Knockdown of DEPTOR induces apoptosis, increases chemosensitivity to doxorubicin and suppresses autophagy in RPMI-8226 human multiple myeloma cells *in vitro*

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Abstract. DEP domain containing mammalian target of rapamycin (mTOR)-interacting protein (DEPTOR) is an mTOR binding protein that is overexpressed in RPMI-8226 human multiple myeloma cells, and plays an important role in maintaining cell survival. However, knowledge on the effects of DEPTOR knockdown on the biological functions of RPMI-8226 human multiple myeloma cells, is limited. This study aimed to determine the role of DEPTOR in the proliferation, apoptosis and autophagy in these cells and to elucidate the mechanisms by which DEPTOR contributes to the chemosensitivity of myeloma cells. RNA interference was used to reduce the expression of DEPTOR. Cytotoxicity was evaluated by MTT assay. Apoptosis was examined by flow cytometry. DEPTOR mRNA and protein expression in RPMI-8226 cells treated with DEPTOR-specific short hairpin RNA (shRNA) was evaluated by RT-PCR, quantitative PCR and western blot analysis. The expression of apoptosis-associated proteins, autophagy-associated proteins, and the activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway were detected by western blot analysis. Autophagy was also measured by transmission electron microscopy and monodansylcadaverine (MDC). In this study, RPMI-8226 cells were transfected with the DEPTOR-specific shRNA, which resulted in the significant inhibition of the transcription and expression of DEPTOR. The downregulation of DEPTOR inhibited proliferation, enhanced the doxorubicin-induced growth inhibitory effects on RPMI-8226 cells, and increased the expression of cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP). Moreover, the downregulation of DEPTOR suppressed autophagy and inhibited the activation of the PI3K/Akt signaling in RPMI-8226 cells. In conclusion, our data demonstrated that the downregulation of DEPTOR induces apoptosis, increases chemosensitivity to doxorubicin, and suppresses autophagy and the activation of the PI3K/Akt signaling pathway in RPMI-8226 human multiple myeloma cells.

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Introduction

Multiple myeloma (MM) is the second most common hematological malignancy. Although patient survival has significantly improved with the development of new treatments, such as bortezomib and lenalidomide, some MM patients experience long-term remission (1-3). Therefore, the development of novel anticancer strategies for the effective treatment of this disease is required.

DEP domain containing mammalian target of rapamycin (mTOR)-interacting protein (DEPTOR) is an mTOR binding protein that normally functions to inhibit the mTOR complex 1 (mTORC1) and 2 (mTORC2) pathways (4). The expression of DEPTOR has been investigated in a number of human tumors; low expression levels have been observed in the majority of tumors (5). However, DEPTOR has been found to be overexpressed in a subset of MM cells, and can mediate the activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway in these cells (5,6). This indirect mode of PI3K/Akt activation is crucial to the survival of myeloma cells.

The PI3K/Akt signaling pathway is frequently activated in many types of cancer and is therefore a major cell survival pathway. Downstream effectors of the PI3K/Akt pathway include caspase-3 and caspase-9 (7). Their activation has long been associated with proliferation, differentiation and apoptosis (8,9).

On the other hand, mTORC1 and PI3K/Akt have been reported to inhibit the induction of autophagy (10-12). In addition, the connection between autophagy and apoptosis has been extensively investigated over the past decade (13-15). Certain studies have found that the inhibition of autophagy induces apoptosis (16-20).

Therefore, in the present study, we aimed to clarify the role of DEPTOR in the proliferation, apoptosis and autophagy in MM cells, and to elucidate the mechanisms by which DEPTOR contributes to the chemosensitivity of MM cells. We used the RPMI-8226 cell line in which DEPTOR is highly expressed and treated the cells with doxorubicin. In our study, we investigated the role of DEPTOR using RNA interference (RNAi) technology *in vitro*. RNAi is a sequence-specific, post-transcriptional gene silencing technique induced by double-stranded RNA, homologous to the target gene (21).

Materials and methods

Cell culture and reagents. RPMI-8226 cells (Wuhan University, Wuhan, China) were cultured in RPMI-1640 medium (Gibco BRL, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Gibco) at 37˚C in a humidified atmosphere containing 5% CO_2. The following reagents were used: anti-cleaved caspase-3 (Asp175), anti-cleaved poly(ADP-ribose) polymerase (PARP; Asp214), anti-Akt, anti-phosphorylated Akt (p-Akt; Ser 473), anti-phosphorylated P70S6K (p-P70S6K) (Thr421/Ser424), anti-phosphorylated eIF4E-binding protein-1 (p-4Ebp-1; Thr70) (Cell Signaling Technology Inc., Danvers, MA, USA), anti-DEPTOR (Millipore, Billerica, MA, USA), anti-autophagy-related 5 (Atg5), anti-light chain (LC)-3 and anti-GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies, as well as doxorubicin (Sigma-Aldrich, St. Louis, MO, USA).

