing cells were treated with hepatocyte induction medium containing 25 ng/mL hepatocyte growth factor (HGF) (Peprotech) and 10⁻⁷ mmol/L dexamethasone (Gibco). The induction protocol was referred to Li's [14] research. The morphology of the cells was observed in the four groups: non-differentiation group as control group, day 0, day 7 and day 14 groups as experimental group. At the same time, samples (at least containing 1×10⁶ cells) in each group were stored in liquid nitrogen container.

DNA isolated and bisulfite conversions

DNA was extracted from 25 cm² culture bottle (about 1×10⁶) in every group, using DNA extract kit (Promega) according to the manufacturer's instructions. DNA concentration and quality were detected by ND-1000 spectrophotometry, and all the samples with OD greater than 1.6 were preserved at -20°C until use. Bisulfite con-
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**Table 2. Characteristics of PCR about ABL AND OCT4**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Annealing temperature (°C)</th>
<th>GC content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB</td>
<td>F 5'TACACCGAAGACACCTCA3'</td>
<td>57.8</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>R 5'TACACCGAAGACACCTCA3'</td>
<td>59.8</td>
<td>55.0</td>
</tr>
<tr>
<td>OCT4</td>
<td>F 5'GGCACCTCGCTTAGACTT3'</td>
<td>59.8</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>R 5'TCCCTCCACAGAATCGTAG3'</td>
<td>60.0</td>
<td>52.4</td>
</tr>
<tr>
<td>GADPH</td>
<td>F 5'ACCAACACGGCTGACATC3'</td>
<td>59.8</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>R 5'TCCACCACCGTGGTTGTA3'</td>
<td>63.7</td>
<td>56.5</td>
</tr>
</tbody>
</table>

**Figure 2. Morphology of RBMLSCs induced into hepatocytes.**

A: RBMLSCs of non-differentiation cultured with base medium, 4×10 magnification; B: RBMLSCs cultured with induction medium, inducing to day 0, 4×10 magnification; C: RBMLSCs induced and cultured to day 7, 10×10 magnification; D: RBMLSCs induced and cultured to day 14, 10×10 magnification.

**RNA isolation and RT-qPCR analysis**

RNA was extracted from 25 cm² culture bottle (about 1×10⁶) in every group using RNA extraction kit (Omega) according to manufacturer’s instruction. RNA concentration and quality were determined using ND-1000 spectrophotometry, and all the samples’ OD was greater than 1.9. RNA analysis was performed with cDNA first strand synthesis kit (Roche) using ABI 9700 PCR and preserved at -20°C until use. RT-PCR primers were designed by Shanghai Sangon company (Table 2). RT-PCR of ALB and OCT4 mRNA was performed using SYBR I (Roche) and each sample were analyzed in triplicates. Relative quantification was used to calculate PCR results. The \( 2^{-\Delta\Delta Ct} \) method was...
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Results

RBMLSCs cultivation and differentiation

RBMMSCs after recovering were spread equally, transparent, uniform and oval in basal medium. After 24 h, most of the spindle-shaped RBMMSCs were clustered, and colonies showed circinate pattern (Figure 2A). After altering induction medium, RBMMSCs did not exhibit remarkable change at day 0 (Figure 2B). On day 7, the cells' differentiated into a spindle or polygon shape without any swirling (Figure 2C). The number of cells presenting more circular and organelles increased with prolonged culture on day 14 (Figure 2D).

OCT4 and ALB mRNA expression during RBMMSCs differentiation

After altering induction medium, no change was observed in ALB and OCT4 mRNA expression at day 0 as compared to non-differentiation group. ALB mRNA expression at day 7 and 14 was 5.22 times ($P = 0.025$) and 14.7 times ($P = 0.003$) higher as compared to the non-differentiation group. However, OCT4 mRNA expression was significantly decreased to 0.23 times ($P = 0.000$) and 0.055 times ($P = 0.000$) respectively (Figure 3).

Analysis of OCT4 promoter methylation frequency

OCT4 promoter was subjected to methylation bisulfite sequencing during Thy-1$^+$Lin RBMMSCs induction into mature hepatocytes. In these bisulfite sequencing maps, sequencing peaks were distinct, with limited background interference and sequence results were identical with that of GeneBank data. Former three CpG sites of upstream presented different methylation frequency in the predicted four CpG sites (Figure 4, Supplemental Figure 1). We set a limit value of 20%, considering CpG methylation frequency above this limit as hypermethylation and below 20% as hypomethylation. The methylation frequency at CpG sites 1-3 was increased constantly during cell differentiation. Methylation frequency at day 7 group was significantly higher ($P = 0.000$) than day 0 group and non-differentiated groups; methylation frequency in day 14 group was significantly higher ($P = 0.000$) than day 0, day 7 and non-

Data analysis

Statistical analysis software SPSS 19.0 was used to calculate experimental results. Gene expression and methylation frequency were calculated by one-way analysis of variance (ANOVA), homogeneity of variance using LSD testing and heterogeneity of variance using Dunnett T3 testing.

