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Lidamycin induces neural differentiation of mouse embryonic carcinoma cells through down-regulation of transcription factor Oct4

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ABSTRACT

Lidamycin is a potential anti-cancer drug, which is widely used in a variety of human cancer types. It has been reported that lidamycin inhibited mouse embryonic carcinoma (EC) cells growth through down-regulation of embryonic stem (ES) cell-like genes. In this study, whether 0.01 nM lidamycin induces neuronal differentiation of mouse EC cells was investigated. It was observed that lidamycin decreased transcription factor Oct4, and increased both p21 mRNA and protein expression in P19 EC cells. Furthermore, luciferase assay showed that lidamycin activated *p21* promoter activity through suppression of Oct4, and Chromatin immunoprecipitation (ChIP) assay confirmed that binding of transcription factor Oct4 to the *p21* promoter decreased in lidamycin-exposed cells. Knockdown of Oct4 resulted in neuron-like differentiation and up–regulation of p21 expression. In accordance, overexpression of Oct4 blocked neural differentiation and down–regulated p21 in lidamycin-treated *P19* cells. Taken together, these results suggested that neuronal differentiation of EC cells induced by lidamycin was associated with the inhibition of Oct4 expression and the activation of p21 transcription. Our results have provided a novel mechanism, in which lidamycin led to cancer cell differentiation.

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1. Introduction

Oct4 is a crucial transcriptional factor for maintenance of self-renewal and pluripotency in embryonic stem (ES) cells, the inner cell mass (ICM) and primordial germ cells [1,2]. Decreased Oct4 leads to the cell differentiation in trophectoderm [3]. These observations were consistent with the fact that during early embryonic development and ES cell self-renewal, Oct4 controlled pluripotency and differentiation by regulating the gene expression pattern [2]. Furthermore, in the process of ES cell self-renewal, the expression of Oct4 promoted cell cycle progression by suppressing its target gene *p21* cyclin-dependent kinase inhibitor (CKI) [4]. In contrast, down-regulation of Oct4 resulted in blocking cell cycle progression followed by differentiation [5].

Embryonic carcinoma (EC) cells are the multi-potential stem cells of teratocarcinoma. Although the origin of EC cells is different from the ES cells, they have many characteristics similar to ES cells, including cell morphology, surface antigen markers and *in vitro* differentiation [6,7]. Both the cell lines served as complementary tools for exploring relation between the stem cell biology and cancer [6,8]. Mouse pluripotent P19 EC cells were used as a model system for studying mechanism of cellular differentiation and identifying small molecule inducers for neuronal, cardiac and skeletal muscle differentiation [8,9]. Retinoic acid (RA) induced neural differentiation of P19 EC cells [9,10], and all-trans-RA induced differentiation, which successfully treated the patients of acute promyelocytic leukemia (APL) [11]. The EC cells were studied to provide a suitable *in vitro* model to identify new differentiation agents in cancer.

Lidamycin (LDM, also known as C-1027) is an enediyne anti-tumor antibiotic. Its labile chromophore caused cytotoxicity in response to DNA damage [12,13], and its non-covalently bound apoprotein got attached to tumor tissue as a deliver carrier for its targets [14]. The *in vitro* and *in vivo* studies demonstrated that lidamycin was extremely potent in activity, and it inhibited growth of many cancers by cell cycle arrest and apoptosis [15,16]. It has been previously reported that suppression of ES cell-like genes in target cells caused by low-dose lidamycin, led to inhibition of self-renewal of P19 EC and E14 ES cells without inducing apoptosis [17]. However, whether lidamycin induces P19 EC cell differentiation was unknown. In this study, it was observed that low-dose lidamycin resulted in neural differentiation of P19 cells, which

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was associated with the inhibition of Oct4 expression and the activation of p21 transcription. The data suggested that lidamycin had an effect on inducing differentiation of cancer cells.

2. Materials and methods

2.1. Chemicals

Lidamycin [14,15] was provided by Dr. Yong-Su Zhen (Institute of Medicinal Biotechnology, Chinese Academy of Medical Science, Beijing, China). Adriamycin and retinoic acid (RA) were purchased from Sigma Aldrich. For the study, the aliquots were prepared by dissolving lidamycin and adiramycin in distill water, respectively, and RA in 100% ethanol, and then aliquots were stored at −20 °C as 1 μM, 1 mM and 0.5 mM, respectively.

