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Original Articles

DEPTOR inhibits cell proliferation and confers sensitivity to dopamine agonist in pituitary adenoma

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ABSTRACT

DEP domain-containing mechanistic target of rapamycin (mTOR)-interacting protein (DEPTOR) is an important modulator of mTOR, a highly conserved kinase whose hyperactivation is critically involved in a variety of human tumors. The role of DEPTOR playing in pituitary adenoma (PA) is largely unknown. Here, we reported that DEPTOR was downregulated in PA tissues, especially dopamine-resistant prolactinomas. Consistently, overexpression of DEPTOR inhibited pituitary tumor GH3 and MMQ cells proliferation *in vitro* and *in vivo*, and sensitized GH3 and MMQ cells to cabergoline (CAB), a dopamine agonist (DA). Conversely, knockdown of DEPTOR promoted GH3 and MMQ cells proliferation, and conferred cells resistance to CAB. Mechanistically, DEPTOR inhibited both mTOR Complex 1 (mTORC1) and 2 (mTORC2) activities in PA cells. In addition, DEPTOR expression level was increased to suppress mTOR kinase activity via decreasing E3 ubiquitin ligase, βTrCP1, in response to CAB. Furthermore, DEPTOR enhanced autophagy-dependent cell death to confer cells sensitivity to CAB. Taken together, our results suggest that DEPTOR may be a potential target for the treatment of PAs.

1. Introduction

Pituitary adenomas (PAs) are benign intracranial and neuroendocrine neoplasms that can cause morbidity due to local invasion and/or excessive or deficient hormone production [1,2]. Prolactinomas, clinically nonfunctioning adenomas and somatotroph adenomas are the three most common subtypes, whose precise mechanisms underlying tumorigenesis are largely unknown [2]. Dopamine agonist (DA), such as cabergoline (CAB) and bromocriptine (BRC), is clinically used as the first-line treatment for prolactinoma and has been recently extended to the treatment of other types of PAs that are nonresponsive to traditional therapeutic approaches [3–6]. Although DA is effective for the most prolactinoma patients, tumor resistance occurs in 10–15% of the patients with prolactinoma; and its efficacy for other types of PAs are limited [7]. Therefore, further research is needed to reveal the mechanisms for tumor resistance to DA.

The mechanistic target of rapamycin (mTOR) is a highly conversed serine-threonine kinase that involves in eukaryotic cell growth and metabolism, and is frequently hyperactivated in neoplasms [8,9]. Several studies have shown that the mTOR signaling pathway is activated

Abbreviations: mTOR, the mechanistic target of rapamycin; DEPTOR, DEP domain-containing mechanistic target of rapamycin-interacting protein; PA, pituitary adenoma; DA, dopamine agonist; DR, dopamine receptor; CAB, cabergoline; BRC, bromocriptine; BafA1, Bafilomycin A1; DPD, domperidone