Lentivirus-mediated gene knockdown. To directly identify the biological function of DEPTOR in MM, an effective RNAi sequence targeting the DEPTOR gene was designed and screened by Genechem (Shanghai, China). Short hairpin RNA (shRNA) was designed using DEPTOR RefSeq cDNA sequence (GenBank accession no. NM_022783.2). The primer sequence was as follows: 5'-CATGACAATCGGAAATCTA-3'. cDNA containing both sense and antisense oligo DNA of the targeting sequence was designed, synthesized and inserted into a GV115-EGFP vector to construct a lentiviral vector that expressed DEPTOR shRNA. Lentiviral DEPTOR shRNA and negative control shRNA were arrested and co-transfected in 293T packaging cells. The negative control sequences have previously been used in a number of studies (22,23), and have no significant homology to any human gene sequences. The lentivirus in the supernatant was collected and filtered and then used to transiently transfect the RPMI-8226 MM cells. Lentivirus production and lentiviral infection were performed by Genechem.

Cell proliferation assay. Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assays following lentiviral infection. Briefly, the cells were seeded at a density of $2x10^3$ cells/well in 96-well plates in 200 μl of medium and cultured for 24, 48 and 72 h. After incubation for a designated period of time, 20 μl of MTT were added to each well and incubated for 4 h. The supernatant was carefully removed and 200 μl DMSO were added to each well. The optical density (OD) of each well was measured at 490 nm. The inhibition rate (%) was calculated as follows: [1 - (OD of the experimental samples/OD of the control)] x100%. The concentration of doxorubicin required to inhibit the growth by 50% (IC_{50}) was calculated. All of the experiments were performed in triplicate.

Flow cytometric analysis of apoptosis. Cells were washed twice with ice-cold PBS and fixed with 70% ethanol at 4˚C overnight. After washing with PBS, cells were incubated in 0.5 ml PBS containing 50 μg/ml RNase A for 30 min at 37˚C, and then propidium iodide (PI) was added to a final concentration of 50 μg/ml and incubated for 30 min in the dark. The resultant cell suspension was then subjected to flow cytometric analysis using a Coulter Epics XL flow cytometer (Beckman Coulter, Inc., Miami, FL, USA). The percentage of apoptotic cells was calculated.

RT-PCR and quantitative RT-PCR (qRT-PCR). Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA (4 μg) was reverse transcribed into cDNA using the Thermoscript RT-PCR System reagent (Gibco) according to the manufacturer's instructions. For quantitative RT-PCR analysis, each 25 μl volume of qRT-PCR was performed using the Applied Biosystems PRISM 7300 sequence detection system (Applied Biosystem, Foster City, USA) in 96-well plates. All reactions were conducted in triplicate. All the threshold cycle (Ct) values were normalized to GAPDH. The $2^{-\Delta\Delta CT}$ method was used to relative quantify the transcriptional level of DEPTOR. Primers were designed for PCR. The primer sequences for human DEPTOR were: 5'-CCTACCCAAACT GTTTTGTCGC-3' (sense) and 5'-CGGTCTGCTAATTTCT GCATGAG-3' (antisense). Primers for the control (GAPDH) were: 5'-TGACTTCAACAGCGACACCCA-3' (sense) and 5'-CACCCTGTTGCTGTAGCCAAA-3' (antisense). The PCR amplification consisted of 35 cycles: 15 sec at 95˚C for denaturation, 30 sec at 60˚C for annealing and 45 sec at 72˚C for elongation, and a final extension of 72˚C for 10 min. PCR was performed for GAPDH as a control to ascertain the amount of the samples. The results were expressed in relation to the control value.

Western blot analysis. Total cell lysates were separated by 6-15% SDS-PAGE gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes, which were blocked with TBST containing 5% non-fat milk at 4˚C overnight and then incubated with anti-cleaved caspase-3, anti-cleaved PARP, anti-Akt, anti-p-Akt, anti-p-P70S6K, anti-p-4Ebp-1, anti-DEPTOR, anti-Atg5, anti-LC-3 and anti-GAPDH antibodies for 2 h. After washing with TBST, membranes were incubated with HRP-labeled secondary antibodies for 2 h at room temperature. The blots were detected using the enhanced chemiluminescence (ECL) reagent kit (Beyotime, Shangshai, China).

Transmission electron microscopy. The treated cells were collected by trypsinization and fixed in 3% glutaraldehyde in 0.1 mol/l phosphate buffer for 1 h at 4˚C. The samples were then fixed with 1% osmium tetroxide in 0.1 mol/l phosphate buffer for 1 h. Ultrathin sections (80 nm) were prepared, stained with uranyl acetate for 15 min, followed by lead citrate for 5 min, and then examined with a Philips EM 208 transmission electron microscope (Philips, Kassel, Germany) at an accelerating voltage of 70 kV.