2.2. Cell culture

Mouse P19 EC stem cells were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO BRL) containing 7.5% fetal bovine serum (HyClone), and incubated at 37 °C.

The differentiation was initiated as per methods mentioned in the previous reports with slight modification, and the P19 cells were treated with lidamycin and RA [10]. Under serum-free conditions, the P19 cells were cultured in DMEM/F-12 after induction, supplemented with ITS supplement (Invitrogen) in the gelatinized tissue culture dishes at a density of 1×10^5 cells/ml.

2.3. RNA interference

Mouse siRNA target Oct4 (siOct4), GAPDH and negative control were purchased from Ambion. siRNA was transfected into P19 cells according to the manufacturer's guidelines using LipofectAMINE 2000(LF2000). The first siRNA transfection was performed 24 h after splitting the cells, followed by medium change 6 h post-transfection. Cells were transfected one more time with siRNA on the beginning of day 2 to maintain the knockdown efficiency. Silencing was assessed by RT-PCR at 48 h and Western blots at 72 h. Twenty-four hours after the second siRNA transfection, cells were cultured in differentiation medium for 6d.

2.4. Generation of lentivirus for overpressing Oct4 and P19 cell infection

Plasmids of FUW-Oct4, FUW-gagpol, FUM-VSV-G and FUW-M2rtTA were gift from Lingsong Li (Department of Cell Biology, Health Science Center of Peking University). The lentivirus overexpressing Oct4 was prepared as described [18].

To overexpress Oct4, lidamycin-induced P19 cells were incubated with the Oct4-expressing lentivirus and FUW-M2rtTA virus at a ratio of 1:1 in the presence of 2 mg/ml of polybrene culture medium was replaced with differentiation medium supplemented with doxycycline (Sigma–Aldrich; 2 mg/ml) 48 h later.

2.5. RNA isolation for PCR and real-time quantitative RT-PCR

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

For PCR, the analysis was performed in triplicate with primers as follows:

For mouse nestin: (sense) 5′-GGATCAGATCGCTCAGATCC-3′ and (antisense) 5′-GCACGACACCAGTAGAACTGG-3′.

For mouse β-Tublin III: (sense) 5′-ACCTCAACCACCTGGTATCG-3′ and (antisense) 5′-GGGATCCACTCCACGAAGTA-3′.
For mouse Map-2: (sense) 5′-CATCGCCAGCCTCGGAACAAACAG-3′ and (antisense) 5′-TGCGCAAATGGAACTGGAGGCAAC-3′.

For real-time quantitative PCR (RT-qPCR), the analysis was performed in triplicate with primers as follows:

For mouse Actin: (sense) 5′-AGTGTGACGTTGACATCCGT-3′ and (antisense) 5′-TGCTAGGAGCCAGAGCAGTA-3′.
For Oct4: (sense) 5′-CTGCAGAAGGAGCTAGAACAGTTTG-3′ and (antisense) 5′-GATGGTGGTCTGGCTGAACAC-3′.
For p21 (sense) 5′-ATGTCCAATCCTGGTGATGT-3′ and (antisense) 5′-TGCAGCAGGGCAGAGGAAGT-3′.

Each PCR mix contained 1 μl of cDNA, a 400 nM concentration of each primer, and 7.5 μl of SYBR® Green Real-time PCR Master Mix (TOYOBO, Osaka, Japan). The relative quantity of Oct4, *p21* mRNA and mouse actin levels were measured in triplicates on a SYBR® Green Real-Time PCR Detection System (Stratagene Mx3000P, USA), where the mouse actin served as a internal control.

2.6. Immunofluorescence staining

For immunofluorecence, the cells were placed in 24-well plates, washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 min and permeabilized using 0.5% Triton X-100 in PBS. After washing with PBS, the cells were incubated with 2% bovine serum albumin for blocking non-specific antigen for 30 min at 37 °C, and labeled overnight with Oct4 (1:100) (Santa Cruz), *p21* (1:100) (Santa Cruz), and SSEA1 (1:100) (Santa Cruz) at room temperature. After washing with PBS, anti-rabbit IgG-TRITC conjugate and anti-mouse IgM-FITC conjugate (1:100) (Pierce) were added. The cells were kept for 1 h in dark and washed again with PBS. The nuclei were stained with DAPI, and confocal microscopy was performed with Leica.

