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Construction and Functional Analysis of Luciferase Reporter Plasmid Containing CD25 Gene Promoter

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Abstract: CD25 is the alpha chain of IL-2R and IL-2R is distributed on the surface of activated T cells, B cells, and NK cell. The transcriptional factor CD25 plays an important role in the proliferation and differentiation of Treg cells. MKL-1 is also called MAL (megakaryocytic acute leukemia) and belongs to the Myocardin family which also includes MKL-2 (megakaryoblastic leukemia 2) and Myocardin. Studies have shown that in the process of mouse megakaryocyte differentiation, the expression of MKL-1 is up-regulated, and MKL-1 promotes the physiological maturation of human and mouse megakaryocytes. However, the exact mechanism of the CD25 on megakaryocytes is unclear. In this study, human CD25 promoter luciferase reporter construct was generated by PCR amplification. Then the PCR fragment was digested and cloned into pGL3 vector, and this promoter sequence was verified by sequencing. The result showed that luciferase reporter with CD25 promoter was successfully constructed. The expression vectors of human MKL-1 were bought from addgene. Then the activation of the CD25 promoter was detected in 293T cells by luciferase reporter assay after transfected expression vectors of human MKL-1. The result showed that transfected the expression vectors can enhance the transcriptional activity of CD25. Our research will reveal the effect of CD25 on megakaryocytes and may provide a theoretical basis and therapeutic method for diseases caused by megakaryocytes.

Keywords: CD25, MKL-1, Megakaryocytes, Luciferase Activity Assay

1. Introduction

CD25, the alpha subunit of the IL2 receptor, is a canonical marker of regulatory T cells (Treg). Regulatory T cell (Treg) ($CD4^+CD25^+FOXP3^+$) defects are thought to play a role in the pathogenesis of a variety of autoimmune diseases which includes thrombocytopenia. [1] The thrombocytopenia characterized by increased platelet destruction and reduced platelet production, resulting in decreased platelet count. [2] Megakaryocytes are bone marrow cells derived from hematopoietic stem cells, and mature megakaryocyte edges break off and form platelets. [3]

MKL-1 (MRTF-A, Myocardinrelated transcriptional factor) is a recently discovered synergistic transcription factor of serum response factor (SRF). [4] It forms a complex with the SRF protein and regulates the transcription of target genes by binding to the specific sequence of CC $(AT)_6GG$ (CArGBox). [5] MKL1 can dimerize with other myocardin family members and is required for RhoA-mediated SRF induction of immediate early and muscle specific genes. [6] (Du KL, Chen M, Li J, Lepore JJ, Mericko P, Parmacek MS. Megakaryoblastic leukemia factor-1 transduces cytoskeletal signals and in-duces smooth muscle cell differentiation from un-differentiated embryonic stem cells. J Biol Chem. 2004; 279: 17578-17586.). MKL-1 was first cloned from blood megakaryocytes. [7] MKL1 inhibits tumor necrosis factor-induced cell death in embryonic fibroblasts, [8] which may be relevant to its role in leukemia. MKL1 is a critical

mediator of the transforming growth factor-β1–induced epithelial-mesenchymal transition via Smad3. [9] Recent studies have shown that over expression of MKL-1 in human erythroleukemia (HEL) cells and human CD34 cells increases the production of megakaryocytes, knocking down SRF, and this effect disappears. The number of megakaryocytes with CD41 knockdown of MKL-1 in mice increased, but the number of polyploid megakaryocyte maturation decreased. It means platelet decrease is due to blocked megakaryocytic differentiation and MKL-1 promotes physiological maturation of human and mouse megakaryocytes. [10]

In this study, luciferase reporter with CD25 promoter was successfully constructed. Then the activation of the CD25 promoter was detected in HEK293T cells by luciferase reporter assay after transfected the expression vector of human MKL-1. The result showed that transfected the expression vectors can enhance the transcriptional activity of CD25. This study demonstrates that MKL-1 promotes the promoter activity of CD25 in vitro, which may regulate the maturation and differentiation of megakaryocytes, and provide the latest theoretical basis for the prevention and treatment of megakaryocyte leukemia.

2. Materials and Methods

2.1. Cell Culture

Human Embryonic Kidney 293T (HEK293T) cells line was obtained from American Type Culture Collection. HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator.