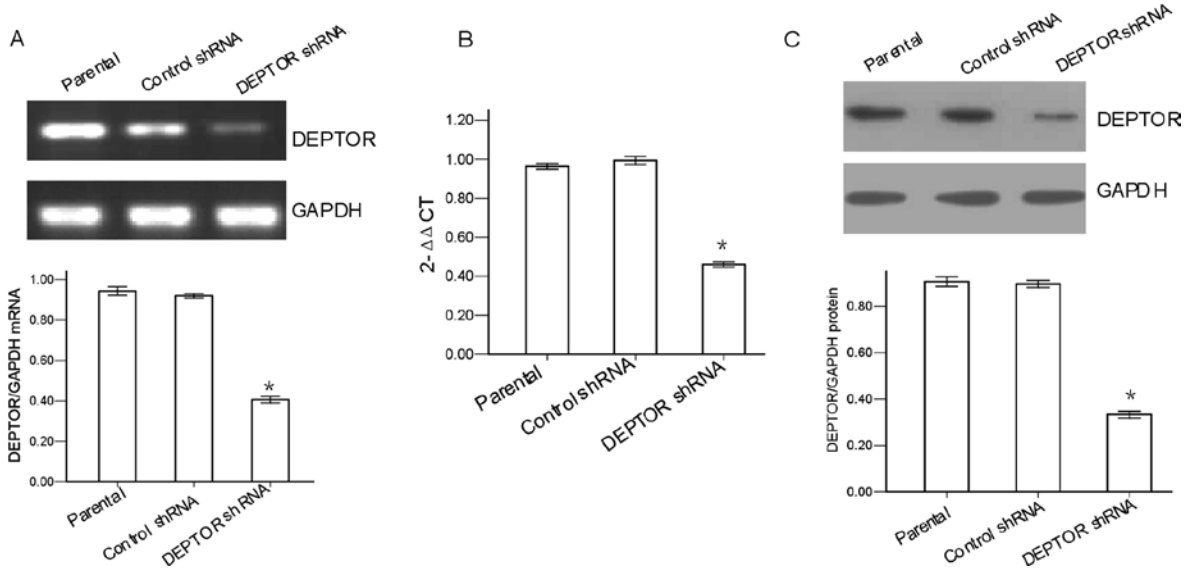

Figure 1. Effect of DEPTOR shRNA on the DEPTOR mRNA and protein expression in RPMI-8226 cells. (A) Relative mRNA levels were analyzed by RT-PCR. (B) Quantitative RT-PCR analysis of the expression levels of DEPTOR mRNA in the 3 groups of RPMI-8226 cells. (C) DEPTOR protein expression analyzed by western blot analysis. GAPDH served as the loading control. *P<0.05 vs. the control shRNA-transfected group or the parental group.

Visualization and quantification of MDC-labeled autophagic vacuoles. Monodansylcadaverine (MDC) staining was also used to detect autophagy and flow cytometry was used for quantification of the autophagosomes. In addition, MDC has been proposed as a special tracer for autophagic vacuoles (24). The autophagic vacuoles were filled with MDC by incubating cell growth on cover-slips with 0.05 mmol/l MDC in PBS at 37˚C for 1 h. Following incubation, the cells were washed twice with PBS and immediately analyzed by fluorescence microscopy using an inverted microscope (Olympus IX-71; Olympus Corp., Tokyo, Japan). The excitation wavelength was 380 nm and the emission filter was 525 nm.

Sensitivity to doxorubicin. The cells were seeded in triplicate on 96-well plates with $1x10^4$ cells/well, then incubated for 24 h. Subsequently, the medium was carefully removed and replaced with fresh medium, containing doxorubicin in reasonable concentration, and with the medium without doxorubicin as the control group. After 24 h incubation, the cells were treated with MTT as described above. The inhibition rate was calculated as follows: 1 - OD490 (doxorubicin +)/OD490 (control) %.

Statistical analysis. The data are expressed as the means ± SD, and one-way analysis of variance (ANOVA) was used to measure statistical significance among the different groups, followed by Student-Newman-Keuls analyses. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

shRNA mediates knockdown of DEPTOR in RPMI-8226 MM cells. RNA and protein were harvested from the cells at 24 h post-transfection for the evaluation of DEPTOR knockdown. The silencing effects of DEPTOR-specific shRNA in the RPMI-8226 cells were first evaluated by RT-PCR. The results revealed that the ratio of DEPTOR/GAPDH mRNA in the DEPTOR shRNA-transfected cells was 40.7±1.5%, significantly lower than that in the control shRNA-transfected cells (92.0±1.0%) or in the parental cells (94.3±2.1%; P<0.05; Fig. 1A). qRT-PCR showed that expression of DEPTOR mRNA was significantly decreased in the DEPTOR shRNA-transfected group (Fig. 1B). The silencing effect of DEPTOR-specific shRNA in the RPMI-8226 cells was also evaluated by western blot analysis. The results revealed that the ratio of DEPTOR/GAPDH protein in the DEPTOR shRNA-transfected cells was 33.3±1.5%, significantly lower than that in the control shRNA-transfected cells (89.7±1.5%) or in the parental cells (90.7±2.1%; P<0.05; Fig. 1C). There was no significant difference observed between the control shRNA-transfected cells and the parental cells (P>0.05). These results demonstrated that DEPTOR was effectively knocked down in the DEPTOR shRNA-transfected RPMI-8226 cells and could be used for following experiments to characterize the role of DEPTOR in MM.