2.7. Western blotting

Cells were lysed by 1 × lysis buffer (Promega), and 20 μg proteins was subjected to 10% SDS–PAGE, transferred onto nitrocellulose membrane (Pierce), and probed with specific antibodies: Oct4 (1:500), p21 (1:200) and GADPH (1:500) (Santa Cruz Biotechnology, CA). The blots were developed with goat anti-mouse IgG (1:10000, IRDye 700; LI-COR) and goat anti–rabbit IgG (1:10000, IRDye 800; LI-COR), then imaged on an infrared scanner (LI-COR).

2.8. Luciferase assay

A 2.4-kb genomic DNA encompassing the promoter region of *p21* in pGL3 Basic vector (Promega, Madison, WI) was provided by Dr. Wei-Guo Zhu (Department of Biochemistry, Health Science Center of Peking University) [19]. The pCMV-BK/Oct-4 expression plasmid was constructed. As per the protocol (Promega), P19 and HEK-293T cells were plated at a density of 1×10^4 cells or 1×10^5 cells per well on a 24-well plate. The co-transfections of HEK-293T cells were performed with 0.25 μg of reporter plasmid and 0, 0.25, 0.5, 0.75 or 1.0 μg of pCMV-BK/Oct-4 expression plasmid using Lipofectamine 2000 (Invitrogen), and luciferase assays were performed using Promega kit. *Renilla* luciferase activities were used to normalize the transfection efficiency.

2.9. Chromatin immunoprecipitation (ChIP) assay

As described primitively, *in vivo* binding of the Oct4 with *p21* promoter was performed [20]. After the precipated protein-DNA adducted P19 or lidamycin, the treated P19 EC cells were reversely cross-linked, and these DNA samples were purified with a QIAquick Spin Kit (Qiagen, CA). For PCR, 1 μl was obtained from a 50 μl DNA extract, and 35 cycles of amplification were used with specific primers:

For *p21* (sense) 5′-AGTGTGGTCCCAGTCAGGTC-3′ and (antisense) 5′-AGACGAGGAAAGCAGTTCCA-3′.
For FGF4 (sense) 5′-AGACTTCTGAGCAACCTCCCGAA-3′ and (antisense) 5′-CAACTGTCTTCTCCCCAACACTCT-3′.
For p53 (sense) 5′-GCAACTTCTAGAAACCCTGGGG-3′ and (antisense) 5′-TTGGGAAATGGAGGCCTGG-3′.

PCR products were resolved on a 1% agarose gel and quantified using the ImageQuant software on a Gel Detector (Bio Rad, USA).

2.10. Statistical analysis

The Student's *t*-test and ANOVA test were used for unvaried analysis. The statistical significance was defined by a two-tailed *P*-value of 0.05.

3. Results

3.1. Lidamycin induced neural differentiation of mouse embryonic carcinoma P19 cells

It is known that P19 cells underwent neural differentiation in the presence of RA *in vitro* [8,10] and lidamycin had similar effect in decreasing expression of ES cell markers in P19 EC cells [17]. It was speculated that lidamycin induced neural differentiation. As shown in Fig. 1A, by day 2 after replating, the cytoplasmic volume of lidamycin-treated P19 cells started to decrease and showed bipolar morphology with short processes as compared to untreated cells. On day 6, lidamycin-treated P19 cells formed long neurite-like processes. From day 8–10, lidamycin-treated P19 cells formed large cell clumps and produced many neurite-like fibers to form networks.

Immunostaining showed that the cells were variable in neuronal morphology during differentiation for nestin (a molecular marker expressed in neural progenitor cells), and Map-2 (molecular markers expressed in mature neural cells) [21,22] (Fig. 1B). The mRNA expression in lidamycin- and RA-induced P19 cells were examined by PCR for three neural genes: *Nestin*, *Tublin-III* and *Map-2* (Fig. 1C). Nestin appeared as early as day 2, other neural genes were seen clearly by day 6 and strongly noticed on day 8 and day 10. The results indicated that lidamycin induced P19 cells neural differentiation.

