Carcinogenesis vol.32 no.12 pp.1890–1896, 2011
doi:10.1093/carcin/bgr221
Advance Access publication October 7, 2011

Ling Zhi-8 mediates p53-dependent growth arrest of lung cancer cells proliferation via the ribosomal protein S7-MDM2-p53 pathway

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Ling Zhi-8 (LZ-8), an immunomodulatory protein, is derived from and has been cloned from the medicinal mushroom *Ganoderma lucidum* (Reishi or Ling Zhi); this protein exhibits immunomodulating and antitumor properties. We investigated the effects of recombinant LZ-8 protein (rLZ-8) on the proliferation of A549 human lung cancer cells. Here, we showed that rLZ-8 inhibits cell growth and that this is correlated with increased G_1 arrest. The treatment of A549 cells with rLZ-8 activated p53 and p21 expression, and both the G_1 arrest and the antigrowth effect were found to be p53 dependent. It was further demonstrated that rLZ-8 inhibited tumor growth in mice transplanted with Lewis lung carcinoma cells. Interestingly, rLZ-8 treatment was found to lead to nucleolar stress (or ribosomal stress) as evidenced by inhibition of precursor ribosomal RNA synthesis and reduced polysome formation in A549 cells. These changes resulted in an increasing binding of ribosomal protein S7 to MDM2 and a decreased interaction between MDM2 and p53. Taking these results together, we have identified a novel rLZ-8 antitumor function that positively modulates p53 via ribosomal stress and inhibits lung cancer cell growth *in vitro* and *in vivo*. Our current results suggest that rLZ-8 may have potential as a therapeutic intervention for the treatment of cancers that contain wild-type p53 and high expression of MDM2.

Introduction

Ganoderma lucidum (Ling Zhi or Reishi), a medicinal fungus and mushroom, has long been used in traditional Chinese medicine (TCM) and in Asia for promotion of health and longevity as well as for the treatment of various diseases (1). *Ganoderma lucidum* has been reported to contain immunomodulating and antitumor agents (1–3). For example, it has been shown that *G.lucidum* inhibits cell proliferation, induces apoptosis or growth arrest and suppresses cell migration of various cancer cells (4–6). *Ganoderma lucidum* also inhibits tumorigenesis and metastasis of human hepatoma cells in both cell and animal models (7); however, the detailed mechanisms by which there is inhibition of tumor proliferation are not well understood.

Ling Zhi-8 (LZ-8), an immunomodulatory protein, was isolated from the mycelia of *G.lucidum* (8) and a complementary DNA and a gene encoded for LZ-8 were cloned (9). This protein is regarded as one of the major bioactive substances of *G.lucidum* (8,9). LZ-8 has excellent thermal and acid stability together with a moderate resistance to alkali and dehydration (10). LZ-8 consists of 110 amino acids and has a molecular mass 12.4 kDa, which is similar to that of the variable region of the immunoglobulin heavy chain (11,12). LZ-8 exerts mitogenic activities *in vitro* and has also been shown to have immunomodulating activities *in vivo* (8,13). Moreover, stimulation of human peripheral blood lymphocytes with rLZ-8-induced cytokines production (14). We demonstrated that a protein kinase-dependent pathway is involved in LZ-8-induced IL-2 production within human T cells (15). It has also been demonstrated that LZ-8 is able to effectively promote the activation and maturation of immature dendritic cells via the nuclear factor-kappaB and mitogen-activated protein kinase pathways (16). Nonetheless, the role of LZ-8 as an antitumor agent and the signal pathways involved in this process are not well understood. In our current report, we dissected the process by which LZ-8-mediated inhibition of tumor proliferation occurs.

The p53 gene is the most frequently mutated gene in human cancers. This results in the loss of p53's function as a transcriptional factor and as a cellular gatekeeper for growth and division (17). In cancers without p53 mutations, the p53 pathway is often inactivated, which partly occurs via MDM2, a negative regulator of p53 (18). Many stress signals, such as genotoxic DNA damage, can disrupt the MDM2-p53 feedback loop, which leads to activation of the p53-dependent response (19). One of the 'stressors' is so-called nucleolar stress (or ribosomal stress) and results from disruption of ribosome synthesis or biogenesis (20,21). In response to ribosomal stress, several ribosomal proteins (RPs), including L11 (22,23), L23 (24), L5 (25), S7 (26,27) and L26 (28), are released from nucleolus and bind to MDM2 in nucleoloplasm. These RPs–MDM2 interactions inhibit MDM2-mediated p53 ubiquitination and degradation; this thereby activates p53-dependent cell cycle arrest or apoptosis (29,30).

In the current study, we used human lung cancer cells A549, and Lewis lung carcinoma (LLC)-1 xenograft-mice, as *in vitro* and *in vivo* models, respectively, to assess the efficacy of rLZ-8 protein as an anticancer agent. Moreover, we investigated the molecular mechanisms involved in this anticancer process. Our findings are the first to show that rLZ-8 regulates p53 stabilization in cancer cells via the RPS7–MDM2 pathway. The current results suggest that rLZ-8 is a potential novel chemoprevention and treatment agent for lung cancer.

Materials and methods

Cell lines and cell culture

The human lung carcinoma cell line A549 and the Lewis lung carcinoma cell line LLC1 were purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). A549 was maintained in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. LLC1 was maintained in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum, 1% NEAA, 1% pyruvate, 1% L-Glutamine and 1% penicillin/streptomycin. All cell lines were cultured in a 5% CO_2 atmosphere at 37°C.