Knockdown of DEPTOR inhibits proliferation and promotes apoptosis in RPMI-8226 cells. We then investigated whether DEPTOR shRNA decreases the proliferation of RPMI-8226 cells. As indicated in Fig. 2A, compared to the parental cells, the proliferation capacity of the DEPTOR shRNA-transfected cells was inhibited by 67.3±1.32% (P<0.05), 61.6±1.35% (P<0.05) and 63.5±1.12% (P<0.05) at 24, 48 and 72 h, respectively. There was no significant difference in the proliferation capacity between the control shRNA-transfected cells and the parental cells (P>0.05). To determine the apoptosis-inducing potential of DEPTOR shRNA in the cells, flow cytometric analysis of the PI-stained cells was performed. As shown in Fig. 2B, the apoptotic rate in the DEPTOR shRNA-transfected group was significantly higher than that in the parental or control shRNA-transfected group. The results showed that the inhibition rate in the DEPTOR shRNA-transfected cells was markedly higher than that in the parental cells or in the control shRNA-transfected RPMI-8226 cells (P<0.05; Fig. 2C).

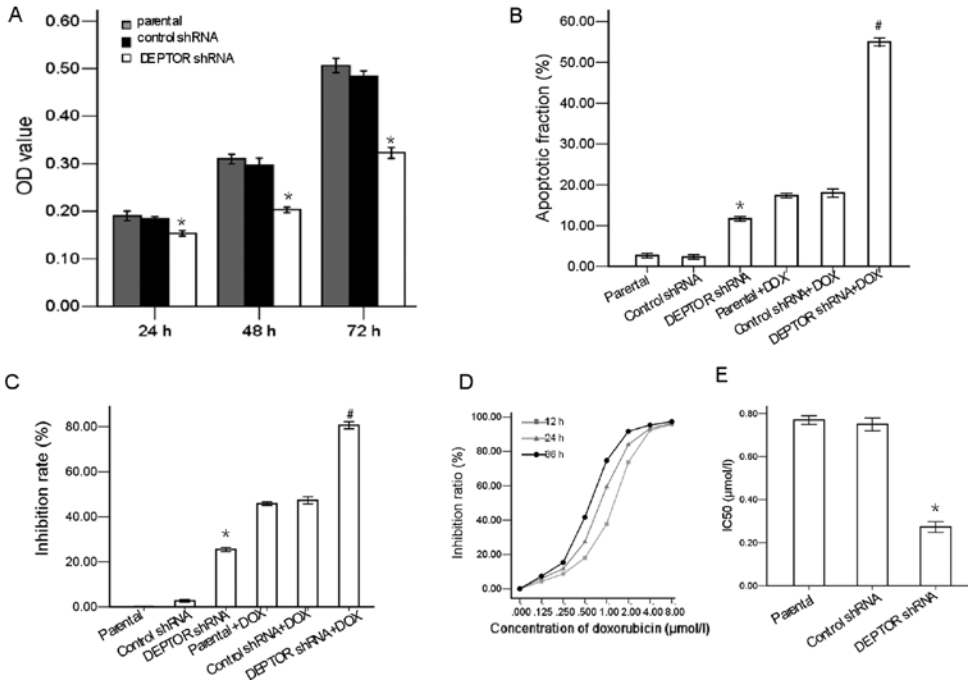

Figure 2. (A) Effect of DEPTOR shRNA on RPMI-8226 cell proliferation. RPMI-8226 cells seeded in 96-well microplates, were cultured for for 24, 48 and 72 h, and the cell numbers were determined by calculating the absorbance. (B) Effect of DEPTOR shRNA on RPMI-8226 cell apoptosis. The apoptotic fraction of RPMI-8226 cells untreated or exposed to 0.75 μmol/l doxorubicin (DOX) for 24 h was quantified by flow cytometry. (C) Effect of DEPTOR shRNA on RPMI-8226 cell growth. RPMI-8226 cells were untreated or treated with 0.75 μmol/l DOX for 24 h. Cell viability was determined by MTT assay. (D) Effect of DOX on the proliferation of RPMI-8226 cells. The cells were treated with DOX at various doses for 12, 24 and 36 h. Viable cells were detected by MTT assay and the proliferation inhibitory ratio (%) was calculated. (E) DEPTOR knockdown enhanced the DOX-induced cytotoxic effects in RPMI-8226 cells. The IC_{50} of DOX was determined by MTT assay following exposure to DOX for 24 h. DEPTOR knockdown decreased the IC_{50} of DOX. *P<0.05 vs. the control shRNA-transfected cells or the parental group cells; $^{\#}$P<0.05 vs. control shRNA-transfected cells treated with DOX or the parental cells treated with DOX.

Doxorubicin inhibits the proliferation of RPMI-8226 cells. MTT assay was employed to detect the cytotoxic effects of various concentrations of doxorubicin (0, 0.125, 0.25, 0.5, 1, 2, 4 and 8 μmol/l) on RPMI-8226 cells for 12, 24 and 36 h. As shown in Fig. 2D, doxorubicin induced a marked inhibition of cell proliferation in a time- and dose-dependent manner with an IC_{50} of 0.77 μmol/l in the RPMI-8226 cells at 24 h.