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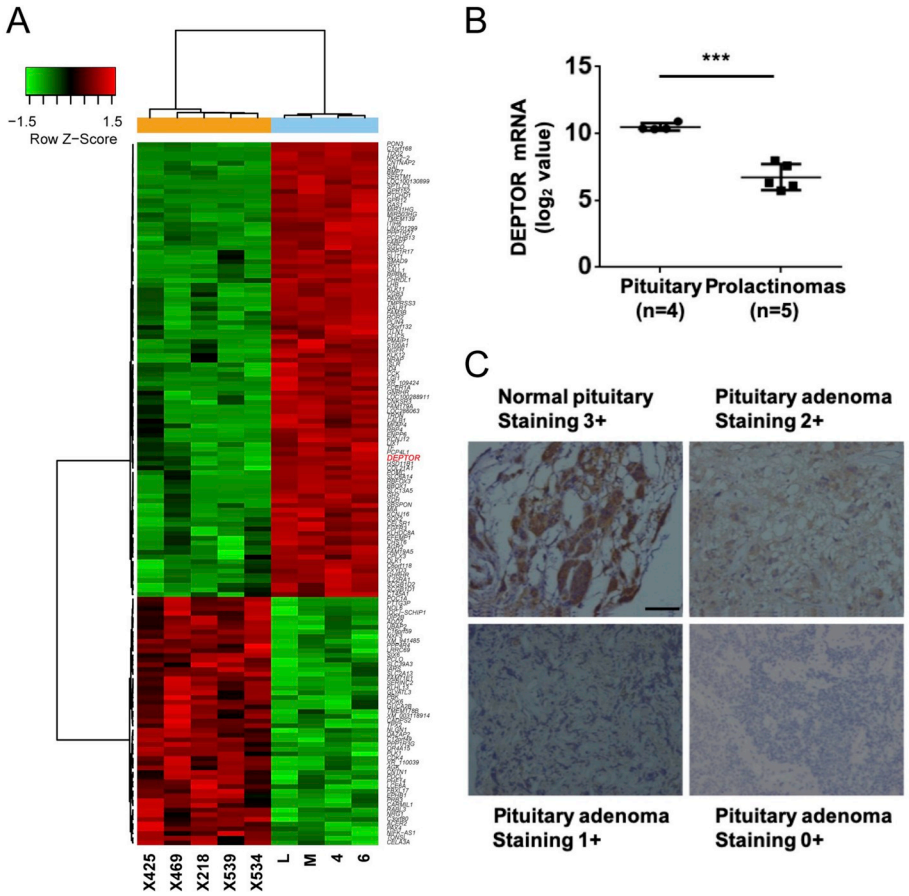

Fig. 1. DEPTOR expression was decreased in PAs. (A and B) DEPTOR expression was decreased in human prolactinomas compared with normal pituitary glands. A microarray was used to profile the gene expression in human normal pituitary glands and prolactinomas. Heat-map showed the top 150 differential expressed genes between normal pituitary glands (n = 4) and prolactinomas (n = 5), where DEPTOR expression was decreased by approximately 14.9-fold in prolactinomas. (C) Representative immunostaining scores of DEPTOR expressed in normal pituitary glands and pituitary tumors. Normal pituitary glands showed high expression of DEPTOR (score = 3+), whereas tumor tissues showed moderate (score = 2+), low (score = 1+), or very week/undetectable expression (score = 0+) of DEPTOR. Scale bar, 100 μm ***$P < 0.001$.

Table 1
Scale of DEPTOR immunoexpression in pituitary glands and all subtypes of pituitary adenomas.

Group	n	DEPTOR Expression				*H* Value	*P* Value
		0+	1+	2+	3+		
Pituitary glands	12	0	0	2	10		
PRL	34	6	18	10	0	−5.087	< 0.001
Nonfunctioning	22	4	12	5	1	−4.527	< 0.001
GH	7	2	3	1	1	−3.214	0.001
GT	7	2	3	2	0	−3.684	< 0.001
ACTH	3	0	2	1	0	−2.940	0.003
TSH	2	0	2	0	0	−2.754	0.006
Mixture	7	1	2	3	1	−3.045	0.002
All tumors	82	15	42	22	3	−5. 400	< 0.001

Table 2
Correlation of the expression of DEPTOR with clinicopathological features of 82 patients.

Feature	n	DEPTOR Expression				*H* Value	*P* Value
		0+	1+	2+	3+		
Sex						0.258	0.611
Male	48	8	25	12	3		
Female	34	7	17	10	0		
Age (years)						0.492	0.483
< 45	37	5	20	11	1		
»45	45	10	22	11	2		
Tumor size(cm)						11.11	0.004
< 1	6	0	1	4	1		
1-3	39	5	21	11	2		
»3	37	10	20	7	0		
Cavernous sinus invasion						2.126	0.145
Knosp 1-2	48	7	24	14	3		
Knosp 3-4	34	8	18	8	0		
Endocrine tumor						2.455	0.873
PRL	34	6	18	10	0		
Nonfunctioning	22	4	12	5	1		
GH	7	2	3	1	1		
GT	7	2	3	2	0		
ACTH	3	0	2	1	0		
TSH	2	0	2	0	0		
Mixture	7	1	2	3	1		