Preparation of plasmids and strains for the recombinant LZ-8 (rLZ-8) production

The rLZ-8 protein from *Saccharomyces cerevisiae* was produced as described previously (31). Briefly, the LZ-8 gene was cloned into the pYEX-S1 plasmid (Clontech) and this was followed by transformation into *S.cerevisiae* DBY747. The cell lysates were collected by centrifugation and purified by AKTA® fast protein liquid chromatography system. The amount of rLZ-8 protein was measured and its purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot.

Generation of two isogenic cell lines containing mutant p53 genes

A549 cells were transfected using Lipofectamine 2000 (Invitrogen) with either of two p53 mutant plasmids, namely pC53-SCX3(R248W) and pC53-

Abbreviations: LLC, Lewis lung carcinoma; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; pre-rRNA, precursor ribosomal RNA; RP, ribosomal protein.

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SCX3(V143A), which encode p53 proteins with dominant-negative mutants (32). The mutant p53 plasmids were kindly provided by B.Vogelstein (Johns Hopkins University, USA). Forty-eight hours after transfection, the cells were selected using medium containing G418 (400 μg/ml) and the G418-resistant cells were pooled for the subsequent studies.

Cell viability assay

Cells were seeded in triplicate on a 96-well plate and incubated overnight before treated with rLZ-8 (5–15 μg/ml) for 12–48 h. After incubation, 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide dye was added and the mixture incubated for 2 h. The medium was then aspirated and replaced with acidic isopropanol (in 0.1 N HCl). The absorbance was determined on a microplate reader at 570 nm.

Flow cytometry analyses

The cells were treated with rLZ8 (0–15 μg/ml) for 12–48 h and then trypsinized; next, the cell pellet was resuspended in cold phosphate-buffered saline (PBS) to which cold ethanol was added for 30 min at −20°C. After this treatment, the cells were centrifuged and suspended in PBS, which was followed by incubation with RNase A (20 mg/ml) for 30 min. After being chilled over ice for 10 min, the cells were incubated with propidium iodide (400 μg/ml) for 30 min and finally analyzed by flow cytometry. Flow cytometry was performed using a FACScan (Becton Dickinson, Germany). A minimum of 10 000 cells per sample were collected and the DNA histograms were further analyzed by Win MDI software.

Western blotting analyses

Total cell lysates, either from a cell culture or from the tumors obtained from xenografts, were subjected to western blotting as described previously (33). The antibodies used were anti-p53 (DO-1) from Oncogene for A549 cell and anti-p53 (PAb421) from CALBIOCHEM for LLC1 cell, anti-p21 (CP74), anti-p27 (DCS-72) and anti-actin (AC-15) from Sigma and anti-MDM2 (SMP14) and anti-S7 (44-K) from Santa Cruz Biotechnology (Santa Cruz, CA)

p53 functional assay

A549 cells were transiently transfected with the p53-Luc reporter and the pRL-TK Renilla luciferase reporter (Promega, Madison, WI) at a ratio of 25:1 using Lipofectamine 2000. One day after transfection, the transfected cells were treated with LZ-8 for 48 h. p53 promoter activity was assayed using a Dual Luciferase Reporter kit from Promega according to manufacturer's instructions. Luciferase activities were measured on Multilabel counter (Perkin Elmer, Waltham, MA).

Analysis of polysome profiles

Polysome analysis was performed as described previously (21). Briefly, cells were treated with cycloheximide (200 μM, 15 min) and lysed in 400 mM K(OAC), 25 mM K-HEPES, 5 mM $Mg(OAC)_2$, 200 μM cycloheximide, 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 40 U/ml RNasin at 4°C for 30 min. After low speed centrifugation (8000*g*, 5 min), the supernatants were centrifuged in an SW41 rotor for 3 h at 35 000 r.p.m. in a 15–50% sucrose gradient containing 400 mM K(OAC), 25 mM K-HEPES, 5 mM $Mg(OAC)_2$, 200 μM cycloheximide, 0.1% Triton X-100 and 10 U/ml RNasin. The optical density of fractions was measured at 260 nm.

RNA interference with sh-p53 or sh-S7

The shRNA target sequence used were 5′-GTCCAGATGAAGCTCCCAGAA-3′ (TRCN0000003755) for p53 (sh-p53) and 5′-CGGAAAGCTATCATAATCTTT-3′ (clone ID: TRCN0000074764) for S7 (sh-S7); pLKO.1-sh-Luc (TRCN0000072243, sh-Luc) was used as a control for the RNA interference experiments. All clones were obtained from TRC library (National RNAi Core Facility, Academia Sinica, Taiwan). VSV-G-pseudotyped lentiviruses were made by transfecting HEK 293T cells with sh-p53 or sh-S7 using JetPEI reagent (Polyplus Transfection, New York, NY) and the plasmids were packaged using the three-plasmid lentivirus packaging system (34,35). Supernatants containing the VSV-G-pseudotyped lentiviruses were collected 36–48 h after transfection. The supernatants were filtered, aliquoted and stored at −80°C. A549 cells were then infected with lentivirus expressing either sh-p53 or sh-S7 together with sh-Luc in the presence of protamine sulfate (0.8 μg/ml) for 24 h followed by protamine sulfate (2 μg/ml) for 48 h. Western blotting was performed to determine the knockdown efficiency.