DEPTOR knockdown enhances the doxorubicin-induced growth inhibitory effect and promotes apoptosis in RPMI-8226 MM cells. We investigated whether the inhibition of DEPTOR by shRNA affected the sensitivity of RPMI-8226 cells to the antitumor drug, doxorubicin. The results demonstrated that the inhibition rate in the DEPTOR shRNA-transfected cells treated with doxorubicin was markedly higher than that in the parental cells treated with doxorubicin or in the control shRNA-transfected cells treated with doxorubicin (P<0.05). There was no significant difference observed between the control shRNA-transfected cells treated with doxorubicin and the parental cells treated with doxorubicin (P>0.05; Fig. 2C).

We performed flow cytometry to evaluate apoptosis in the RPMI-8226 cells treated with doxorubicin. The results showed that upon exposure to doxorubicin, apoptosis was significantly increased in the RPMI-8226 cells in which the expression of DEPTOR had been knocked down compared with the control cells (Fig. 2B).

The RPMI-8226 cells were exposed to 0-8 μmol/l doxorubicin for 24 h. The IC_{50} calculated based on the data from MTT cytotoxicity assay showed that DEPTOR knockdown enhanced the sensitivity of RPMI-8226 cells to doxorubicin. The DEPTOR knockdown decreased the IC_{50} of doxorubicin. The IC_{50} of doxorubicin in the RPMI-8226 cells decreased from 0.77 μmol/l to 0.27 μmol/l (Fig. 2E).

Taken together, these data suggest that DEPTOR knockdown enhances the doxorubicin-induced growth inhibitory effect, promotes apoptosis, and increases the chemosensitivity of RPMI-8226 human multiple myeloma cells to doxorubicin.

DEPTOR knockdown leads to changes in the expression of apoptosis-associated proteins in RPMI-8226 MM cells. To determine the apoptosis-inducing potential of DEPTOR shRNA in RPMI-8226 cells, we performed western blot analysis to detect the expression of apoptosis-associated proteins. As shown in Fig. 3, the expression levels of cleaved caspase-3 and cleaved PARP in the DEPTOR shRNA-transfected group were significantly higher than those observed in the control shRNA-transfected and in the parental group. Following exposure to doxorubicin for 24 h, the expression levels of both proteins were markedly increased in the DEPTOR shRNA-transfected cells, but not in the control shRNA-transfected cells. These results suggest that DEPTOR knockdown induces the upregulation of caspases, which then leads to apoptosis.

Knockdown of DEPTOR inhibits cell autophagy in RPMI-8226 MM cells. Evidence indicates that autophagy can be detected morphologically and biochemically (25,26). In this study, transmission electron microscopy and the fluorescence of MDC observations revealed that the number of

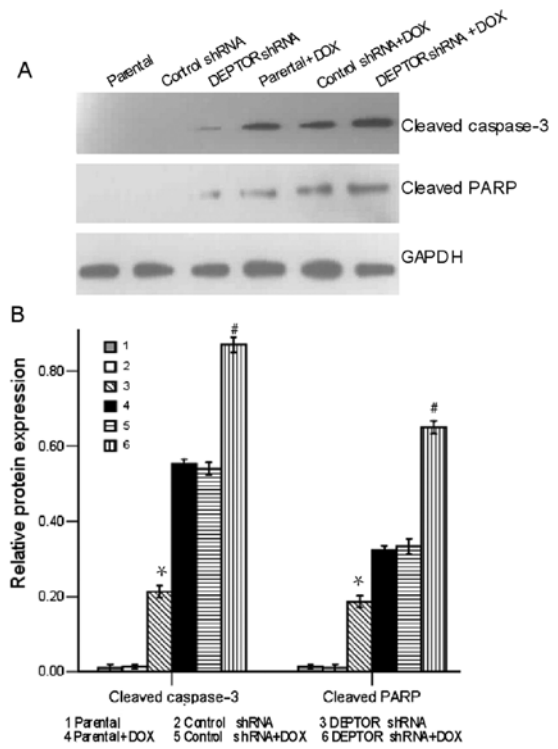

Figure 3. DEPTOR knockdown alters the levels of apoptosis-associated proteins in RPMI-8226 cells. RPMI-8226 cells were unexposed or exposed to 0.75 μmol/l DOX for 24 h and then subjected to western blot analysis. (A) Representative blots showing the protein levels of cleaved caspase-3 and cleaved PARP in the 6 groups of cells. (B) Densitometric analysis of the results shown in (A). *P<0.05 vs. the control shRNA-transfected cells or the parental group cells; $^{\#}$P<0.05 vs. control shRNA-transfected cells treated with DOX or parental cells treated with DOX.

autophagic vacuoles in the DEPTOR shRNA-transfected cells was markedly lower than that in the parental or the control shRNA-transfected cells (P<0.05; Fig. 4). Consistent with these findings, the different treatments also induced the conversion of LC-3 I to LC-3 II.

