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Overexpression of the c-Myb but not its leukemogenic mutant DNA-binding domain increased adipogenic differentiation in mesenchymal stem cells

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ABSTRACT

The c-Myb protein is a vital transcription factor that regulates the differentiation of hematopoietic cells. Previous works have noticed that c-Myb is involved in an epigenetic control mechanism, in which the c-Myb DNA-binding domain (DBD) binds to the N-terminal histone tail of H3 to facilitate it acetylation and activate endogenous differentiation genes, while the leukemogenic mutant of c-Myb does not have these functions. However, whether c-Myb has corresponding biologic functions on the differentiation of other cells except for hematopoietic cells has not been explored. In our studies, we constructed the c-Myb wild type and its leukemogenic mutant DBD recombinant adenovirus with replication-defective adenoviral vectors carrying the GFP gene. We compared their roles on adipogenic differentiation efficiency in human bone marrow-derived mesenchymal stem cells (hMSCs). Our results demonstrated that the overexpression of c-Myb could enhance adipogenic differentiation in hMSCs, while the overexpression of its leukemogenic mutant blocked the adipogenic differentiation to a certain extent. These suggest that c-Myb play an important role in the hMSCs differentiation too, which is consistent with the epigenetic control mechanism of c-Myb.

1. Introduction

The Myb gene family is first recognized in the form of the v-Myb oncogene of the avian myeloblastosis virus (AMV) [1]. This acutely transforming retrovirus causes rapidly fatal monoblastic leukemia in chickens. The Myb has a highly conserved DBD composed of three consecutive SANT helix-turn-helix-like motifs (R1, R2, R3) [2], two of which (R2, R3) are sufficient for the recognition of specific DNA sequences [3,4]. The c-Myb protein is an important transcription factor, as well as a proto-oncogene that is the cellular homologue of the oncogene v-Myb [5]. Comparing with c-Myb, v-Myb leukemia protein of AMV carries four amino acid substitutions in its DBD (three in R2-I91N, L106H, V117D, and one in R3-I181V). The three mutations in the R2 of v-Myb act in concert to achieve a block in cell differentiation and an acute myeloblastosis [6]. The previous study has demonstrated that c-Myb-W (wild type), and c-Myb-2M (double amino acid mutations in the R2 domain), bind to the H3 tail to position its acetylation. But the leukemogenic cousins including c-Myb-3M (three amino acid mutations in the c-Myb R2), and c-Myb-4M (three mutations in R2 and one mutation in R3), can not bind to H3 [7] (Fig. 1). Both histone tail binding and acetylation are prerequisites for the activation of differentiation genes. In addition, crystal structural analysis has shown that these three critical amino acid mutations in v-Myb have cooperatively reduced DNA binding activity, probably due to the positional changes around the α1–α2 loop and DNA minor groove interaction [8]. These provide a compelling explanation for the failure of v-Myb in participating in cell maturation and differentiation.

The c-Myb transcription factor is required at multiple differentiation steps of hematopoietic precursor cells [9,10]. Also, it is prominently involved in vascular smooth muscle cells differentiation from stem cells derived population [11]. However, its role on hMSCs differentiation has not been investigated. hMSCs have been initially identified in the bone marrow as multipotent non-hematopoietic progenitor cells that can give rise to osteoblasts, chondrocytes, myocytes, adipocytes and so on [12,13]. In our study, we have examined the adipogenic differentiation functions of the c-Myb and its leukemogenic mutant cousins in hMSCs. We also have detected the change of peroxisome proliferator-activated receptorγ2 (PPARγ2), as it plays a crucial role among various transcription factors involved in adipogenic differentiation [14]. Here, we show that forced expression of c-Myb-W and c-Myb-2M (I91N, L106H in R2) have up-regulated the fat-related gene expression and enhanced adipogenic differentiation efficiency in hMSCs, while the leukemogenic mutation counterparts (c-Myb-3M and c-Myb-4M) have decreased the adipogenic efficiency. These results

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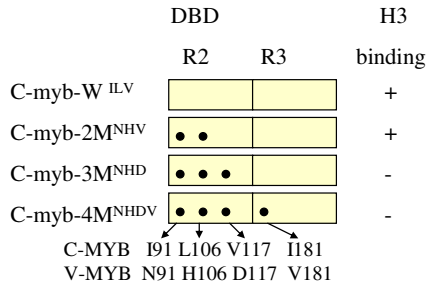

Fig. 1. Configuration of the c-Myb DBD and H3 tail binding. c-Myb-W and c-Myb-2M can bind to the H3 tail to position it acetylation, but the leukemogenic cousins including c-Myb-3M and c-Myb-4M abrogate binding to H3.

firstly suggest that c-Myb plays an important role on the hMSCs differentiation.