Reverse transcription–polymerase chain reaction analysis

Cells were treated with rLZ-8 (10 μg/ml) for 4–12 h. Total RNA was isolated using RNeasy mini kits (QIAGEN). A total of 2 μg of RNA was reverse transcribed with random primers using a reverse transcription system (Invitrogen). Polymerase chain reaction (PCR) was performed in RBC *Taq* DNA polymerase Mastermix (RBC Bioscience, Taipei, Taiwan). The reverse transcription–PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. The primers 5′-GCTCTACCT-TACCTACCTGG-3′ and 5′-TGAGCCATTCGCAGTTTCAC-3′ were used to amplify a fragment containing the 112 bp precursor ribosomal RNA (pre-rRNA) encompassing the 5′-external transcribed sequence (ETS) and part of the 18S rRNA (36), whereas the primers for the 5S rRNA were 5′-GGCCA-TACCACCCTGAACGC-3′and 5-CAGCACCCGGTATTCCCAGG-3′. The primers for the GAPDH controls were 5′-GGTGGTCTCCTCTGACTT-CAAC-3′ and 5'-TCTCTCTTCCTCTTGTGTTCTTG-3′.

Coimmunoprecipitation

In total, 500–1000 μg of cell lysate was immunoprecipitated with either MDM2 or S7 antibodies overnight at 4°C. Protein G-bound agarose (Millipore, Billerica, MA) was then added to each sample and incubated for 2 h at 4°C. The beads were washed with PBS buffer, and the immunoprecipitate was separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which was followed by immunoblot analysis using specific antibodies against p53, MDM2 or S7.

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Anti-S7 was used as primary antibody followed by Alexa Fluor 488 (green) goat anti-mouse antibody (Molecular Probes, Eugene, OR). 4′, 6-diamidino-2-phenylindole (DAPI) was used for DNA staining. The stained cells were analyzed using a laser scanning confocal microscope (Olympus FV1000, Center Valley, PA).

In vivo *LLC1 tumor mice model*

Male C57BL/6 (H-2b; 6–8 weeks of age) mice were purchased from National Laboratory Animal Center (Taipei, Taiwan). The mice were weighed and randomly distributed across the different experimental groups ($n = 3$). At day 0, approximately 2×10^5 LLC1 cells suspended in 100 μl serum free Dulbecco's modified Eagle's medium were subcutaneously implanted into the right flank of all mice. The mice consisted of three groups, each of three animals. Group one animals were treated with intraperitoneally PBS and served as controls. The animals of Groups 2 and 3 were treated with intraperitoneally rLZ8 at doses of either 4 or 12 mg/kg. Treatment took place on days 4, 8, 12 and 16. Tumor size was measured for each mouse everyday using a set of digital calipers, and the tumor volume was calculated by the formula $L1 \times L2 \times H$, where L1 is long diameter, L2 is the short diameter and H is the height of the tumor. The mice were killed on day 28 and the tumors were then excised, weighted and stored at −80°C for further analysis.

Results

The rLZ-8 protein and rLZ-8 inhibits proliferation of human A549 lung cancer cells

The purity of rLZ-8 protein was >98% as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by Coomassie Blue staining (see Supplementary Figure S1A available at *Carcinogenesis* Online) and western blotting (see Supplementary Figure S1B available at *Carcinogenesis* Online). To test whether rLZ-8 treatment might affect cancer cell growth, A549 human lung cancer cells were treated with various concentration of rLZ-8 for various times and cell growth analyzed. As shown in Figure 1, rLZ-8 inhibited cell growth significantly at the higher doses (10 and 15 μg/ml) after 48 and 72 h of incubation time. At a lower concentration of rLZ-8 (5 μg/ml), growth inhibition was not apparent until after 72 h of incubation. Similar results were observed when cell growth inhibition was measured by the trypan blue exclusion assay (data not shown).

rLZ-8 enhances G_1 arrest, expression of p53 and p53 transactivational activity

The signal pathway involved in antigrowth effect of rLZ-8 on A549 cells was assessed by first examining cell cycle distribution in the rLZ-8-treated cells. When the cells were treated with rLZ-8 for 24 h, the percent of G_1 arrest cells was increased from 64.9% in the control cells to 80.5% in the cells treated with rLZ-8 (15 μg/ml); whereas, the sub-G_1 population was increased only slightly from 1 to 6% under similar circumstances (Figure 2A). The expression of three proteins, p53, p21/Cip1 and p27/Kip1, was increased after rLZ-8 treatment (Figure 2B). Both p21 and p27 are cyclin-dependent kinase inhibitors that are associated with the G_1 checkpoint; furthermore, p21 is directly regulated by p53 (37). When a p53 reporter system was transiently transfected into A549 cells, the transactivational activity of p53, measured in terms of

relative luciferase activity, was increased in the rLZ-8-treated cells (Figure 2C). UV treatment of A549 cells, which is known to cause DNA damage and activate p53, served as a positive control.

rLZ-8 induced growth arrest is p53 dependent

To determine whether rLZ-8 induced G_1 arrest is p53 dependent, the p53 gene was knocked down using a sh-p53-expressing lentivirus and this was followed by rLZ-8 treatment (see Materials and Methods). We demonstrated that the level of p53 protein in these A549 cells was significantly reduced by sh-p53 but not by sh-Luc as a control (Figure 3A). The increase in G_1 arrest caused by rLZ-8 was abrogated (Figure 3B). We further generated isogenic A549 cells that contained either a R248W p53 mutant gene or a V143A p53 mutant gene. Both p53 mutants are known to exhibit a dominant-negative effect on wild-type (wt) p53 protein (32). As expected, the p53 R248W and V143A transfectants showed high levels of the mutant p53 proteins (Figure 3A, right) but were not activated by rLZ-8 treatment. The failure of p53 activation in these p53 mutant isogenic cell lines was confirmed by the fact that these cells had low levels of p21 protein expression. When the cells were treated with rLZ-8, the G_1 arrest induced by rLZ-8 was reduced in the transfectants compared with vector-only cells (Figure 3B, right). These results support the hypothesis that rLZ-8 induced growth arrest in A549 cells is p53 dependent.