3.2. Lidamycin suppressed expression of transcriptional factor Oct4 and activated p21 gene in mouse P19 EC cells

In the current study, the expression of Oct4 was detected by immunofluorescence staining as previously described [17], after exposure of EC cells to low-dose lidamycin, adiramycin and RA, respectively (Fig. 2A). The inhibitory effect of lidamycin on Oct4 expression in EC cells was similar to that of RA. However, *an* anti-tumor *drug* adiramycin [23] showed little effect on Oct4 expression of EC cells. This result was confirmed by Western blot (Fig. 2B). The protein level of Oct4 was markedly inhibited in lidamycin- or RA-exposed cells, but there was no change of Oct4 expression in the adiramycin-exposed cells. These results

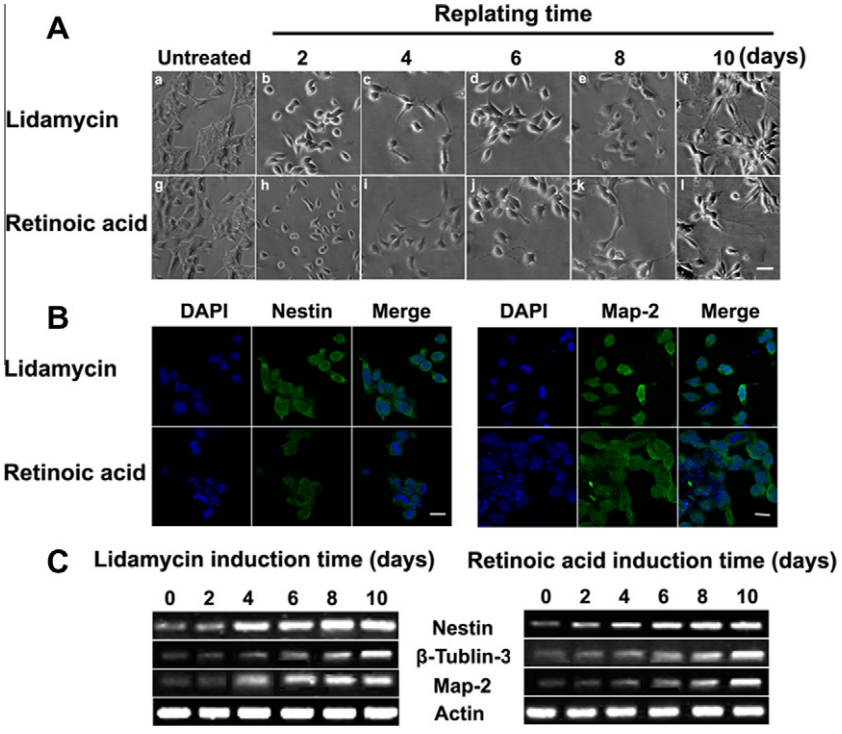

Fig. 1. Induction of neural differentiation of P19 EC cells by lidamycin. (A) Morphology of lidamycin- induced P19 cells undergoing neural differentiation. Scale bar: 40 μm. (B) Expression of neural marker nestin and Map2 by immunostaining. Lidamycin- induced P19 cells immunolabeled with anti-nestin and anti-Map2 antibody and TRITC-conjugated secondary antibody (red). The nuclei were stained with DAPI (blue). The cells were examined with confocal microscopy. Scale bar: 50 μm. (C) Gene expression of nestin, tublin III and Map2 by PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

suggested that lidamycin suppressed Oct4 expression in EC cells. p21 is a cyclin-dependent kinase inhibitor (CKI). It played roles in biological functions of cell cycle control and apoptosis, and additionally played unexpectedly complex role in differentiation [24,25]. After exposure of EC cells to lidamycin, adiramycin and RA, the effect on p21 expression was observed by Immunostaining and Western Blot (Fig. 2C and D). The kinetics of Oct4 expression was tested to demonstrate the relationship between the reduced Oct4 and the increased p21 expression. RT-PCR analysis showed that when EC cells were exposed to lidamycin, the levels of Oct4 were down-regulated within 6–72 h, coincidently, there was relative increase in levels of p21 within 6–72 h (Fig. 2E and F). These results suggested that lidamycin suppressed Oct4, and simultaneously induced the activation of p21 gene (Fig. 2G).