2.2. Plasmid Construction

The human genome was extracted from HEK293T cells. CD25 promoter fragment from -451 to +35 is amplified by PCR using the primers (F: 5'-GGGTACCAGTTCGCCGCATCCTTCTCCATTAT -3'; R: 5'- CCTCGAGTGTGGGTCCATCCAGTCTCTATCGG -3'). PCR condition is as follows: Pre-degeneration for 5 min at 94°C, denaturation for 1 minute at 94°C, annealing for 1 min at 56°C, and extension for 1.5 minute at 72°C. PCR reaction was carried out for 35 cycles and PCR products were visualized in 2% agarose gels stained with ethidium bromide under UV transillumination.

The PCR product and pGL3-Basic vehicle plasmid were digested with restriction enzyme KpnI and XhoI. pGL3-Basic vehicle plasmid were mixed with 2 μL T4 ligase buffer and 1 μL T4 DNA ligase and added water to 20μL, incubated at 16°C for 24h, and then transformed into competent E. coli. Monoclonal colony was well separated by incubating in 5 mL LB which contains ampicillin at 37°C overnight. A monoclonal colony was picked and cultured in LB. The plasmid was extracted with the small extraction of plasmid kit (Solarbio) according to the manufacturer's instructions and sequenced.

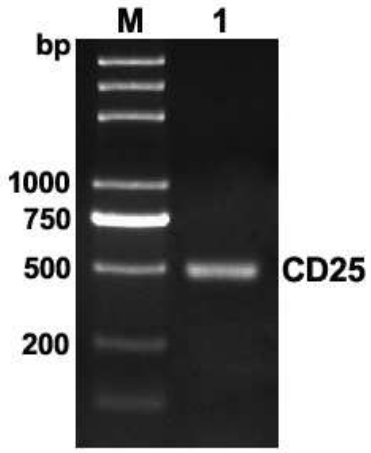
2.3. Luciferase Reporter Assays

After transfection 24 hours, luciferase activity was measured by on a Synergy™ 4 (Bioteck). Transfection efficiencies were normalized by total protein concentrations of each luciferase assay preparations. All experiments were performed at least three times with different preparations of plasmids and primary cells, producing qualitatively similar results. Data in each experiment are presented as the mean ± standard deviation of triplicates from a representative experiment.

3. Result

3.1. Construction of CD25 Promoter Luciferase Reporter Plasmid

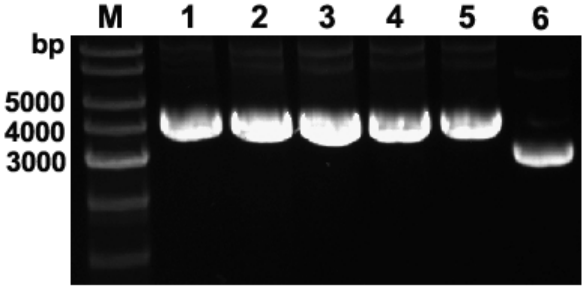
To estimate the PCR amplification of CD25 promoter, agarose gel electrophoresis was performed. As shown in Figure 1, a single band emerged at the site of 500bp, which represents the PCR products of CD25 promoter.



1: CD25 gene promoter.

Figure 1. *Agarose gel electrophoretic analysis of PCR product.*

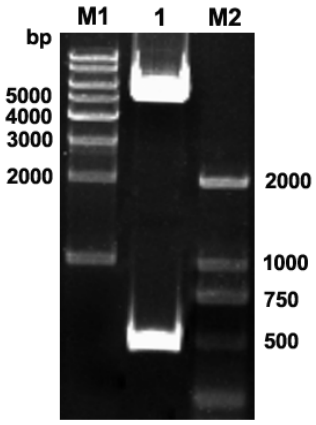
Then, the PCR product was digested by double restriction enzymes and cloned to the pGL3-Basic vector. After that, the recombinant plasmid was extracted and purified, and the agarose gel electrophoretic analysis was performed. Figure 2 showed the size of recombinant plasmid that was purified.



1-5: recombinant plasmid 6: pGL3-Basic vehicle plasmid;

Figure 2. *Agarose gel electrophoretic analysis of plasmids.*

To confirm the recombinant plasmids, we used double restriction enzymes to digest them and then electrophoresed through agarose gel. As shown in Figure 3, the recombinant plasmid was cut into two bands. One band was at about 500bp, which represents CD25 promoter, and another band was at 5,000 bp, which represents pGL3-Basic vehicle plasmid. The luciferase reporter plasmid of CD25 gene promoter was further confirmed by sequencing. The result of DNA sequence alignment showed that luciferase reporter plasmid containing CD25 promoter was constructed successfully.