in PAs and plays an important role in PA development [10–12]. Preclinical studies have demonstrated that mTOR inhibitor significantly reduces tumor size, indicating the therapeutic effects of targeting the mTOR signaling pathway in PAs [10,12,13]. DEP domain-containing mTOR-interacting protein (DEPTOR) is recently identified naturally occurring inhibitor of mTOR, which inhibits the kinase activities of both mTOR Complex 1 (mTORC1) and 2 (mTORC2), two subcomplexes of mTOR [14]. DEPTOR inhibits cell proliferation and invasion by blocking the activation of mTOR, which has been confirmed in several tumor types [8,15–17]. However, the function of DEPTOR in PA is unknown.

Here, we reported that DEPTOR was downregulated in PA tissues, especially dopamine-resistant prolactinomas. The ectopic expression of DEPTOR inhibited GH3 and MMQ cells proliferation by inactivation of mTOR kinase, and promoted PA cells sensitivity to CAB by enhancing autophagy-dependent cell death. Taken together, DEPTOR may be a promising target for the treatment of PAs.

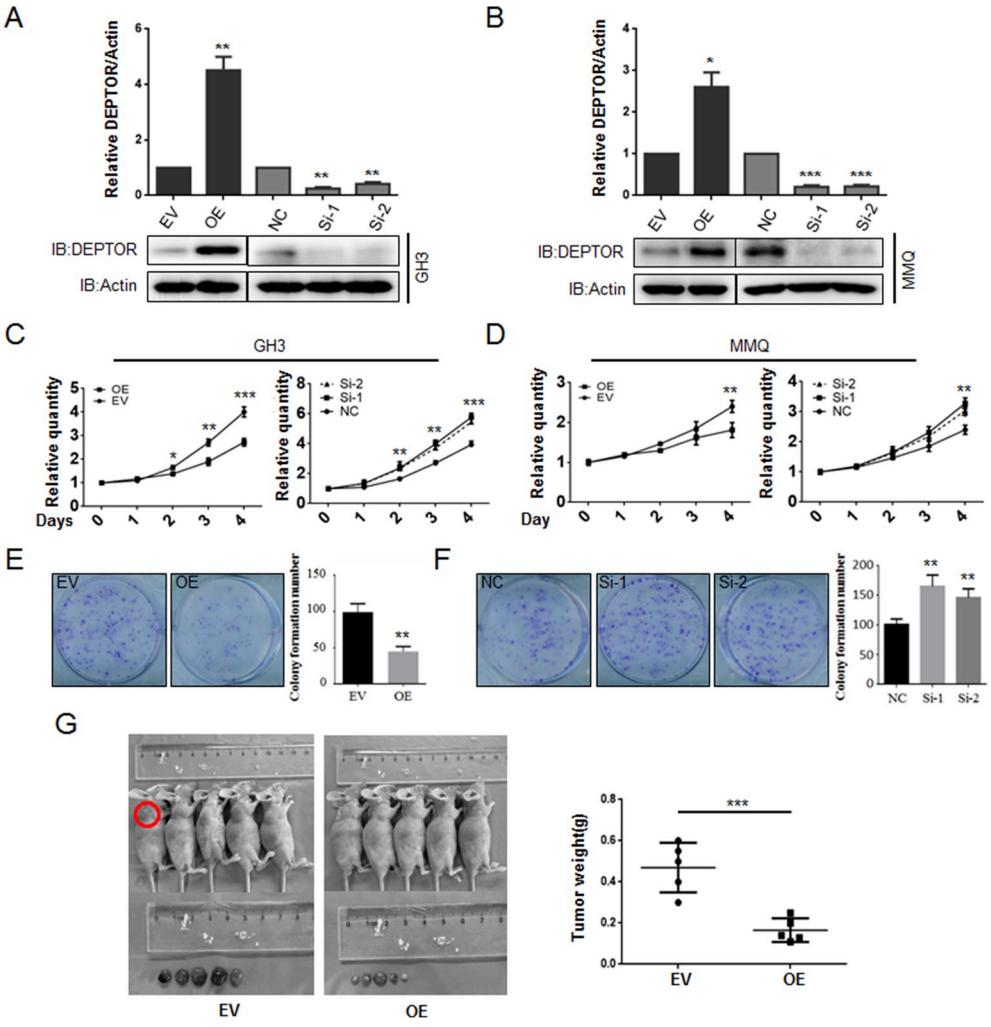

Fig. 2. DEPTOR expression inhibited the proliferation of PA cell lines *in vitro and in vivo*. (A and B) The effect of DEPTOR overexpression and knockdown. GH3 or MMQ cells were transfected with the indicated plasmids or siRNA. Then, 36 h after transfection, the cells lysates were subjected to western blotting analysis. Actin was used as a loading control. (C and D) MTS assays were performed to evaluate the proliferation of transfected cells each day for 4 days. DEPTOR overexpression