3.3. Knockdown of Oct4 increased p21 expression and induced neural differentiation of P19 cells

To confirm whether the down regulation of Oct4 is sufficient to promote neuronal differentiation, we used siRNA against Oct4 (siOct4) to suppress Oct4 expression in P19 cells. Transient transfection of siOct4 in P19 cells efficiently reduced the endogenuous Oct4 protein expression, with a concomitant increase in basal p21 levels by immunoblotting of crude lysates, as compared to that of GAPDH siRNA or negative siRNA-transfected cells (Fig. 3A).

To evaluate whether knockdown of Oct4 induces neural differentiation, siRNA targeting Oct-4 was transfected into P19 cells and we found that knockdown of Oct4 caused neural differentiation at 48 h after the cells were transfer to differentiation medium (Fig 3B). Immunochemical staining showed that after Oct4 was depleted 6 days, expression of p21 and neuronal markers Nestin and Map2 was up-regulated (Fig. 3C). Further, RT-PCR analysis was performed to evaluate mRNA level of Oct4, p21, Nestin and Map2 after Oct4 was silenced by siRNA. As shown in Fig. 3D, mRNA level of p21, Nestin and Map2 was up-regulated in Oct4-depleted cells. Taken together, our data indicated that Oct4 deficiency induced activation of p21 and promote neuronal differentiation of P19 cells.

3.4. Overexpression of Oct4 blocked neural differentiation and decreased p21 expression in lidamycin-induced P19 cells

To investigate whether over-expression of Oct4 could antagonize lidamycin-induced neural differentiation and decreased p21 expression, Oct4 were over-expressed in lidamycin-induced P19 cells. p21 protein expression was decreased and an undifferentiation state was maintained by over-expression of Oct4, which is tightly controlled with lentivirus-mediated Tet-On system (Fig. 3E and F). Immuno-fluorescence showed a strong positive staining for Oct4, and in contrast, negative or very weak staining for p21, Nestin and Map2 were observed at day 6 in Oct4 over-expressing cells (Fig. 3G). This was confirmed by an experiment of RT-PCR analysis (Fig. 3I). These results indicated that over-expression of Oct4 blocked differentiation and decreased p21 expression in lidamycin induced-P19 cells.

3.5. Repression of Oct4 activated p21 transcription during lidamycin-induced p19 cell differentiation

The expression patterns of Oct4 and p21 in lidamycin-treated P19 EC cells were opposite (Fig. 3). It was assumed that Oct4 suppressed the expression of *p21* gene. To monitor promoter activity of *p21* gene, a reporter luciferase plasmid (*p21-luc*) (Fig. 4A, upper panel) and Oct4 expression vector (Fig. 4A, lower panel) were co-transfected into HEK-293T cells. As shown in Fig. 4B, the reporter luciferase activities were inhibited by expression of Oct4 in a dose-dependent manner (lanes 1–5). Effection of inhibition of Oct4 on *p21* gene was reversed by lidamycin (lanes 6–10). Similar results were observed in P19 EC cells, the reporter luciferase activity increased to 330-folds in the presence of lidamycin, as compared to 140-folds in control (pGL3-Luc-transfected) cells (Fig. 4C). This suggested that lidamycin activated *p21* gene transcription.

It was recently observed that *p21* gene was negatively regulated by Oct4 [4]. The binding of Oct4 to *p21* promoter was studied using chromatin immunoprecipitation (ChIP) assays. As shown in Fig. 4D,