2. Materials and methods

2.1. Isolation and culture of hMSCs

Bone marrow aspirates were obtained from a female volunteer (aged 18). Mononuclear cells were isolated with density gradient centrifugation and cultured in 25 cm^2 flasks at a concentration of 3×10^7 cells in 5 ml high glucose-Dulbecco Modified Eagle Medium (H-DMEM), with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 mg/ml streptomycin, and maintained at 37 °C in 5% CO_2. The cells were allowed to attach for 4 days before the removal of nonadherent cells. Thereafter medium was changed twice per week until 70–80% confluence (10–12 days) and the cells attached were defined as passage zero (P0) hMSCs. P0 hMSCs were subsequently detached using 0.25% trypsin, replaced in 75 cm^2 flask and cultured until confluence to generate a homogenous cell population defined as first passage (P1) hMSCs. P1 hMSCs were then frozen in liquid nitrogen in H-DMEM consisting of 20% FCS and 10% dimethyl sulfoxide or further expanded. To establish serial passages of hMSCs, the plated cells were carefully maintained to reach 90% confluence and serially subcultured at a ratio of 1:4. All cells used for the following experiments were P3–P5.

2.2. Flow cytometry analysis of hMSCs

hMSCs were immunolabeled at 4 °C for 30 min with the following antihuman antibodies: phycoerythrin (PE) conjugated CD45, CD34, and CD90. For indirect assays, cells were immunolabeled with antihuman CD73. As the secondary antibody, goat anti-mouse IgG–PE was used. Labeled cells were analyzed by FC 500 with the use of CXP software.

2.3. Plasmid construction and adenoviral gene transfer

The recombinant adenoviruses were made using the AdEasy system (Stratagene). DNA fragments encoding human c-Myb or its mutants DBD were generated, respectively, by PCR from the pCDNA3.1-c-Myb-W, pCDNA3.1-c-Myb-2M, pCDNA3.1-c-Myb-3M, and pCDNA3.1-c-Myb-4M (a gift from Xianming Mo, Amino residues within the c-Myb DBD were mutated by site-directed mutagenesis), and inserted into the XbaII/SalI sites of the shuttle vector pAdTrack-CMV. The primer sequences of human c-Myb were forward 5′ aaa tct aga atg gcc cga aga ccc cgg cac agc 3′, and reverse (flag tagged) 5′ aaa gtc gac tcg ctt gtc atc gtc gtc ctt gta gtc cat gac cag cgt ccg ggc tga 3′. Recombinant adenoviruses were produced by performing homologous recombination in BJ 5183 with this shuttle vector and pAdEasy-1. Correct recombinants were firstly selected with kanamycin and then determined by restriction endonuclease digestion. Simultaneously we constructed a recombinant without gene insert as a control. The linearized recombinant plasmids were transfected with Exgen 500 (Fermentas) into AD293 cells to produce infective adenovirus. Viral stocks were amplified in AD293 cells on 15 cm plates and purified using Adeno-X™ Virus Mini Purification Kit (Clontech). The titer of the virus was 1×10^{10} PFU/ml.

2.4. Adenovirus infection and adipogenic differentiation of hMSCs

Twenty-four hours after seeding the P3 hMSC cells in 6-well dishes in expansion medium, we respectively infected these cells with Ad-C (adenovirus control), Ad-c-Myb-W, Ad-c-Myb-2M, Ad-c-Myb-3M, and Ad-c-Myb-4M at a multiplicity of infection (MOI) of 100 PFU/cell. Another twenty-four hours later, the culture medium was replaced with an induction medium suitable for adipogenic differentiation. The adipogenic induction medium consisted of the expansion medium as above, with adipogenic stimulants consisting of 100 mg/ml 3-isobutyl-1-methyl-xanthine (Sigma), 10^{-6} M dexamethasone, 10 mg/ml insulin, and 50 mM indomethacin (Sigma). The medium exchange was carried out twice per week.

2.5. Oil Red O staining

Oil Red O staining was used to identify adipocytes. Following 21 days of cultivation with adipogenic culture medium, hMSCs fixed with 4% formaldehyde were incubated with 0.3% Oil Red O (Sigma) for 1 h at room temperature, and then rinsed with deionized water. The stained triglyceride droplets in the cells were visualized and photographed. The numbers of Oil Red O positive cells were then manually counted in each view to determine the frequency of adipogenic differentiation. The numbers of adipogenic cells were counted and averaged over five fields of microscopic observation in each sample.