1: recombinant plasmid.

Figure 3. *Agarose gel electrophoretic analysis of plasmids.*

3.2. Formula Fluorescence of the Efficiency of Transduction

The transfection efficiency was demonstrated using a EGFP (enhanced green fluoresent protein) expression plasmid. As shown in Figure 4, the transfection efficiency was approximately 60%, demonstrating that the MKL-1 plasmid was transfected efficiently into HEK293T cells.

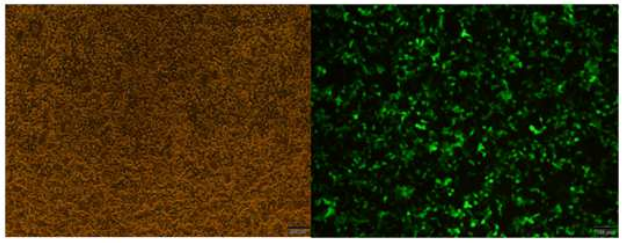


Figure 4. *The transfection efficiency of HEK293T cells.*

3.3. Luciferase Assay

MKL-1 can enhance the transcriptional activity of CD25 promoter obviously. Luciferase Reporter Assays were performed after HEK293T cells were transfected with MKL-1 and CD25 promoter. Contrasting with control group transfected with pcDNA3.1, MKL-1 showed a significant effect to enhance the transcription activity of CD25 promoter (Figure 5). Taken together, MKL-1 dose dependent promoted the the transcription activity of CD25 in HEK293 cell.

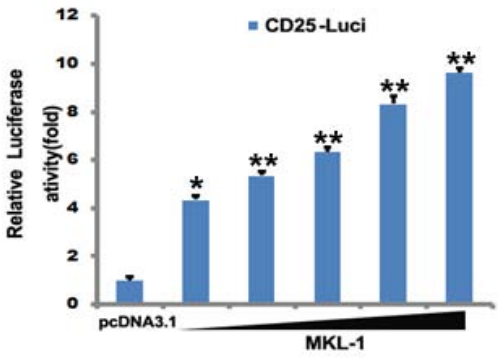


Figure 5. *MKL-1 can enhance the transcriptional activity of CD25. (**P< 0.01, *P < 0.05).*

4. Conclusion

$CD25^+$ is the alpha chain of IL-2 receptor, which constitutes a low affinity receptor, which can not transmit to the signal, but is important for the formation of high affinity receptors. [11] IL-2R is distributed on the activated T cells, B cells and NK cell surface. [12] Idiopathic thrombocytopenic purpura (ITP) is a chronic acquired organ-specific autoimmune hemorrhagic disease. [13] Decreased number or disfunction of Treg cells may be the mechanisms of ITP. [14] ITP is characterized by increased platelet destruction and reduced platelet production. [15] $CD4^+CD25^+$ T regulatory (Treg) cells suppresses T-cell proliferation and Reduce immune responses to autonomous antigens and foreign antigens. [16-18] We hypothesized that the CD25 gene has an effect on the maturation and differentiation of megakaryocytes.

Myocardin-related transcription factors also play a central role in differentiation of vascular smooth muscle cells, [19, 20] which naturally undergo polyploidization. [21] It will be of interest to determine whether MKL1 has the same role in mediating polyploidization in vascular smooth muscle cells. MKL-1 is a recently discovered synergistic transcription factor of serum response factor (SRF). [4] Studies have shown that MKL-1 can induce a variety of cell differentiation, including fibroblasts and smooth muscle cells, skeletal muscle cells, epithelial cells and other differentiation. [22] In the process of mouse megakaryocyte differentiation, the expression of MKL-1 is up-regulated, and MKL-1 promotes the physiological maturation of human and mouse megakaryocytes. [10] It will be of interest to determine whether MKL-1 acts via SRF to enhance megakaryocyte differentiation.

In this study, luciferase reporter with CD25 promoter was successfully constructed. Then the activation of the CD25 promoter was detected in 293T cells by luciferase reporter assay after transfected the expression vectors of human MKL-1, the transfection efficiency was demonstrated using an EGFP (enhanced green fluorescent protein) expression plasmid. The result showed that MKL-1 can obviously enhance the transcriptional activity of CD25 promoter. This study may provide a new mechanism for further research on the influence of MKL-1 on the maturation and differentiation of megakaryocytes, and also provide a theoretical basis for

the treatment of diseases caused by megakaryocyte abnormalities, such as ITP.

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