To determine the autophagy-inducing potential of DEPTOR shRNA in RPMI-8226 cells, we performed western blot analysis to detect the expression of autophagy-associated proteins. As shown in Fig. 4, the expression levels of Atg5 and LC-3 in the DEPTOR shRNA-transfected group were significantly lower than those observed in the control shRNA-transfected group and the parental group. Following exposure to doxorubicin for 24 h, the expression levels of Atg5 and LC-3 II/LC-3 I, which indicated the activation of autophagy, were markedly reduced in the DEPTOR shRNA-transfected cells, but not in the control shRNA-transfected cells.

Thus, taken together, these data indicate the inhibition of autophagic response in the DEPTOR shRNA-transfected RPMI-8226 cells. Our data suggest that the knockdown of DEPTOR inhibits autophagy in MM cells.

DEPTOR knockdown reduces PI3K/Akt activity in RPMI-8226 MM cells. We detected the PI3K/Akt activity in the RPMI-8226 MM cells unexposed or exposed to doxorubicin. Western blot analysis showed that the level of p-Akt was markedly reduced in the RPMI-8226 cells in which the expression of DEPTOR had been knocked down, but not in the control RPMI-8226 cells; however, the level of total Akt remained unaltered between the cells in which DEPTOR expression had been knocked down and the control RPMI-8226 cells (Fig. 5). In addition, doxorubicin markedly activated the mTOR complex 1 targets, p-P70S6K and p-4Ebp-1, in the RPMI-8226 cells in which DEPTOR expression had been knocked down, but had no such effects on the control shRNA-transfected RPMI-8226 cells (Fig. 5). Collectively, these results demonstrate that DEPTOR knockdown reduces the PI3K/Akt activity in the RPMI-8226 MM cells.

Discussion

Our results demonstrated the the knockdown of DEPTOR, a recently identified inhibitor of mTOR complexes (5), induces apoptosis, increases the chemosensitivity to doxorubicin, and suppresses autophagy and PI3K/Akt signaling in RPMI-8226 cells. Our data have demonstrated that targeting DEPTOR can be used for the treatment of MM. shRNA is an effective and efficient technique for the study of tumors, as well as treatment (27). In our study, we successfully transfected shRNA targeting the DEPTOR gene into the human MM cell line, RPMI-8226, and DEPTOR expression was effectively inhibited at the protein level.

A recent study found that high expression levels of DEPTOR are predictive of response to thalidomide in myeloma. However, there was no survival benefit for thalidomide in the low DEPTOR expression group (28). The results showed that the level of DEPTOR expression is crucial to the survival of myeloma patients. The results from our study also indicate that the level of DEPTOR expression is crucial to the survival of myeloma cells.

We examined the effect of DEPTOR silencing on the autophagic capacity of RPMI-8226 cells. The autophagic capacity of DEPTOR shRNA-transfected cells was suppressed compared to the control shRNA-transfected cells or the parental cells. At the same time, mTORC1 and the PI3K/Akt pathway plays an important role in cell autophagy (10-12). Since DEPTOR is an mTOR-interacting protein that normally functions to inhibit the mTORC1 pathway, the knockdown of DEPTOR inhibits cell autophagy.

Certain evidence indicates that the inhibition of autophagy may induce apoptosis (16-20). An earlier study suggested a role for autophagy as a potential pro-survival mechanism in MM cells (29). Recently, another study suggested that the suppression of autophagy significantly augments the *in vitro* and *in vivo* antimyeloma activity of DNA-damaging chemotherapy (30). Thus, apoptosis may be enhanced by the inhibition of autophagy using shRNA targeting the DEPTOR gene in MM cells.

Active caspases play a vital role in the induction of apoptosis. Following the activation of caspase-3, PARP was cleaved (31). The cleavage of PARP has often been viewed as an indicator of apoptosis. In our study, DEPTOR shRNA-transfected RPMI-8226 cells demonstrated a higher level of cleaved caspase-3 fragments and cleaved PARP. These results reveal that the DEPTOR knockdown by shRNA is sufficient to trigger caspase-dependent apoptosis, which could be the reason for the decrease in cell viability. We found that the