2.6. Real-time RT-PCR for detection of adipogenic differentiation marker

hMSCs, infected with Ad-C, Ad-c-Myb-W, Ad-c-Myb-2M, Ad-c-Myb-3M, and Ad-c-Myb-4M were cultured under adipogenic differentiation condition. After 21 days, we detected gene expression of PPARγ2 at the mRNA level, using real-time RT-PCR. Total RNA was isolated using the RNeasy mini kit (QIAGEN). Reverse transcription was performed with 1 μg RNA in a total volume of 20 μl per reaction. The mRNA levels were normalized to β-actin (internal control) and gene expression was presented as -fold changes.

2.7. Statistical analysis

Results were expressed as means ± standard deviation. Data were evaluated by Dunnett in ONE-WAY ANOVA. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Characteristics of primary hMSCs

Mononuclear cells were isolated from bone marrow and cultured on plastic flasks in H-DMEM with 10% FCS to gain hMSCs. By passage 3, a homogenous population of rapidly dividing cells with spindle-shaped morphology was obtained. Flow cytometry analysis showed the phenotype was $CD34^-$, $CD45^-$, $SH3^+$ ($CD73^+$), Thy-1^+ ($CD90^+$) (Fig. 2).

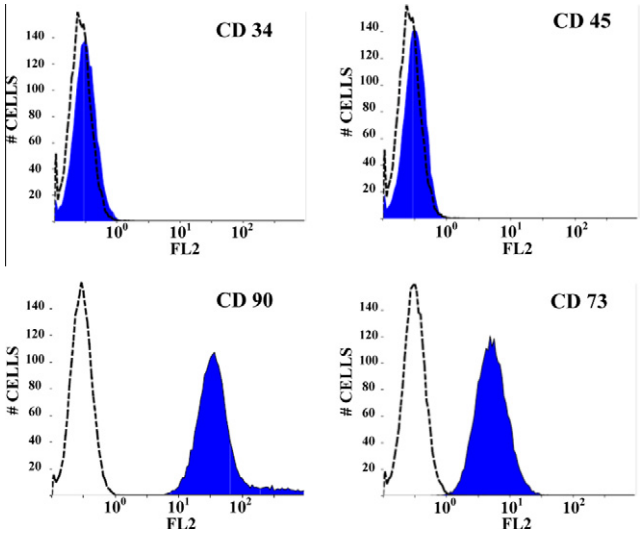

Fig. 2. Flow cytometric analysis of surface antigen expression on primary hMSC. The cells were immunolabeled with PE-conjugated monoclonal antibody specific for the indicated surface antigen. Dead cells were eliminated by forward and side scatter.

3.2. Adipogenic differentiation program in infected hMSCs

hMSCs were infected with Ad-C, Ad-c-Myb-W, Ad-c-Myb-2M, Ad-c-Myb-3M, and Ad-c-Myb-4M, respectively. The cells successfully transferred with these recombinant adenovirus simultaneously expressed GFP. Therefore, the infection efficiency could be monitored directly under fluorescence microscopy. The results showed that more than 50% cells expressed GFP 24 h after infection, when the MOI of 1:100 was empirically utilized. Nearly all cells were able to express GFP at 48 h.

We further observed the lipid formation course in our hMSCs highly expressing recombinant adenovirus under fluorescence microscopy (Fig. 3). No lipid vacuole was observed in hMSCs before cultured in adipogenic medium. Lipid vacuoles were appreciable when the cells cultured in the adipogenic medium on day 3 and continued to increase. As the differentiation program proceeded, most of the small lipid vacuoles syncretized into bigger and bigger vacuoles. On day 21, even after dyed with Oil Red O, the bright green fluorescence in the infected cells was still observed.

3.3. The effects of c-Myb and its leukemogenic mutants on adipogenic differentiation in hMSCs

The three amino acid substitutions in R2 of the c-Myb DBD achieved a block in cell differentiation and an acute myeloblastosis in hematopoietic cells. Here, we explored the effects of c-Myb and its leukemogenic mutants DBD on adipogenic differentiation in hMSCs. Interestingly, we found that overexpression of c-Myb-W and c-Myb-2M resulted in more cells to differentiate into adipocytes which filled with lipid droplets when the cells matured (on day 21), while the overexpression of c-myb-3M and c-Myb-4M led to sharp decrease of cells differentiating into adipocytes. This result was distinctly visualized by the Oil Red O staining of lipid droplets (Fig. 4A–F). To confirm these findings, we further examined the mRNA expression of PPARγ2, a early stage adipocyte lineage marker, by real-time RT-PCR. Consistent with the Oil Red O staining, the mRNA levels of PPARγ2 were enhanced significantly in hMSCs overexpressing c-Myb-W and c-Myb-2M, and decreased in cells overexpressing c-myb-3M and c-Myb-4M (Fig. 4G). These results supported our hypothesis that according to the epigenetic control mechanism, c-Myb could promote the adipogenic differentiation of hMSCs in the adipogenic condition but the leukemogenic mutants could block this function.