rLZ-8 exhibits antitumor activity in vivo

It seemed likely that rLZ-8 would inhibit the growth of mouse LLC1 cells *in vitro* and therefore the *in vitro* and *in vivo* effects of rLZ-8 on these cells were explored. Initially, the effect of rLZ-8 treatment on the LLC1 cells *in vitro* was examined. rLZ-8 treatment resulted in a significant dose-dependent decrease in the growth curve of LLC1 at the doses of 10–15 μg/ml (Figure 4A and Supplementary Figure S2B which is available at *Carcinogenesis* Online). This treatment also increased the percentage of G_1 phase slightly after 24 h exposure (Figure 4B). Western blotting analysis showed that rLZ-8 increased levels of p53, p21, MDM2 and p27 in LLC1 cells (Figure 4C). These results are similar to those of A549 cells and suggest that p53 protein is also involved in antigrowth effect of rLZ-8 on LLC1 cells.

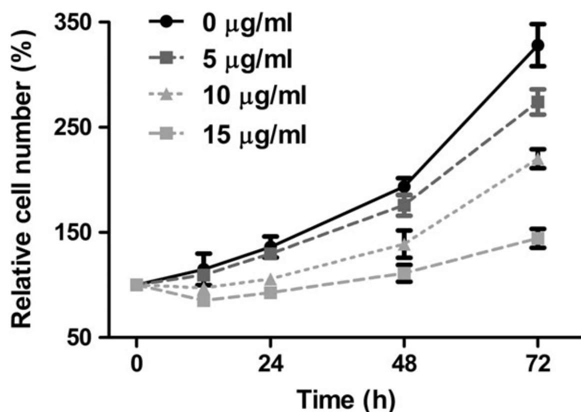

Fig. 1. rLZ-8 inhibits proliferation of A549 lung cancer cells. A549 cells were treated with various concentrations of rLZ-8 as indicated for 12–72 h. The relative cell numbers were measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay. The results are expressed as percentages of cell numbers relative to the control and represent the mean ± standard error of the three experiments.

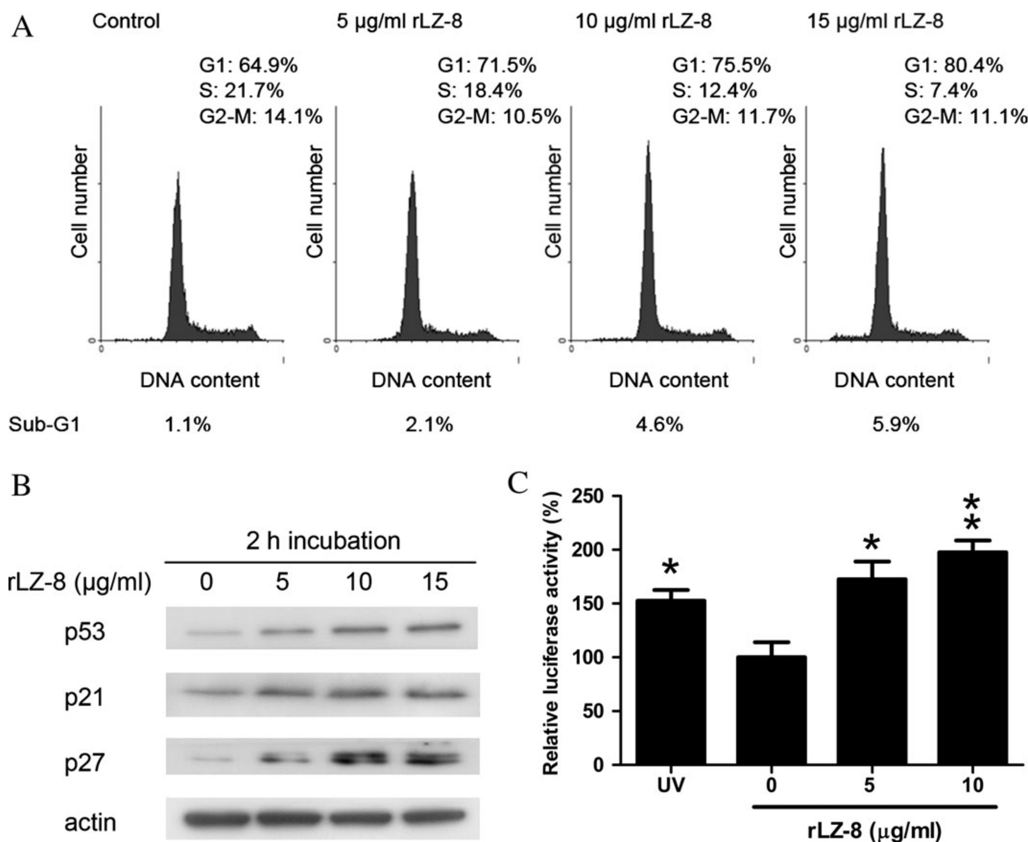

Fig. 2. rLZ-8 increases G_1 arrest and enhances p53 transactivational activity in A549 cells. (**A**) Cells were treated with rLZ-8 for 24 h, stained with PI and analyzed by flow cytometry. The dataset is representative example of duplicate experiments. (**B**) p53 protein levels were examined by western blotting. (**C**) Cells were cotransfected with p53 luciferase reporter and Renilla luciferase vector for 24 h followed by rLZ-8 treatment for 6 h. UV-treated (10 J/m^2) cells were used as a positive control. The luciferase activity was normalized against the internal control of Renilla luciferase. The data were expressed relative to the cells without rLZ-8. Columns mean from three independent experiments. (Column, mean (n = 3); bars, SD; $^*P < 0.05$, $^{**}P < 0.005$).