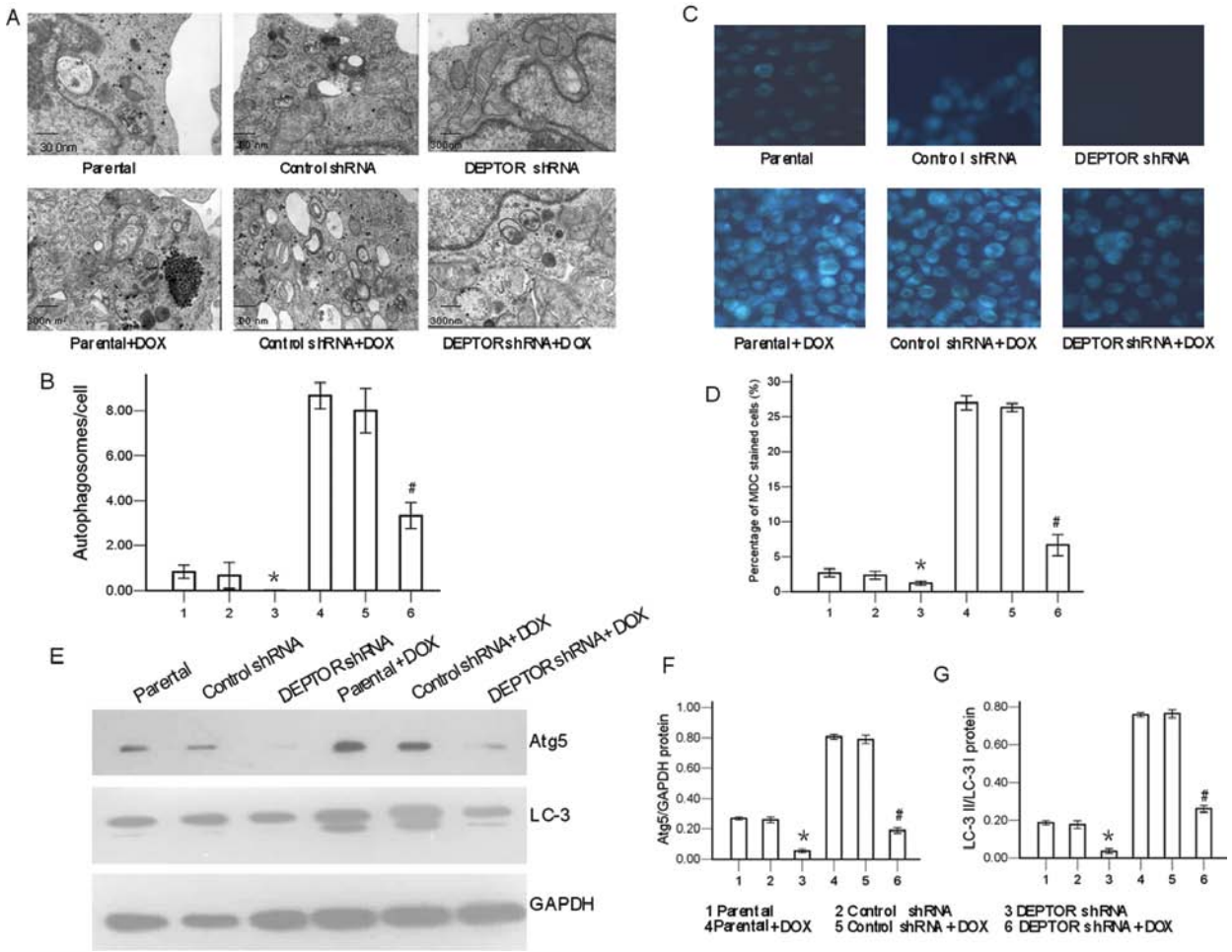

Figure 4. Knockdown of DEPTOR inhibits autophagy in RPMI-8226 cells. (A) The autophagic vesicles were observed with transmission electron microscopy. RPMI-8226 cells were unexposed or exposed to 0.75 μmol/l DOX for 24 h. Electron microscopy images were taken in the 6 groups of cells. Scale bars, 300 nm. Original magnification, x25,000. (B) Quantification of autophagic vesicles was determined. The data obtained from a minimum of 50 independent cells were averaged (means ± SD). (C) DEPTOR knockdown reduced the quantification of monodansylcadaverine (MDC)-labeled autophagic vacuoles in RPMI-8226 cells. RPMI-8226 cells were unexposed or exposed to 0.75 μmol/l DOX for 24 h. The autophagic vacuoles were labeled with MDC in 6 cell lines. (D) Different ratios of MDC-positive stained cells are shown between groups (%). Results shown are the means of 3 independent experiments. (E) DEPTOR knockdown altered the levels of autophagy-associated proteins in RPMI-8226 cells. RPMI-8226 cells were unexposed or exposed to 0.75 μmol/l DOX and then subjected to western blot analysis. Representative blots showing the protein levels of Atg5 and LC-3 in the 6 groups of cells. (F and G) Densitometric analysis of the results shown in (E). *P<0.05 vs. the control shRNA-transfected cells or the parental group cells; $^{\#}$P<0.05 vs. control shRNA-transfected cells treated with DOX or parental cells treated with DOX.