4. Discussion

The balance between self-renewal and differentiation in stem cells must be tightly regulated. Excess self-renewal may induce large numbers of proliferating progenitors, leading to mutation and tumorigenesis; whereas over differentiation may cause stem

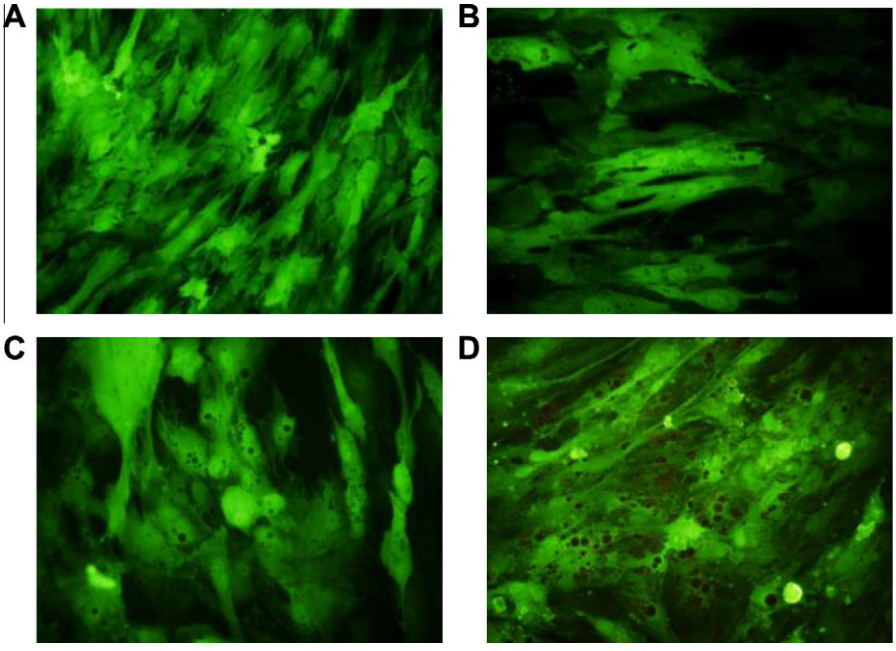

Fig. 3. The lipid formation course in the recombinant adenoviruses infected hMSCs under fluorescence microscopy. (A) hMSCs were infected by adenoviruses at a MOI of 1:100 and observed at 48 h. High efficiency of adenovirus transfection was observed. Cells were cultured in adipogenic medium and observed by fluorescence microscopy (B) on day 5, (C) on day 10, (D) on day 21 stained with Oil Red O.