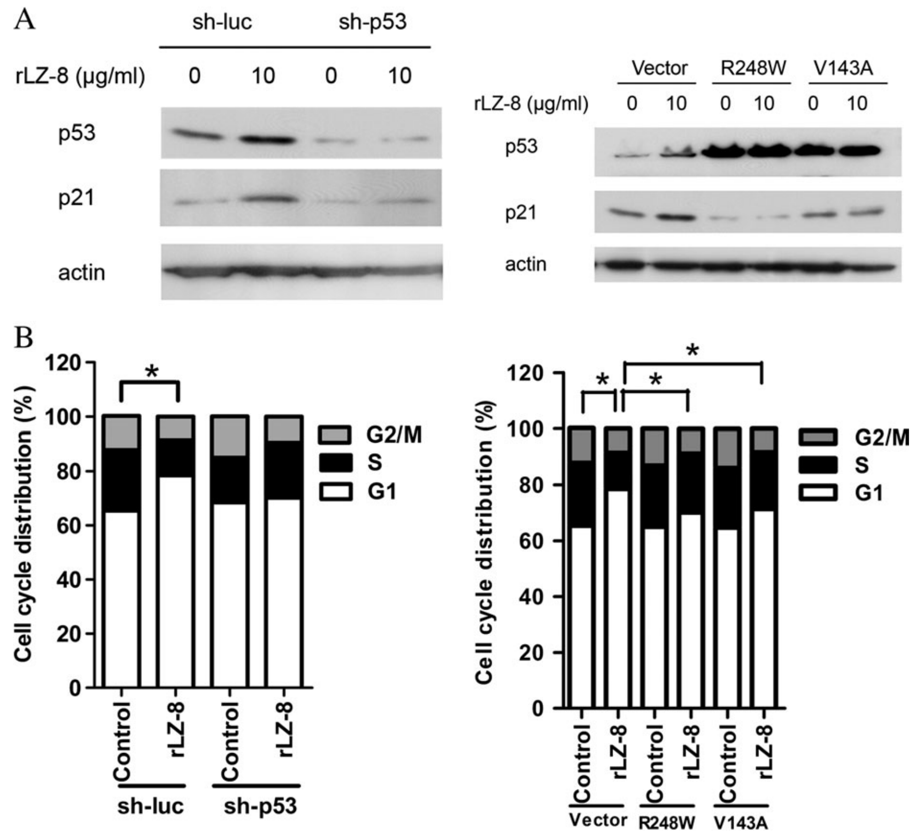

Fig. 3. rLZ-8 enhances G_1 arrest in A549 but this is less apparent in p53 knockdown or A549 isogenic cells. Cells were infected with lentiviruses expressing sh-p53 or sh-luc (vector control) for p53 knockdown. A549 R248W or V143A isogenic cells carry transfected R248W or V143A p53 mutant plasmids where G418-resistant cells were pooled as A549 isogenic cells. (see Materials and Methods). (**A**) Protein expression was analyzed by western blotting after treatment with rLZ-8 for 2 h. (**B**) Cell cycle distribution was measured by flow cytometry after treatment with rLZ-8 (10 μg/ml) for 24 h. (Column, mean ($n = 3$); bars, SD; $^*P < 0.05$).

Next, we injected LLC1 cells subcutaneously into C57BL/6 mice. Four weeks after implantation, the mice bearing tumors were injected intraperitoneally with rLZ-8. No apparent differences in body weights were observed between LCC1 mice that received rLZ-8 and those that received the PBS control (Figure 5A). In contrast, the tumor volumes in the LLC1-transplanted mice were significantly decreased in mice receiving rLZ-8 treatment compared with the mice receiving PBS treatment (Figure 5B). The mice were killed when the tumor volume reached 2000 mm^3 at ∼20 days for the control group. Western blot analysis of the cell lysates derived from tumor tissue in the mice receiving rLZ-8 treatment showed that the levels of p53, p21, MDM2 and p27 were increased (Figure 5C).

rLZ-8 inhibits pre-rRNA synthesis and alters the polysome profile of cells

Recently, perturbations of ribosomal biogenesis have been shown to trigger a stress signal mediated by RPs and this inhibit MDM2 and activates p53 (29). To investigate whether treatment with rLZ-8 affects ribosomal biogenesis in A549 cells, we first examined the effect of rLZ-8 on pre-rRNA synthesis. As shown in Figure 6A, rLZ-8 treatment (4–12 h) reduced the levels of pre-rRNA as measured by reverse transcription–PCR assay. When the polysome profile of rLZ-8-treated cells was compared with that of untreated control cells, rLZ-8 was found to reduce the level of polysomes, of 80S ribosomes and of other ribosomal subunits (Figure 6B). Such an alteration to the cells' polysome profile strongly suggests that there is perturbation of ribosome biogenesis due to the presence of rLZ-8 and that this is likely to cause nucleolar stress (21,36).

