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Vitamin D directly regulates Mdm2 gene expression in osteoblasts

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ABSTRACT

While Mdm2 is an important negative regulator of the p53 tumor suppressor, it also possesses p53-independent functions in cellular differentiation processes. Mdm2 expression is alternatively regulated by two P1 and P2 promoters. In this study we show that the P2-intiated transcription of Mdm2 gene is activated by 1,25-dihydroxy vitamin D3 in MC3T3 cells. By using P1 and P2-specific reporters, we demonstrate that only the P2-promoter responds to vitamin D treatment. We have further identified a potential vitamin D receptor responsive element proximal to the two p53 response elements within the Mdm2 P2 promoter. Using cell lines that are p53-temperature sensitive and p53-null, we show requirement of p53 for VDR-mediated up regulation of Mdm2 expression. Our results indicate that 1,25-dihydroxy vitamin D3 and its receptor have a role in the regulation of P2-initiated Mdm2 gene expression in a p53-dependent way.

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1. Introduction

The murine double minute (Mdm2) gene was originally identified as being gene-amplified on double-minute chromosomes in transformed mouse fibroblast [1]. Mdm2 is a p53 inducible gene and encodes a type E3 ubiquitin ligase responsible for the degradation of p53 in the 26S proteosome [2]. A well established role for MDM2 is as a key negative regulator of p53 activity: p53 binds to the p53 responsive elements (p53REs) in the secondary promoter of the *Mdm2* gene and activates *MDM2* expression, while the subsequent increase of MDM2 protein results in its binding to p53 at the N-terminal 1–52 residues and leads to p53 degradation [3]. However, MDM2 also possesses numerous p53-independent activities, and is also known to interact with a number of other proteins (Numb, RB, p300, insulin like growth factor receptor, estrogen receptor, androgen receptor, etc.) involved in different cellular activities such as cell fate determination, differentiation and signaling [4–7]. In the case of bone, it has been reported that targeted disruption of *Mdm2* in this tissue causes skeletal abnormalities, osteopenia and osteoporosis [5].

Transcription of the *Mdm2* gene is believed to be controlled by two distinct promoters (referred to as P1 and P2) [8,9]. The P1 promoter, which is located at the upstream of the first exon, is responsible for the basal expression of Mdm2. The P2 promoter is situated in the first intron and is responsible for inducible expression. The two transcripts initiated from the P1 and P2 promoter encode identical full-length Mdm2 proteins by using the same translation start codon which is located in exon 2, while there are some differences in the 5′-untranslated regions (UTR) of these transcripts. A number of transcription factor binding sites have been identified in the P2 promoter region, including the two well-established p53RE sites [8,9], AP-1/ETS [10], Smad 2/3 [11], and an Sp1 site within a GC box cluster [12]. Recently, it has been reported that a tissue-specific RXR can bind to its recognition site within the P2 promoter and activate expression of the Mdm2 gene in retinal cone cells [13]. Vitamin D is an important bone anabolic agent and several bone specific genes are directly regulated by this hormone. 1,25-Dihydroxy vitamin D3, the active form of vitamin D exerts its action through its vitamin D receptor (VDR) a ligand dependent transcription factor, belonging to the nuclear receptor family of transcription factors. Vitamin D mediates its action through a homodimer of VDR or as a heterodimer with retinoic acid receptor in the regulation of bone specific genes.

Our work revolves around understanding the role of tumor suppressor gene p53 in osteoblast differentiation. We believe that Mdm2 under some conditions synergizes with p53 in the regulation of bone specific gene expression. In our previous study we have shown that MDM2 aids in the expression of osteocalcin, a bone-specific gene, during osteoblastic differentiation [14]. In this study we conducted *in vitro* and *in vivo* analyses to further investigate the regulation of the Mdm2 gene expression in osteoblast cells. We observed that Mdm2 expression could be upregulated by vitamin D3 and further identified a vitamin D receptor (VDR) responsive element in the P2 promoter of the Mdm2 gene.

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2. Materials and methods

2.1. Cell lines, plasmids and cell culture

The mouse osteoblast cell line MC3T3 (American Type Culture Collection, Manassas, VA) and rat osteosarcoma cell line ROS17/2.8 (kindly provided by Dr. Rodan, Merck Research Laboratory, West Point, PA) were used for these studies. A cell line stably expressing a temperature sensitive p53 plasmid was created in a p53 null osteosarcoma cell line [15]. The p53-null cell lines were also obtained from calvaria of p53 null mice as described earlier [16]. The two luciferase reporter plasmids, P1-Luc (containing Mdm2 P1 promoter) and P2-Luc (containing Mdm2 P2 promoter), were provided by Dr. Wu (University of California School of Medicine, Las Angeles, CA) [17]. The p53 expression vectors were a kind gift from Dr. Oren (Weisman Institute, Israel). Cell lines were grown in DMEM/F-12 with 10% fetal bovine serum in a modified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.2. Transient transfections and reporter assays

The cell lines were transfected with P2-Luc or other expression vectors using Superfect transfection reagent (Qiagen, Valencia, CA). Luciferase activity was measured using equal amounts of cell lysates prepared from the transfected cells. All measurements were carried out on triplicate samples and experiments were repeated at least thrice.