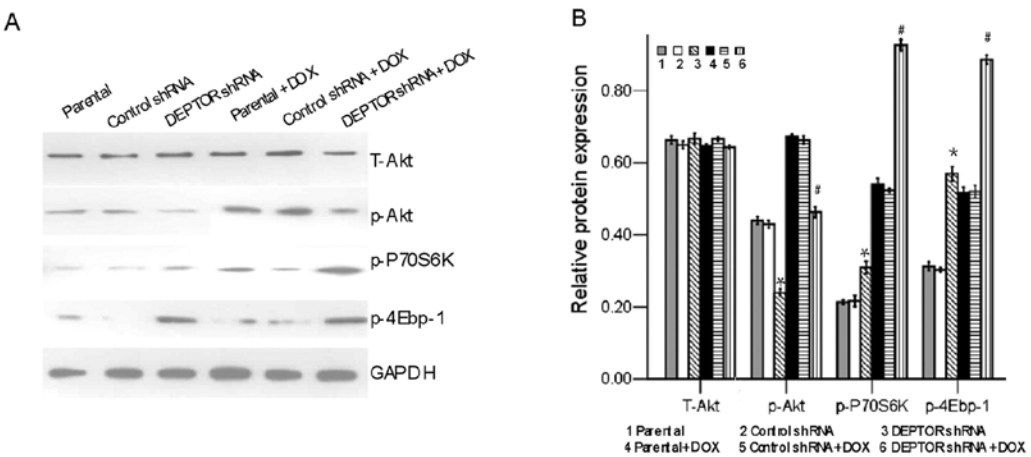

Figure 5. DEPTOR knockdown inhibits the activation of the PI3K/Akt signaling pathway in RPMI-8226 cells. RPMI-8226 cells were unexposed or exposed to 0.75 μmol/l doxorubicin (DOX) for 24 h and then subjected to western blot analysis. (A) Representative blots showing the protein levels of total Akt (T-Akt), phosphorylated Akt (p-Akt), phosphorylated P70S6K (p-P70S6K) and phosphorylated eIF4E-binding protein-1 (p-4Ebp-1) in the 6 groups of cells. (B) Densitometric analysis of the results shown in (A). *P<0.05 vs. the control shRNA-transfected cells or the parental group cells; $^{\#}$P<0.05 vs. control shRNA-transfected cells treated with DOX or parental cells treated with DOX.

inhibition of DEPTOR expression led to increased levels of cleaved caspase-3 and cleaved PARP, which contributed to doxorubicin-induced apoptosis in RPMI-8226 cells. These results are consistent with those from a previous study, reporting that doxorubicin-induced apoptosis in MM cells was associated with the activation of caspase-3 and PARP (30,32).

We then identified the signaling pathway through which DEPTOR modulates the effects of chemotherapy in MM. Recently, DEPTOR was identified as a regulator of the PI3K/Akt pathway. DEPTOR appears to play a specific role in upregulating the PI3K/Akt pathway in myeloma (5). The PI3K/Akt signaling pathway plays an important role in cell proliferation, development and apoptotic resistance and survival (8,9,28,33). At the same time, PI3K/Akt inhibition has been found to induce chemosensitization in MM cells (34,35). The constitutive activation of the PI3K/Akt pathway has been accepted as an important molecular event that contributes to the malignant phenotype of MM cells (36).

Our results have demonstrated that DEPTOR shRNA suppressed PI3K/Akt activity in the RPMI-8226 cells, indicating the involvement of the PI3K/Akt signaling pathway downstream of DEPTOR. Akt can also phosphorylate procaspase-3 to inhibit apoptosis (37). Akt is a major mediator of cell survival, either directly by inhibiting pro-apoptotic proteins, such as caspase-9 and Bad, or indirectly by modulating regulators of cell death including p53 and nuclear factor-κB (NF-κB) (38-42). Activated Akt modulates the function of many substrates involved in cell cycle progression, cell growth and the regulation of cell survival (43-45). The major upstream regulator of Akt is PI3K, which is activated by a variety of transmembrane receptors (46). DEPTOR also acts as an oncogene by relieving the feedback inhibition from S6 kinase 1 (S6K1) to PI3K, thus activating Akt (47).

Our study has demonstrated that the doxorubicin-induced increase in the level of p-Akt level was markedly reduced in the DEPTOR shRNA-transfected cells, but was unaffected in the control shRNA-transfected cells or in the parental RPMI-8226 cells. Moreover, Akt regulates cell proliferation through its effects on the mTOR/P70S6 kinase pathway (8,36,48). mTORC1 controls cell proliferation partly by phosphoylating S6K1 and 4Ebp-1, key regulators of protein synthesis (49). In our study, we found that doxorubicin markedly activated the mTORC1 targets, S6K1 and 4Ebp-1, in the DEPTOR shRNA-transfected RPMI-8226 cells. Collectively, these results suggest that the suppression of PI3K/Akt activity following DEPTOR knockdown is responsible for the increased sensitivity of RPMI-8226 cells to doxorubicin.

In conclusion, our study demonstrates that the knockdown of DEPTOR by RNAi induces apoptosis, increases the chemosensitivity to doxorubicin, suppresses autophagy and inhibits the activation of the PI3K/Akt signaling pathway in RPMI-8226 cells. To the best of our knowledge, this the first study to demonstrate a possible correlation between DEPTOR gene expression and autophagy in MM cells. Our results provide evidence that DEPTOR is an important therapeutic target for the treatment of MM. These findings raise the possibility that DEPTOR inhibitors may be used to enhance the effectiveness of doxorubicin in the treatment of myeloma. Animal experiments should be performed to further confirm the effects of DEPTOR knockdown on the proliferation, apoptosis and autophagy of MM cells. The anticancer effects induced by DEPTOR knockdown require further investigation.

Acknowledgements

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