inhibited GH3 and MMQ cells proliferation; DEPTOR knockdown promoted GH3 and MMQ cells proliferation. (E and F) GH3 cells were infected with lentiviral DEPTOR overexpressing or shRNA construct. Infected cells were seeded into a 6 well plate with 500 cells per well and cultured for 1 week, followed by crystal violet staining and colony counting. DEPTOR overexpression inhibited the colony formation rate of GH3 cells. DEPTOR knockdown enhanced the colony formation rate of GH3 cells. (G) Female nude mice were subcutaneously injected with empty vectors containing GH3 cells (EV) or DEPTOR-overexpressing GH3 cells (OE). Mice were sacrificed in the ninth day and the xenograft tumors were dissected and weighted. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. EV, empty vector; OE, over expression; NC, negative control.

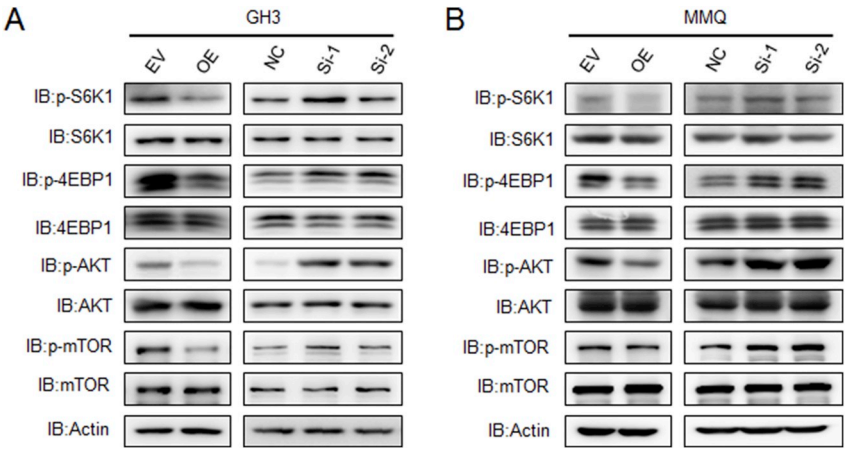

Fig. 3. DEPTOR suppressed the mTOR signaling pathway in GH3 and MMQ cells. GH3 and MMQ cells were transfected with the indicated plasmids or siRNA. Then, the cells lysates were subjected to western blotting analysis for the levels of the indicated proteins and phosphorylation states at 48 h post-transfection. Actin was used as a loading control. (A) DEPTOR overexpression in GH3 cells led to inhibition of mTOR signaling pathway as evidenced by downregulation of phosphorylation of mTOR, AKT, 4EBP1 and S6K1 in this pathway; while DEPTOR knockdown led to activation of the pathway. (B) DEPTOR alterations had the similar effect on MMQ cells. $^{*}P < 0.05$. EV, empty vector; OE, over expression; NC, negative control.