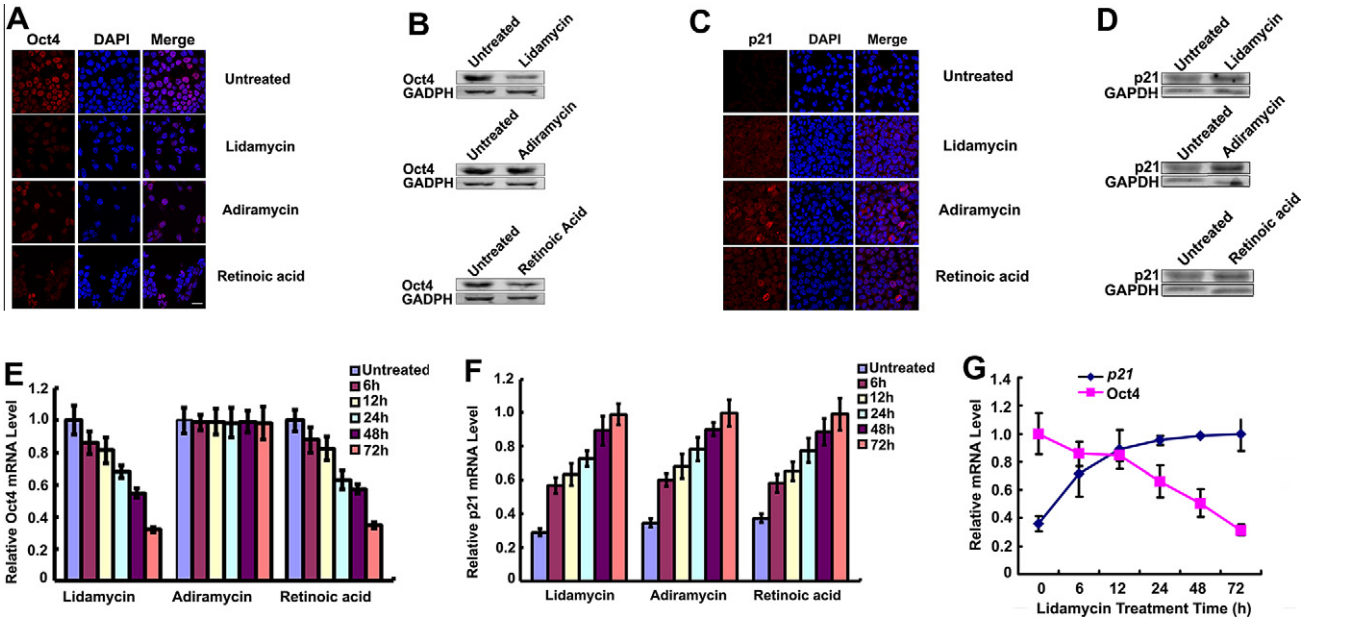

Fig. 2. Suppression of Oct4 expression and up-regulation of p21 in EC cells by lidamycin. (A) and (C) Expression of Oct4 and p21 in mouse P19 cells. The cells were exposed to 0.01 nM lidamycin, 0.5 μM adiramycin and 0.5 μM RA for 48 h. Immunofluorescence staining was performed with anti-Oct4 antibody and TRITC-conjugated secondary antibody (red). The immuno-complex signals were observed under confocal microscopy after nuclei were stained with DAPI (blue). Scale bar: 50 μm. (B) and (D) Western blotting detection of Oct4 and p21 expression. (E) and (F) Real-time PCR of Oct4 and p21 mRNA in EC cells. Actin was use as an internal control. Data was represented as mean ± S.D. ($n = 3$). (I) The relationship between Oct4 and p21 expression. Data was from the (E)/(F) experiments, and presented as mean ± S.D. ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

