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# Lidamycin regulates p53 expression by repressing Oct4 transcription

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ABSTRACT

Antitumor antibiotic lidamycin (LDM) is widely used in the treatment of a variety of cancers. Here we demonstrated that LDM up-regulates the expression of the tumor suppressor p53 gene by repressing Oct4 transcription. We showed that low dose LDM-induced increase of p53 expression and *decrease of Oct4 expression* in P19 and HCT116-p53$^{+/+}$ cells. Knockdown of Oct4 expression by siRNA led to activation of p53 in both cell lines, whereas ectopical expression of Oct4 significantly inhibited p53 expression in P19 cells. LDM-induced p53 activation was blocked by ectopical *expression of Oct4.*



## 1. Introduction

Lidamycin (LDM, also known as C-1027) is one of the most potent members of the enediyne family, which can be dissociated into an apoprotein (LDP) and an active enediyne chromophore [1–3]. The labile chromophore is responsible for its cytotoxicity through its DNA damage activity [2,4], the non-covalently bound LDP serves as a vehicle to deliver the enediyne chromophore to tumor tissues [3]. Previous studies showed that LDM exhibited its anti-cancer effect through a variety of mechanisms in a dose-dependent manner, including chromosomal aberrations, telomere dysfunction, cell cycle arrest, and apoptosis [5,6]. A high dose (1 pM) of LDM induced unusual DNA damage response to double-strand breaks [4], whereas low dose (0.1 nM) of LDM induced cell cycle arrest in a p53-dependent manner [5]. However, the molecular mechanisms of p53 activation induced by low dose-LDM are still largely unknown.p53 functions as a transcription factor and coordinates in a wide variety of cellular processes. The steady-state level of p53 is usually kept low by its negative regulator HDM2 (mouse ortholog is mdm2) and HDMX (mouse ortholog is mdmX) [7]. However, p53 is stabilized and activated as a central mediator to respond to stimuli in a promoter-specific manner, including the induction of cell cycle arrest, senescence, differentiation, and apoptosis [7,8]. During embryonic stem (ES) cell differentiation, p53 directly suppresses the expression of several genes, such as the pluripotency factor Nanog [9] and expression of p53 is regulated by another pluripotency factor Oct4 [10].

Oct4, a homeodomain transcription factor of the POU family [11], plays a crucial role in the maintenance of self-renewal and pluripotency in ES cells, inner cell mass (ICM), and primordial germ cells [11,12]. In the process of ES cell self-renewal, the expression of Oct4 promotes cell cycle progression by suppressing its target gene p21, a cyclin-dependent kinase inhibitor [13]. In contrast, downregulation of Oct4 results in blocking of cell cycle progression followed by differentiation [13]. Oct4 has been proposed as a biomarker for cancer stem cell (CSC)-like cells [14]. Oct4 is detectable in a variety of cancers such as breast, bladder, and lung cancers [14,15]. Growing evidence shows that Oct4 participates in malignancy and is associated with CSCs and poor prognosis of human cancers [14,15]. Forced expression of Oct4 gene causes dedifferentiation of cancer cells, which acquire cancer stem cell phenotypes [16]. Repression of Oct4 expression promotes differentiation of human endometrial adenocarcinoma cells through upregulation of microRNA-145 [17]. Therefore, Oct4 may be a useful target in cancer stem cell therapy.

Our previous study showed that LDM inhibited cancer cell growth through downregulation of ES cell-like genes [18]. LDM activates p53 and p21 through dowregulating Oct4 and consequently induces differentiation of mouse EC cells P19 [19]. However, the molecular mechanism by which low-dose LDM regulates the Oct4-p53 pathway is still poorly understood. In this report, we demonstrated that LDM up-regulated p53 by repressing Oct4 transcription.

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## 2. Materials and methods

### 2.1. Chemicals

LDM [3,5] was provided by Dr. Yong-Su Zhen (Institute of Medicinal Biotechnology, Chinese Academy of Medical Science, Beijing, China). LDM (1 μM) were prepared in distilled water and then stored at −70 °C.

### 2.2. Cell culture

Mouse EC P19 cells and human colon cancer HCT116-p53$^{+/+}$ cells were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO/BRL) containing 10% fetal bovine serum (FBS, HyClone) and incubated at 37 °C.

Floating multicellular spheroids were described previously [20,21]. The CD133$^{high}$/CD44$^{high}$ fraction of HCT116-p53$^{+/+}$ colon cancer cells was sorted by fluorescence activated cell sorting (FACS) analysis. Cells with the particular phenotype were seeded at a density $4 \times 10^2$/well on a 6-well tissue culture plate in serum-free MSCB medium (supplement with 20 ng/ml EGF, 10 ng/ml FGF-2, 10 ng/ml LIF and 25% Matrigel matrix). After 1 week in culture, fresh medium of normal culture (DMEM/F12 supplement with 10% FBS) was added and maintained for another week. Primary spheres were gently disaggregated by repeated pipetting and transferred into tissue culture flasks for further propagation and maintenance.