2. Materials and methods

2.1. Patients and tissue samples

The study was approved by the Ethical Review Board in Ruijin Hospital of Shanghai Jiao Tong University School of Medicine. Patients diagnosed as PA were given informed consent according to the institutional guidelines. Twelve normal pituitary tissues were from autopsy, and eighty-two PA tissues including thirty-four prolactinomas were obtained at surgery. Suitable parts of the tissues were embedded in paraffin or stored at liquid nitrogen until use. In line with the other reports, resistance to BRC was defined as prolactin levels failing to be normalized despite the administration of more than 15 mg of BRC daily for at least 3 months (normal PRL level is defined as less than 25 ng/ml) [18–20]. There were thirteen dopamine-resistant and twenty-one dopamine-sensitive patients with prolactinoma in our study. The twenty-one dopamine-sensitive patients took BRC after surgical excision and subsequently achieved PRL normalization.

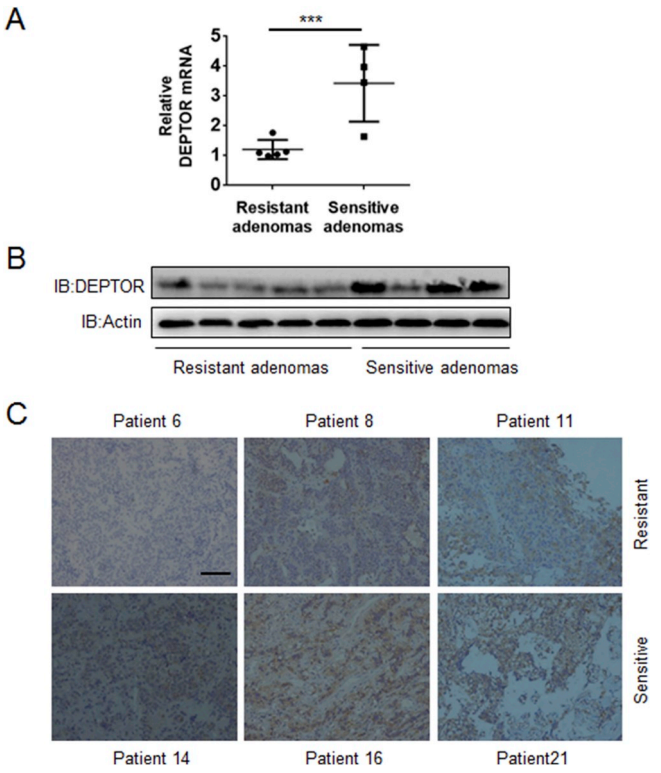

Fig. 4. DEPTOR was downregulated in dopamine-resistant prolactinomas. (A) Quantitative real-time PCR and (B) western blotting showed DEPTOR expression was lower in dopamine-resistant prolactinomas(n = 5) than sensitive tumors(n = 4). (C) Immunohistochemical analyses also showed that DEPTOR was expressed lower in dopamine-resistant prolactinomas than sensitive tumors. Scale bar, 100 μm ***$P < 0.001$.

Table 3
Scale of DEPTOR immunoexpression in prolactinoma.

Scale	DEPTOR		*P* value
	Dopamine-sensitive	Dopamine-resistant	
Negative(0 +)	1/21	5/13	0.005
Low(1 +)	11/21	7/13	
Moderate(2 +)	9/21	1/13	

2.2. Microarray profiling and analysis

Total RNA extracted from four normal pituitary glands and five prolactinoma tissues was subjected to a SurePrint G3 Human GE 8 × 60K Microarray platform assay performed by BioGenius Co. Ltd (Shanghai, China). The gene expression level was quantified with the software package RSEM. Differentially expressed genes with fold changes > 1 or < −1 were chosen for further analysis. The list of significance was operated by setting the *p* value threshold at 0.05.