Figure 3. ALB and OCT4 mRNA expression. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$ versus 0 day.

Figure 4. OCT4 promoter methylation frequency. *$P < 0.001$ versus non-differentiation and 0 d groups, **$P < 0.001$ versus non-differentiation, 0 d and 7 d groups.
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Table 3. Methylation frequency of OCT4 gene promoter CPG sites (%)

<table>
<thead>
<tr>
<th>CpG sites</th>
<th>1 p value</th>
<th>2 p value</th>
<th>3 p value</th>
<th>4 p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-differentiation</td>
<td>14.33 ± 1.528</td>
<td>13.33 ± 2.517</td>
<td>16.67 ± 0.577</td>
<td>19.33 ± 1.528</td>
</tr>
<tr>
<td>Day 0 group</td>
<td>18.00 ± 3.000</td>
<td>0.132</td>
<td>14.00 ± 1.000</td>
<td>0.691</td>
</tr>
<tr>
<td>Day 7 group</td>
<td>47.33 ± 2.082</td>
<td>0.000</td>
<td>46.33 ± 0.577</td>
<td>0.000</td>
</tr>
<tr>
<td>Day 14 group</td>
<td>72.67 ± 1.528</td>
<td>0.000</td>
<td>85.67 ± 1.528</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Note: CpG sites methylation frequency was presented mean ± s. a, P < 0.001 versus 0 d groups; b, P < 0.001 versus 7 d groups.

OCT4 is the most important gene underlying embryonic stem cell pluripotency, proliferation and differentiation [21]. OCT4 along with various target genes collectively mediate the regulation of LIF, Wnt, Tgf-β and others in conjunction with SOX-2 and NANOG to maintain pluripotency and self-renewal of stem cells [22]. OCT4, SOX-2 and NANOG may regulate JAK-STAT3 pathway via STAT3 regulation, cell proliferation and differentiation. In addition, they also regulate the PRC2 target gene to control cell differentiation [23]. Rodda’s research [24] suggested that OCT4 gene was the most critical regulatory gene underlying cellular pluripotency, acting in concert with NANOG and various target genes downstream. OCT4 deficiency in embryonic stem cells failed to sustain its undifferentiated status even after NANOG expression [25]. OCT4 also maintains pluripotency of adult stem cells, for example, human epithelial and melanin stem cells that express OCT4 play a role in self-renewal and differentiation of stem cells [26]. However, rat adult stem cells without OCT4 gene still maintained its pluripotency [27].

OCT4 expression level depends on promoter methylation in embryonic stem cells [28]. Ju W [29] also reported that OCT4 expression was related to its promoter methylation in RBMLSCs differentiation. Based on the foregoing studies, we performed pyrosequencing of OCT4 promoter. However, this method was applicable only to shorter sequences and therefore, the CpG sites was divided into three fragments to ensure successful sequencing. The result showed that CpG site methylation frequency increased on days 7 and 14, without altering the GC box status.

In summary, this study has successfully established a method of culturing rat RBMLSCs in vitro and differentiation into mature hepatocytes. We observed that RBMLSCs induced on day 7 expressed ALB and possessed character-
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istics of hepatocytes. They further matured until day 14 of differentiation. Pyrosequencing was conducted for OCT4 promoter. CpG sites methylation frequency in OCT4 promoter was increased; however, its mRNA expression was decreased during RBMLSCs differentiation. Pluripotency of stem cells disappeared. This study had not only supplemented the epigenetic mechanism of RBMLSCs directional differentiation, but also provided further theoretical and experimental data underlying the role of OCT4 gene in the regulation of RBMLSCs differentiation.

Summary

We studied the epigenetic alteration of OCT4 promoter in RBMLSCs induced into hepatocytes. The OCT4 promoter hypermethylation was negatively correlated with mRNA expression. The extinction of RBMLSCs pluripotency was related to OCT4 promoter hypermethylation. However, GC box may not mediate the regulation of RBMLSCs differentiation. These results also emphasize the complexity of the regulation in RBMLSCs.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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Supplemental Figure 1. Pyrosequencing maps of OCT4 promoter methylation. A-C: Methylation of 4 CpG sites in non-differentiation group; D-F: Methylation of 4 CpG sites in 0 d group; G-I: Methylation of 4 CpG sites in 7 d group; J-L: Methylation of 4 CpG sites in 14 d group.