2.3. Semi-quantitative RT-PCR

Total RNAs were isolated from MC3T3 cells using TRI reagent (Sigma Chemical Company, St. Louis, MI). The primers for P1-initiated and P2-initiated Mdm2 transcripts were listed as follows: P1-Mdm2-F (5′-CTCGTCGCTCGAGCTCTGGA-3′), P1-Mdm2-R (5′-AGGTGCTTGCAGCACCCTCG-3′), and P2-Mdm2-F (5′-CTGGGGGA CCCTCTCGGATC-3′), P2-Mdm2-R (5′-TGTGCTGCTGCTTCTCGTCA-3′). Reverse transcription-PCR was carried out using Onestep RT-PCR kit (Qiagen, Valencia, CA) as follows: 50 °C for 30 min, 95 °C for 10 min, 45 cycles × 95 °C for 15 s, and 60 °C for 1 min, followed by the dissociation stage. PCR products were run on 2% agarose gel and semi-quantification of gene expression was determined by relative pixel densitometry using UNSCAN-IT (Silk Scientific, Orem, UT) for Mdm2 after normalization to β-Actin.

2.4. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted to investigate the occupation of VDR to the P2 promoter of the Mdm2 gene in ROS17/2.8 cells by using an EZ-ChIP™ Assay Kit (Upstate, Temecula, CA) as described previously [18]. Mouse antibody N-20 against VDR (Santa Cruz Biotech, CA) was used in ChIP assays. The PCR primers used to detect target sequences were 5′-AGGGAAGAGCGGGGGTCTC-3′ (forward) and 5′-ACCAGGCACCTGTCACCTCCT-3′ (reverse) which span from −436 to −450 bp position within the P2 promoter region of the Mdm2 gene and generate a 101-bp fragment in PCR amplification.

2.5. Western blot assay

Protein lysates were prepared using MPER reagent (Pierce, Rockford, IL). A Bradford assay was performed to determine protein concentration. The protein (25–50 μg) was run out on an SDS page gel, transferred onto a nitrocellulose membrane, and blocked in 5% milk solution. Primary antibodies used in this study were mouse monoclonal p53 (Pab240) IgG (Santa Cruz Biotech, CA), mouse monoclonal Mdm2 (SMP14) antibody (Santa Cruz Biotech, CA), rabbit VDR (N-20) antibody (Santa Cruz Biotech), and rabbit β-Actin antibody (Imgenex, San Diego, CA). The secondary antibodies used in Western blot were ImmunoPure Antibodoies (Thermo Scientific, Rockford, IL) against rabbit and mouse IgGs, respectively. The blot was developed using SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific). Relative pixel densitometry was conducted using UNSCAN-IT for p53, MDM2 and VDR protein after normalization to β-Actin.

2.6. Statistical analysis

Statistical analysis was performed using software GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The values given are mean ± S.E.M. Statistical analysis between two samples was performed using Student's *t*-test. In all cases, $P < 0.05$ was considered as significant.

3. Results

3.1. Mdm2 expression is upregulated by 1,25-dihydroxy vitamin D3 in MC3T3 cells

To investigate the effect of vitamin D3 on the Mdm2 gene expression, MC3T3 cells were treated with 1,25-dihydroxy vitamin D3 at a final concentration of 20 nM and Mdm2 transcripts were measured after different periods (0, 0.5, 2, 6, and 24 h) by using semi-quantitative RT-PCR. As shown in Fig. 1A, vitamin D3 treatment displayed a positive effect on the Mdm2 expression. The level of Mdm2 mRNA peaked at 2 h with an 11-fold increase compared to the control. Western blot further revealed an increase in Mdm2 protein after vitamin D3 treatment (Fig. 1B, top panel). Vitamin D receptor levels were also measured during the same period of time and showed responsiveness to vitamin D treatment as expected (Fig. 1B, bottom panel).

3.2. Vitamin D3 mediates upregulation of Mdm2 expression through the Mdm2 P2 promoter

The Mdm2 gene has two alternative promoters to initiate two different full length isoforms of mRNA transcripts. To further investigate the effect of vitamin D3 on the Mdm2 expression, we detected levels of the two Mdm2 mRNA transcript isoforms generated by P1 and P2 promoter in MC3T3 cells after different periods of 1,25-dihydroxy vitamin D3 treatment. Two pairs of primers specific to the P1-initiated and the P2-initiated Mdm2 mRNA transcripts were used in semi-quantitative RT-PCR. As shown in Fig. 1C, the P2-initiated transcript levels were increased significantly after treatment of vitamin D3, with the highest level of about 5.6-fold increasing at the 4-h compared to the control. However, changes of the levels of P1-initiated Mdm2 transcripts were not observed during the vitamin D3 treatment (Fig. 1C).

3.3. A potential VDR responsive element is located in the Mdm2 P2 promoter

To further investigate the effect of vitamin D3 on the Mdm2 P1 and P2 promoter activity, we introduced P1 and P2 reporter plasmids into MC3T3 cells, and measured the promoter activation 2 h after 1,25-dihydroxy vitamin D3 treatment. As shown in Fig. 2, no change of P1 promoter activity was observed after vitamin D3 treatment (Fig. 2A), but the P2 promoter displayed about 56-fold increased activity after vitamin D3 treatment compared to the control (Fig. 2B). We further detected a time dependent activation of the P2 promoter activity when measured after different intervals (0, 15, 30, 120, and 240 min). We observed that the P2 promoter

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