rLZ-8 enhances the interaction of MDM2 with RPS7

To further determine whether the nucleolar stress caused by rLZ-8 leads to an accumulation of free forms of RP in the nucleoplasm, the cellular localization of the S7 ribosomal protein (RPS7 or S7) in response to rLZ-8 was explored. S7 has been identified as an MDM2-interacting partner and the level of S7 is able to modulate the interaction of MDM2 and p53 in response to ribosomal stress (26,27). As shown in Figure 6C, S7 was detected mainly in the nucleolus in the control cells and it was found that rLZ-8 treatment led to a redistribution of S7 from the nucleolus into the nucleoplasm. To further demonstrate whether this redistribution of S7 affects its interaction with MDM2, coimmunoprecipitation experiments were carried out. rLZ-8 treatment enhanced the interaction of MDM2 with S7 as evidenced by the increased levels of S7 protein when anti-MDM2 antibodies were used for immunoprecipitation. In contrast, p53 protein decreased in the S7–MDM2–p53 complex (Figure 6D, middle panel). The increased binding of MDM2 to S7 was also true in a reciprocal experiment where anti-S7 antibodies were used for immunoprecipitation (Figure 6D, right panel). Total amount of S7 protein in the rLZ-8-treated cells remained relatively unchanged (Figure 6D, left panel). Together, these results support the hypothesis that rLZ-8-induced p53 activation involves a suppression of MDM2–p53 interaction by RPS7 and further supports the involvement of the RPS7–MDM2–p53 pathway as a response to rLZ-8 treatment.

We further performed a shRNA-mediated RPS7 knockdown experiment. A reduction in S7 protein resulted in an inhibition of rLZ-8-induced p53 activation compared with cells treated with sh-Luc (see Supplementary Figure S3, available at *Carcinogenesis* Online). In addition, the rLZ-8 induced G_1 arrest in A549 cells was decreased from 78% in sh-luc control cells to 66% in sh-S7 cells (see supplementary Figure S3B is available at *Carcinogenesis* Online). These results suggested that RPS7 is required for rLZ-8-mediated p53 activation. In conclusion, we have shown that rLZ-8 inhibits the proliferation of lung cancer cells *in vitro* and *in vivo* via p53-dependent cellular arrest. This p53-dependent arrest probably results from

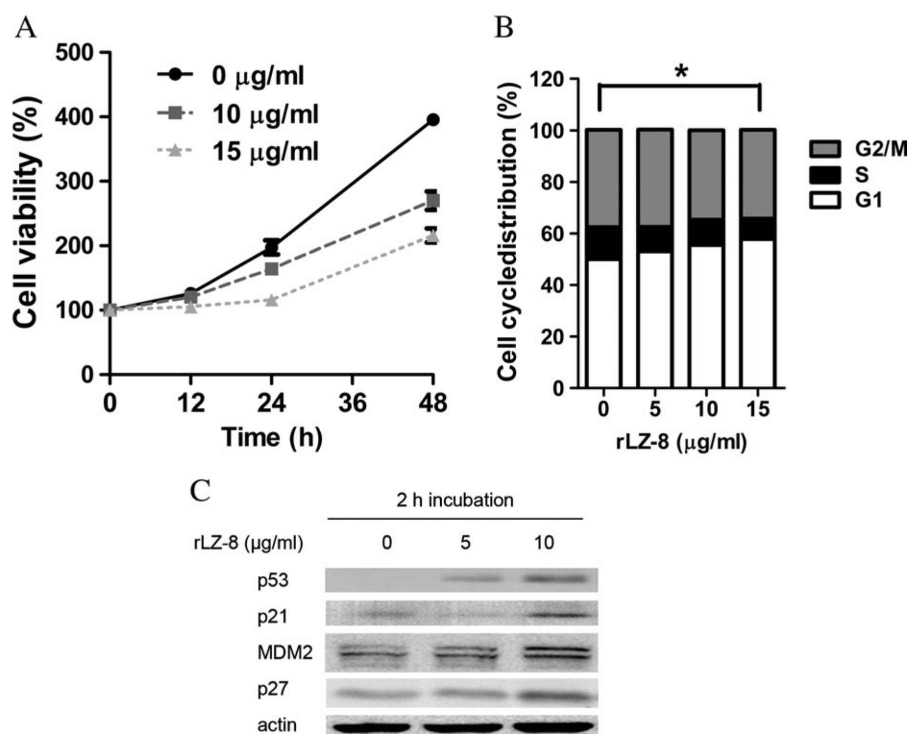

Fig. 4. rLZ-8 reduces cell proliferation and increases p53 protein level and G_1 arrest in LLC1 cells. (**A**) LLC1 cells were treated with rLZ-8 for 12 to 48 h. Relative cell number was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay. (**B**) Cell cycle distribution was measured by flow cytometry after treatment with rLZ-8 for 24 h. ($^*P < 0.05$) (**C**) p53, p21, p27 and MDM2 protein levels were examined by western blotting.

a perturbation of ribosome biogenesis that is induced by rLZ-8. Thus, it would seem that there is a cross talking between rLZ-8 and the S7–MDM2–p53 pathway that provides a novel molecular mechanism whereby rLZ-8 exerts its anticancer function.

Discussion

Previously, rLZ-8 has been shown to exert immunomodulation activity on cells involved in various immune responses (8,13,15). However, this protein's effect on the growth of tumors has not been properly explored. Here, we have demonstrated that rLZ-8 exhibits an anticancer growth effect both *in vitro* and *in vivo* via p53-dependent G_1 arrest. This p53-dependent arrest is probably due to a perturbation of ribosome biogenesis, which is presumably induced by rLZ-8 and results in the interaction of MDM2 with S7 and the stabilization of p53. Nevertheless, how rLZ-8 exerts an effect that perturbs ribosome biogenesis remains to be explored.