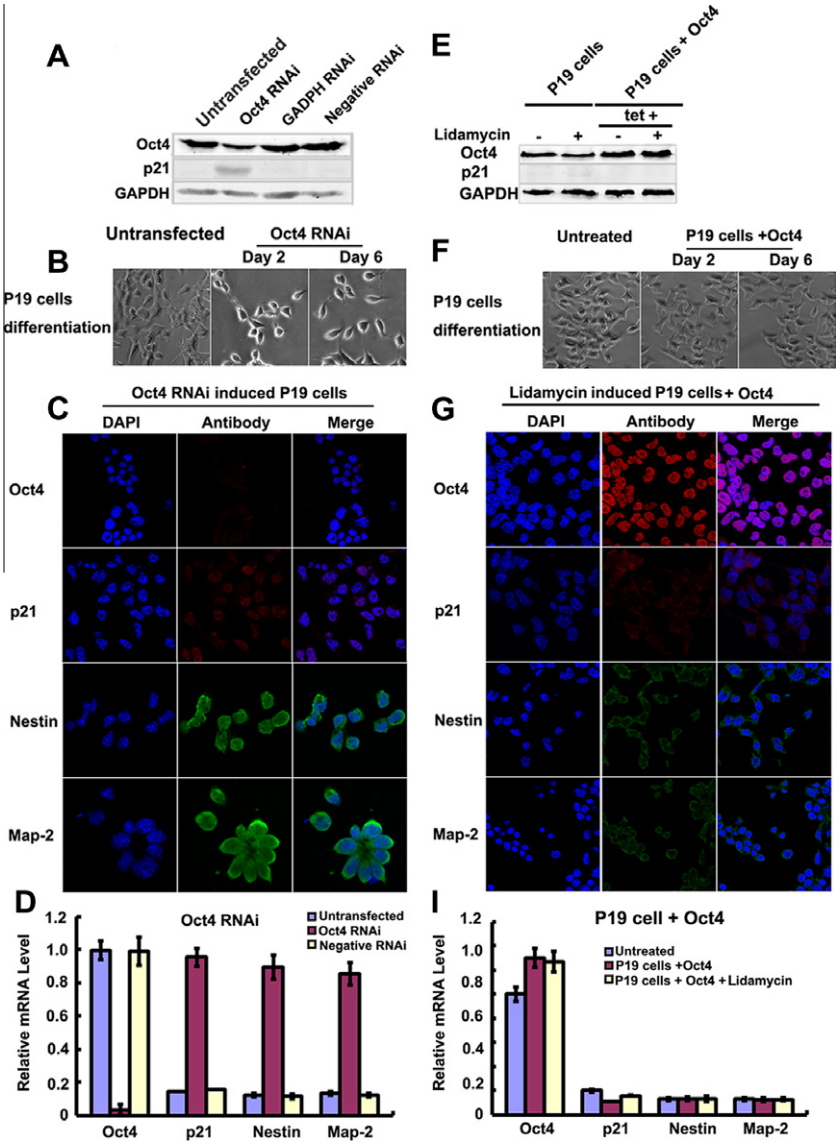

Fig. 3. Knockdown of Oct4 induced cell differentiation and over-expression of Oct4 blocked Lidamycin-induced neural differentiation. P19 cells were transfected with siOct4 (A) and the expression of Oct4, p21, and GAPDH were determined by Western blot in the transfected cells using the indicated antibodies. (B) Representative microscopy images of siOct4 transfected P19 cells. siOct4 transfected P19 cells were cultured in differentiation medium and photos were taken at day 2 or day 6. (C) Molecular changes in siOct4-transfected P19 cells. Expression of Oct4, p21, Nestin, and Map2 was examined by immunofluorescence staining using indicated antibody at day 6 posttransfection of siOct4. The photos were taken with confocal microscopy. (D) RT-PCR analysis of Oct4, p21, Nestin, and Map2 mRNA in siOct4 targeted p19 cells. Data was represented as mean ± S.D. (n = 3). (E) P19 cells were infected with inducible lentivirus system expressing Oct4. The expression of Oct4, p21, and GAPDH in the infected cells were determined by Western blot using the indicated antibodies. (F) Morphological changes of lidamycin-induced P19 cells by over-expression Oct4. P19 cells infected with lentivirus vector expressing Oct4 were cultured in differentiation medium at day 2 or day 6. (G) Molecular changes in P19 cells infected with lentivirus vector expressing Oct4. Expression of Oct4, p21, Nestin, and Map2 was examined by immunofluorescence staining using indicated antibody at day 6 after infection of Oct4 lentivirus. The photos were taken with confocal microscopy. (I) RT-PCR analysis of Oct4, p21, Nestin, and Map2 mRNA in Oct4 lentivirus infected cells. Data was represented as mean ± S.D. (n = 3).

Oct4 binding to *p21* promoter was detected in P19 cells (0 h). After exposure of lidamycin for 12–72 h, Oct4 binding to p21 promoter gradually reduced with time lapse. Consistently, Oct4 binding to the promoters of FGF4 and *p53* genes (as positive controls) was also decreased. This showed that Oct4 binding to *p21* gene promoter was decreased. These results suggested that suppression of Oct4 by lidamycin led to activation of the *p21* gene.

4. Discussion

Lidamycin, derived from a *Streptomyces globisporus* C1027 strain, was a potential anti-cancer drug [16,26], and has been evaluated in a phase II clinical trial for cancer chemotherapy in China [16]. The mechanisms of lidamycin was complicated, and involved in several biological processes including cell cycle arrest, apoptosis, mitotic cell death, DNA damage and chromosome aberrations [15,16,26]. It was observed that low-dose lidamycin inhibited self-renewal capability of mouse P19 EC cells without inducing apoptosis and also suppressed ES cell-like genes [17]. In this study, it was observed that lidamycin induced P19 EC cells neural differentiation was associated with inhibition of Oct4 expression and activation of p21 transcription.