2.3. Cell culture and reagents

Rat pituitary cell lines GH3 and MMQ cells were purchased from the American Type Culture Collection (Manassas, VA, USA), whose passage numbers are CCL-82.1™ and CRL-10609™, respectively, and were cultured in Dulbecco's modified Eagle medium and F12 medium, respectively (Gibco, Grand Island, NY, USA), supplemented with 2.5% fetal bovine serum (Gibco) and 15% horse serum (Gibco). All cell lines were maintained in humidified atmosphere with 5% CO2 at 37 °C. CAB, everolimus, Bafilomycin A1 (BafA1) and domperidone (DPD) was purchased from MedChemExpress (Shanghai, China) and dimethyl sulfoxide was purchased from Sigma (St. Louis, MO, USA).

2.4. Quantitative real-time PCR

Total RNA of tumor tissues or cells was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA was reversely transcribed into cDNA using the cDNA Synthesis Kit (Takara Bio, Shiga, Japan). The mRNA expression level of DEPTOR was quantified using a real-time RT-PCR with the SYBR Green real-time PCR Master Mix kit (Takara Bio). PCR primers used were as follows: human DEPTOR forward, 5′-ACAGAGGCTATATGAAAAGC TGA-3’; reverse, 5′- CCTTCCTGAACCAGCCAGTC-3’; human GAPDH forward, 5’ –CTCTCTGCTCCTCCGTTCGAC-3’; reverse, 5′-TGAGCGAT GTGGCTCGGCT-3’ [14]; rat DEPTOR forward, 5′- AAGTCCTAGTTAC GGGAGAGCAG-3’; reverse, 5′- TGGTGCCGTCTATCTTTAATAACCT-3’; rat GAPDH forward, 5’ -ACCCTGTTGCTGTAGCCATATTC-3’; reverse, 5′-ACCCTGTTGCTGTAGCCATATTC-3’. An ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Warrington, UK) was used to carry out the amplification reaction. Each experiment was carried out in triplicate, and the date was analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blotting

Total proteins of tissue samples were extracted using the Total Protein Extraction Kit (Millipore Corporation, Billerica, MA, USA). As for cultured tumor cells, the cells were lysed with RIPA Lysis Buffer (Beyotime, Shanghai, China). Equivalent proteins (30 mg per sample) were electrophoresed in SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% nonfat milk in TBST buffer for 1 h and incubated overnight at 4 °C with primary antibodies. The antibodies used were as follows: DEPTOR (Novus Biologicals, Littleton, CO, USA), p62, PRL (Abcam, Cambridge, MA, USA), GH (Proteintech Group, Chicago, IL, USA), LC3 (Sigma-Aldrich, St. Louis, MO, USA), mTOR, phospho-mTOR (Ser2448), AKT, phospho-AKT (Ser473), 4EBP1, phospho-4EBP1(Thr37/46), S6K1, phospho-S6K1 (Thr389), βTrCP1, ATG7, Beclin1 and Actin (all from Cell Signaling Technology, Danvers, MA, USA). Signals were detected using ECL detection reagent (Millipore) according to the manufacturer's instructions.

2.6. Immunohistochemistry and scoring

Tissue samples were fixed in 4% formalin and embedded in paraffin. Tissue slices were cut into 5 μm thick. Immunohistochemistry was conducted using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Rabbit primary antibodies for DEPTOR (1:500) and phospho-4EBP1 (Thr37/46, 1:50) were purchased from Novus Biologicals and Abcam, respectively. Slices were developed with DAB and counterstained with hematoxylin. The stained slides were observed under a Nicon Eclipse Ti inverted microscope (Nicon, Tokyo, Japan). Clinicopathological characteristics of stained slices were assessed by two pathologists blinded to the patients’ clinical features. DEPTOR immunostaining of PA tissues was scored as negative (0 +), low (1 +), moderate (2 +), and high (3 +), as described in the previous study [21].