The biogenesis of ribosomes is a highly co-ordinated process that involves the synthesis and process of ribosomal RNA, the production of RPs and the assembly of ribosomal subunits. Importantly, this process is tightly linked to cell growth and proliferation including in cancer cells (38), and upregulation of rRNA has been correlated with uncontrolled growth in cancer cells (39–41). We have shown that rLZ-8 reduces pre-rRNA expression, which suggests that rLZ-8 may affect pathways associated with either RNA polymerase I activity or the various transcriptional factors that are involved in rRNA transcription (21,41). Since p53 regulates the transcription of many genes including rRNA (42), we hypothesize that one possibility is that rLZ-8 activated p53 may directly regulate the transcription of rRNA. Moreover, from the results of the polysome study, rLZ-8 may also affect the assembly of either the 40S or 60S ribosome subunits or, alternatively, the assembly of polysomes.

It is well known that various stresses can activate p53 through disruption of p53–MDM2 interaction (19). For instance, DNA damaged caused by ionizing radiation or by genotoxic chemicals is known to trigger activation of ataxia-telangiectasia-mutated kinase; this leads to phosphorylation of p53 and the disruption of the p53–MDM2 interaction (43). In our current study, we did not observe any activation of ataxia-telangiectasia mutated (data not shown). Recent studies have shown that perturbation of ribosome biogenesis leads to ribosomal stress (20). In response to ribosomal stress, several RPs, including L5, L11, L23 and S7 (29), can bind to MDM2 and block MDM2-mediated p53 ubiquitination and degradation. This results in p53-dependent cell cycle arrest and/or apoptosis. This RP-MDM2-p53 signaling pathway is thought to provide a surveillance system that monitors the integrity of ribosomal biogenesis (29,30). It has been suggested that each of these RPs may be involved in various pathways in response to a variety of stresses (30). One of these RPs, namely S7, was identified during a screen of MDM2-binding proteins using the yeast two hybrid assay (26). Another study has found that S7 is both a regulator and a substrate of MDM2 (27). Our results indicate that rLZ-8 is a positive regulator of p53 via the S7–MDM2 interaction. Consistent with previous findings (26), knockdown of S7 alone is able to reduce p53-mediated functions and cause p53-dependent growth arrest. (see Supplementary Figure S3, available at *Carcinogenesis Online*).

In addition to the above effects, we also observed downregulation of S6K1 following rLZ-8 treatment for 1–2 h in A549 cells (see Supplementary Figure S4 available at *Carcinogenesis* Online). S6K1 is an activator for RP S6 via phosphorylation, and S6K1 inhibition impairs tumor growth (39). S6K1 is also a downstream effector of mTOR, and down-regulation of S6K activity has been shown to affect the assembly of the translation pre-initiation complex (44). Moreover, knock down of S6 expression impairs 40S biogenesis and the induction of p53 in A549 cells, where these effects are also mediated by another RP L11 (45). It is possible that rLZ-8 may reduce S6 expression via decreased S6K1 and this subsequently results in p53 activation.

Ganoderma lucidum (Ling Zhi), an oriental medicinal mushroom, has long been reported to exhibit antitumor properties. Here, we

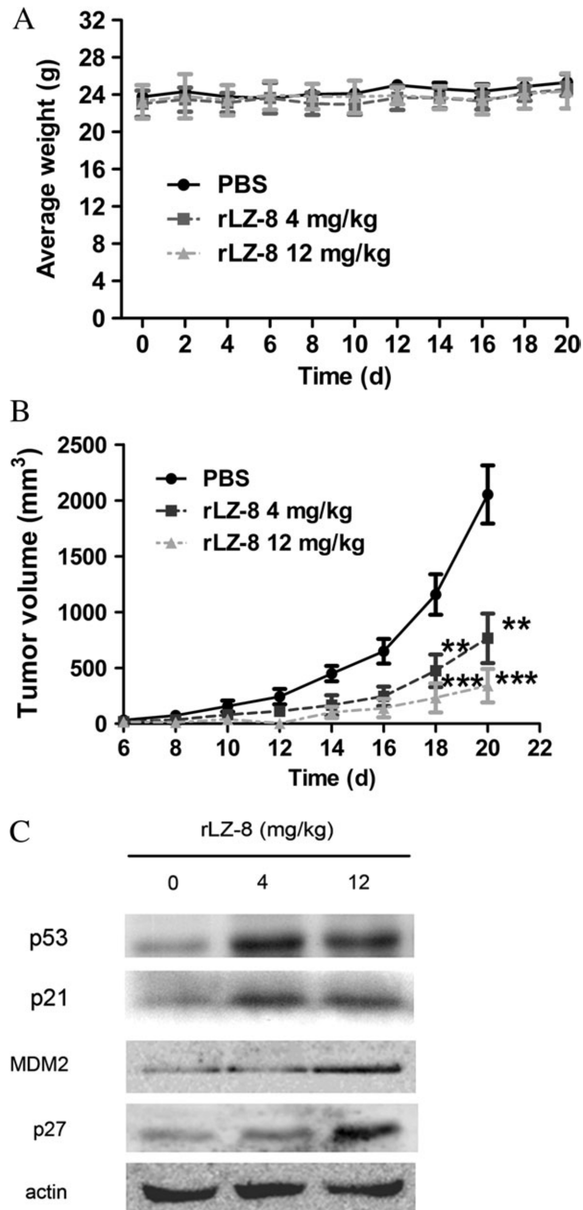

Fig. 5. Inhibition of LLC1 xenograft growth by rLZ-8. LLC1 cells were injected subcutaneously in each flank of C57BL/6 mouse. Four days after implantation, mice were given intraperitoneal injections every 4 days with rLZ-8. The control group received equal volume of PBS. The mice ($N = 9$) were randomly divided into three groups. (**A**) Body weight of the mice over time. (**B**) tumor growth over time. (Column, mean ($n = 3$); bars, SD; $^{**}P < 0.005$, $^{***}P < 0.001$, relative to control). (**C**) p53, p21, p27 and MDM2 protein levels were analyzed from the lysates of the LLC1 xenograft tumors by immunoblotting.

demonstrate that one protein, LZ-8, from *G.lucidum* has antitumor activity. Importantly, the effect of LZ-8, whereby it inhibits cancer cell growth, is not due to DNA damage but rather involves a ribosomal stress activation of p53-mediated growth arrest. Dissection and map of the domains of rLZ-8 that are involved in the RP–MDM2–p53 pathway may provide a clue as to the structure of potential small molecules that might act as therapeutic interventions against cancers in patients that have wild-type p53.