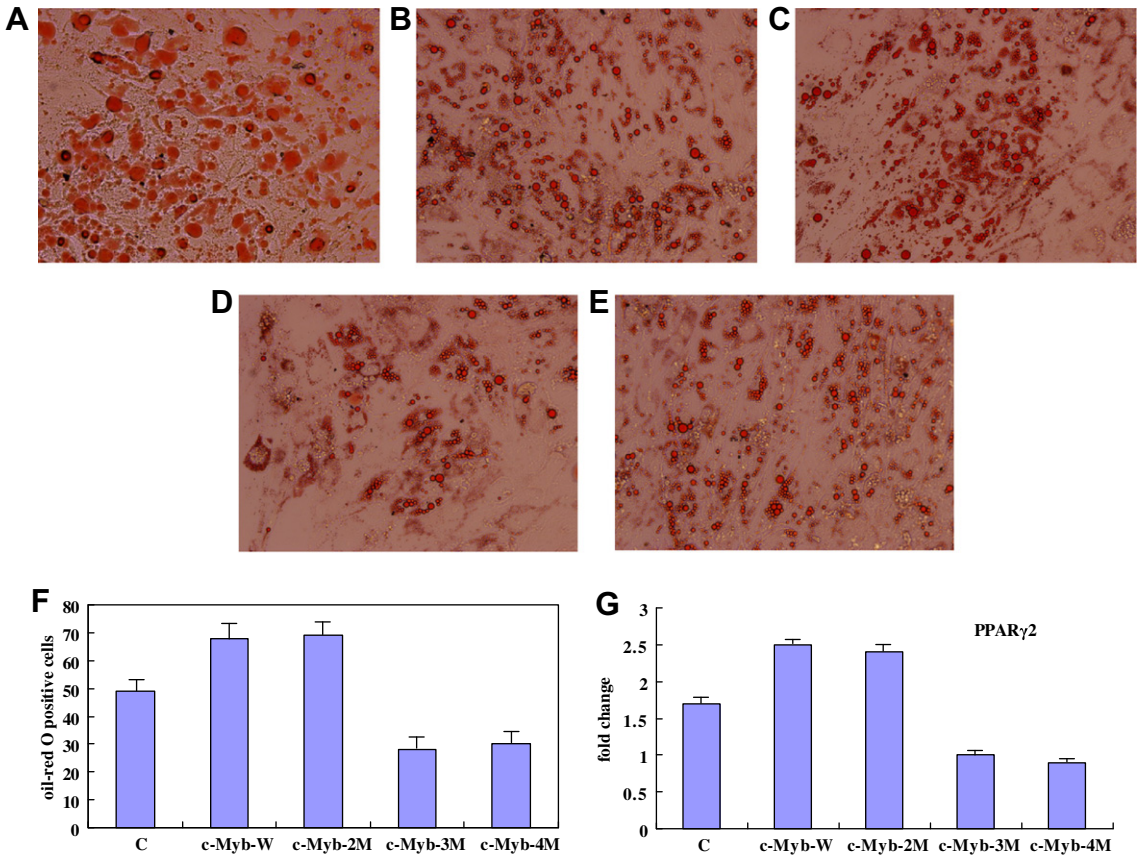

Fig. 4. Effects of c-Myb and its leukemogenic mutation counterparts DBD on adipogenic differentiation of hMSCs. The hMSCs were infected with the recombinant adenoviruses (A) Ad-C (B) Ad-c-Myb-W (C) Ad-c-Myb-2M (D) Ad-c-Myb-3M (E) Ad-c-Myb-4M, respectively. After 24 h they were grown under adipogenic conditions. On day 21, the cells were stained with Oil Red O. (F) Summaries of the Oil Red O positive cells per well. (G) On 21 d, real-time RT-PCR analysis of adipogenic-specific gene expression was performed.

cell depletion. Single-point mutation in the homeodomain of the paired gene product can cause a change in the spectrum of regulated genes [15]. Previous findings have demonstrated that c-Myb and its non-leukemogenic mutant DBD bind to the N-terminal histone tail of H3, and facilitate histone tail acetylation, which is mandatory during activation of prevalent differentiation genes. But leukemogenic mutations in c-Myb eliminate the interaction with H3 and acetylation of H3 tails and abolish activation of endogenous differentiation genes [7]. The question we ask in this study is whether the c-Myb and its leukemogenic mutants have the corresponding biologic function on hMSCs adipogenic differentiation. To address this question, we infected hMSCs with Ad-C, Ad-c-Myb-W, Ad-c-Myb-2M, Ad-c-Myb-3M, and Ad-c-Myb-4M respectively and then cultured the cells in adipogenic medium. We found that c-Myb-W and the non-leukemogenic mutant (c-Myb-2M) stimulated adipogenic differentiation in hMSCs, and this function was blocked by the leukemogenic mutants(c-Myb-3M, c-Myb-4M). The function of c-Myb during differentiation is involved in H3 tail acetylation and mediating modifications in succession. Our results are consistent with the epigenetic control mechanism of c-Myb.

c-Myb is a transcription factor that is essential to the differentiation of hematopoietic precursor cells. In our studies, we have first investigated the c-Myb function in hMSCs. hMSCs are pluripotent and in addition to adipocytes, they can give rise to several distinct cell lineages, such as osteoblasts, chondrocytes, myocytes, and even neurons under appropriate conditions. In our future studies, we need to further explore the functions of c-Myb on the other differentiation aspects in hMSCs. Our data now provide novel evidences suggesting an important role of c-Myb as differentiation regulating gene in hMSCs. The hMSCs exhibit versatile functions and are considered to be important for prospective cell-based therapy. The result could help us to further understand the mechanisms regulating hMSCs to differentiate and self-renew, and manipulate hMSCs for therapeutic use.

Acknowledgments

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