### 2.3. Cell cycle arrest

The cells ($2 \times 10^4$) were seeded into 60 mm dishes. For synchronization, the cells were incubated in serum-free DMEM for 24 h, and then 10% FBS was added into the culture medium. After cells were exposed to LDM for 24 h, samples were collected and fixed with 500 μl of 70% ethanol at 4 °C for 1 h. Subsequently, cells were resuspended in PBS with RNase A (50 mg/ml) and PI (2 mg/ml) at 37 °C for 30 min. The stained cells were analyzed on a flow cytometer.

### 2.4. RNA isolation for PCR and real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of RNA was subjected to reverse transcription by M-MLV reverse transcriptase (Promega, Madison, WI).

For real-time quantitative PCR, the analysis was performed with primers as previously described [18].

Mouse p53: (sense) 5′-GCAACTTCTAGAAACCCTGGGG-3′ and (antisense) 5′-TTGGGAAATGGAGGCCTGG-3′.

Human p53: (sense) 5′-GCGAGCACTGCCCAACAACA-3′ and (antisense) 5′-GGATCTGAAGGGTGAAATATTCT-3′.

Each 15 μl PCR mixture contained 1 μl of cDNA, 0.15 μl of each primer (400 nM), 6.2 μl of ddH2O and 7.5 μl of SYBR® Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan). Relative mRNA expression levels of Oct4 and p53 were determined in triplicate on a SYBR® Green Real-Time PCR Detection System (Stratagene Mx3000P, USA) and normalized to actin levels.

### 2.5. RNA interference

Small interfering (si) RNAs targeting Oct4 in mouse and human were purchased from Ambion. P19 and HCT116-p53$^{+/+}$ cells were transfected for 6 h with LipofectAMINE 2000 (LF2000) according to the manufacturer's guidelines. After recovery in fresh cell culture medium, cells were transfected again at 24 h to maintain knockdown efficiency. Silencing was assessed by RT-PCR at 48 h and by Western blots at 72 h.

### 2.6. Generation of lentivirus for overexpressing Oct4 and P19 cell infection

The lentivirus overexpressing Oct4 was prepared and infected as previously described [19].

### 2.7. Western blot analysis

Cells were lysed with 1× lysis buffer (Promega) by centrifugation. Equal amounts of Proteins were electrophoresed on SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Pierce). The membranes were probed with primary antibodies. The blots were developed with goat anti-mouse IgG (IRDye 700; LI-COR) and goat anti-rabbit IgG (IRDye 800; LI-COR), and imaged on an infrared scanner (LI-COR). Antibodies to Oct4, p53, and GADPH were from Santa Cruz Biotechnology.

### 2.8. Luciferase assay

#### 2.8.1. p53 transactivity luciferase reporter assay

Luciferase activity assays were performed using PG13-luc and MG15-luc reporter plasmids (Addgene) in HEK-293T cells. The cells were plated in a 24-well plate and split 24 h before transfection. Co-transfection of reporter plasmid was performed as previously described in [22]. After 24 h post-transfection, cells were treated with 0.1 nM LDM and cultured for another 24 h. The transfected-cells were variously treated and analyzed using a Dual-luciferase Reporter Assay kit (Promega). Results showed that the firefly luciferase activity normalized to *Renilla* luciferase activity.

#### 2.8.2. Oct4 promoter reporter assay

A 2.2 kb genomic DNA encompassing the promoter region of *Oct4* in the pGL3 vector (Promega, Madison, WI) was constructed by insertion into HindIII and XhoI restriction enzyme sites upstream of luciferase. The transfection and luciferase activity detection procedures were the same as the p53 transactivity luciferase reporter assay.

### 2.9. ChIP assay

As described before, in vivo binding of the Oct4 with *p53* promoter or LDP with the Oct4 promoter was performed [23].

Mouse

- For Nanog (sense) 5′-TGTTTTAGTGTGGGTATGGGCC-3′ and (antisense) 5′-TGTGGTCCCTCCTCTTTC-3′.
- For Oct4 (sense) 5′-GGAACTGGGTGTGGGGAGGTTGTA-3′ and (antisense) 5′-AGCAGATTAAGGAAGGGCTAGGACGAGAG-3′.
- For Sox2 (sense) 5′-CCCTGTTCCAAGTCTCTTTCTGCTAGTCA-3′ and (antisense) 5′-CACCGATTTCAATCCAACACCATCATAG-3′.

Human

- For p53 (sense) 5′-CGGATTACTTGCCCTTACT and (antisense) 5′-AATCCAGGGAAGCGTGTC-3′.
- For Nanog (sense) 5′-GAGGATGCCCCCTAAGCTTTCCCTCCC-3′ and (antisense) 5′-CCTCCTACCCTACCCACCCCCTATTCTCCC-3′.
- For Oct4 (sense) 5′-GGGGAACCTGGAGGATGGCAAGCTGAGAAA-3′ and (antisense) 5′-GGCCTGGTGGGGGTGGGAGGAACAT-3′.
- For Sox2 (sense) 5′-GGATAACATTGTACTGGGAAGGGACA-3′ and (antisense) 5′-CAAAGTTTCTTTATTCGTATGTGTGAGCA-3′.