Supplementary material

Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

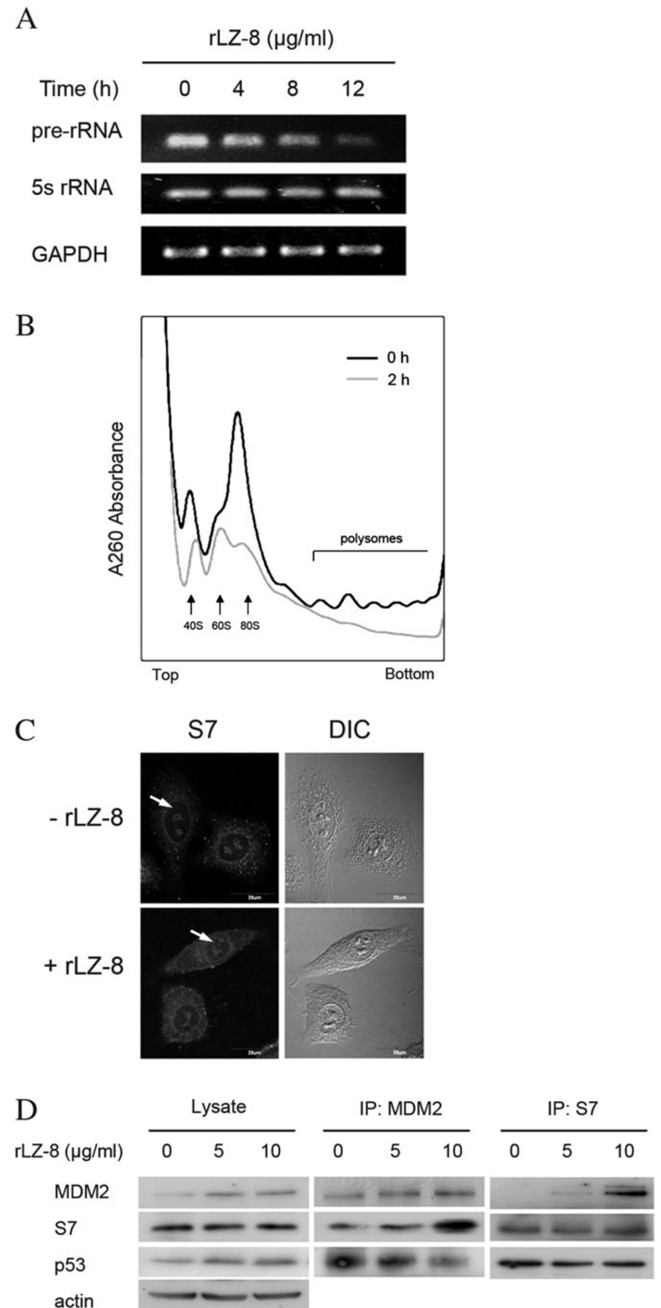

Fig. 6. rLZ-8 treatment causes a perturbation of ribosome biogenesis and enhances RPS7–MDM2 interaction in A549 cells. (**A**) Total RNA was isolated from rLZ-8-treated cells for pre-rRNA expression using reverse transcription–PCR analysis and normalized against GAPDH messenger RNA. (**B**) Cells were treated with rLZ-8 (15 μg/ml), and cytoplasmic extracts were centrifuged on sucrose gradient for polysome analysis. (**C**) Redistribution of RPS7 (S7) into nucleoplasm in rLZ-8-treated cells. Cells were treated with rLZ-8 (10 μg/ml) for 6 h followed by immunofluorescent staining. Arrows indicate the nucleolus. (**D**) Cells were treated with rLZ-8 for 2 h, and cell lysates were either immunoprecipitated with anti-MDM2 or anti-S7 antibody followed by western blotting analysis.

Funding

This work was supported by research grants from National Science Council, Taiwan, (NSC 95-2320-B-010-033) (to E.I.-T.C.) (NSC99-2320-B-010-010-MY3, NSC 99-2627-M-010-003-, NSC 100-2627-M-010-003- , NSC 100-2627-M-010-004- and The Ministry of Education, The Top University Project: 100AC-BI1) (to H.-Y. H) and a grant from

the National Yang-Ming University (Ministry of Education, Aim for the Top University Plan), Taiwan. (to H.-Y. H. and E.I.-T.C).

Acknowledgements

We thank Dr Ralph Kirby (National Yang-Ming University) for critical review of the manuscript and Mr Jason Hsu (Fordham University, NY, USA) for helpful editing of the manuscript; Mr Shu-Ming Tsao (National Yang-Ming University) for preparing parts of the figures in the manuscript; Dr Alan Lin (National Yang-Ming University) for experimental assistance. RNAi reagents were obtained from the National RNAi Core facility (The Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan).

Conflict of Interest Statement: None declared.

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Received May 17, 2011; revised September 19, 2011; accepted October 2, 2011