Oct4 played a pivotal role in maintaining undifferentiated pluripotent state in ES and EC cells [1,3]. Loss of Oct4 caused ES and EC cell differentiation. Mouse pluripotent EC P19 cells differentiated into neurons in the presence of RA [9,10]. In this study, it

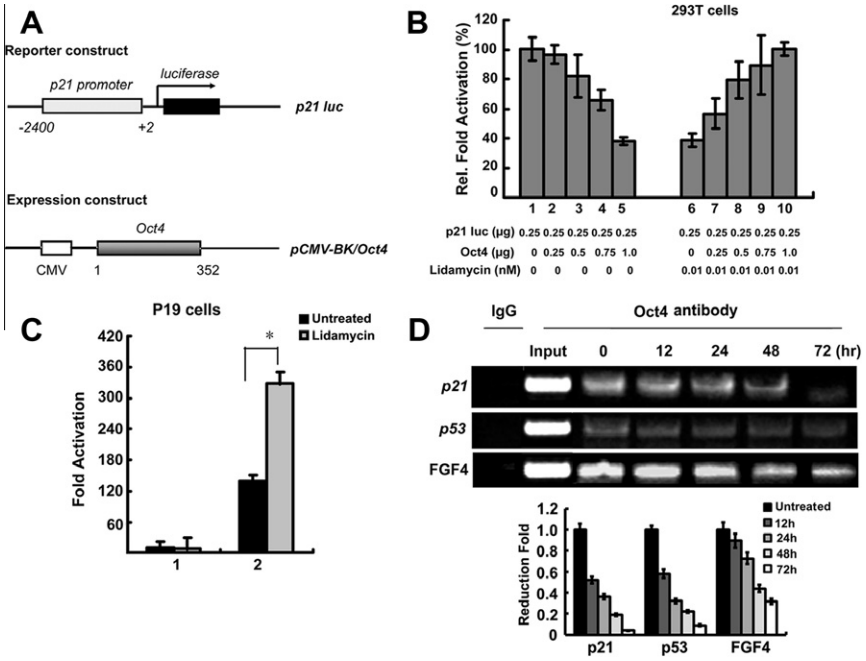

Fig. 4. Down-regulation of Oct4 expression leads to up-regulation of the p21 gene by lidamycin. (A) Schematic diagram of p21-luc reporter construct (upper panel) and Oct4 expression vector (pCMV-BK/Oct4) (lower panel). (B) Transcriptional activation of the p21 promoter by down-regulation of Oct-4 in P19 EC cells. Data was represented as mean ± S.D. (n = 3). (C) Transcriptional regulation of the p21 promoter by lidamycin via decrease of Oct4. (D) ChIP assay for Oct4 binding to the p21 promoter, p53 and FGF4 genes were amplified as positive control.

was observed that low-dose lidamycin had the similar effect as compared to RA, on inducing neuron-like differentiation of P19 EC cells (Fig. 1). This was further confirmed by suppression of Oct4 (Figs. 2 and 3), the molecular markers of ES and EC cells, after lidamycin exposure and Oct4 knockdown. It was concluded that low-dose lidamycin induced neural differentiation in EC cell.

Activation of p21 played an important role in oligodendrocyte maturation and cell differentiation [27,28]. It has been recently reported that Oct4 is a negative regulator of *p21* [4]. It was also found that *p21* gene was activated when NT2/D1 human EC cells underwent neuronal differentiation by genistein [29]. In this study, it was observed that *p21* gene was inhibited by transcriptional factor Oct4, but this inhibition was reversed by lidamycin or knock down of Oct4 (Figs. 3A,C,D, 4B and C). The specific binding of Oct4 to *p21* promoter was decreased in lidamycin-treated P19 cells (Fig. 4D). The data suggested that *p21* gene was negatively regulated by Oct4, and lidamycin exposure induced *p21* gene activation through suppressing Oct4. Thus, our results indicated that lidamycin induced neuronal differentiation by down regulating Oct4. If activation of *p21* plays a key role in lidamycin-induced neuronal differentiation needs further investigation. It is likely that activation of p21 is at least involved in lidamycin-induced neuronal differentiation. Since many cancer cells re-express Oct4, which is an ES cell-like gene [30,31], the data provided a novel mechanisms that lidamycin induced differentiation of cells in chemotherapy. The further studies will be required to understand exact mechanism by which lidamycin induced differentiation of EC cell.

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