2.7. Immunofluorescent microscopy

Cultured cells were washed with PBS once, and fixed with cold 4% formaldehyde for 20 min, and then rinsed with PBS for three times. Cells were permeabilized with cold 0.2% triton X-100 for 15 min and incubated with the primary antibodies overnight at 4 °C after 5% BSA blocking. The cells were incubated with secondary antibodies conjugated with Alexa 488 or Alexa 594, (Invitrogen) in 5% BSA for 1 h at 37 °C. Nuclei were stained with DAPI (Vector Laboratories). A confocal microscope (Nikon, Tokyo, Japan) was used to observe all stained slices.

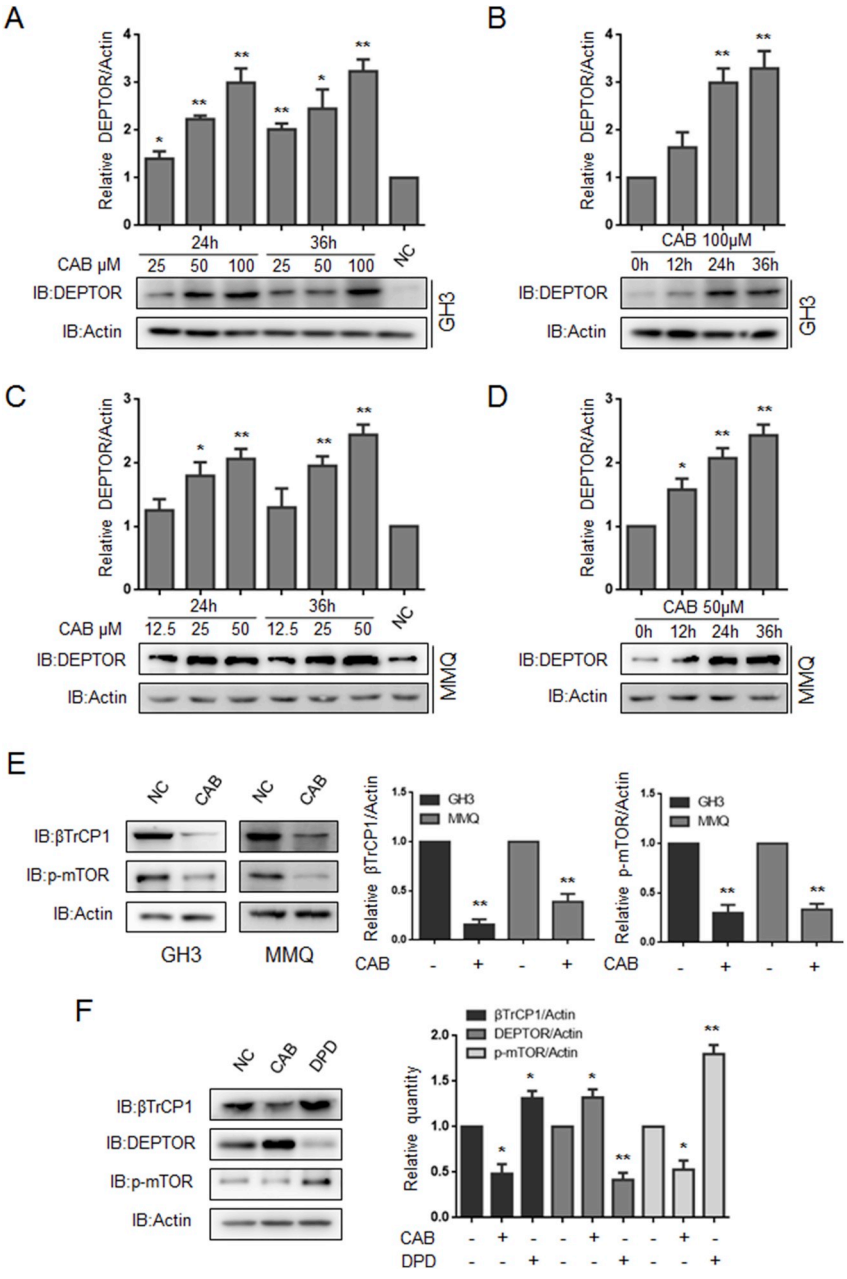

Fig. 5. CAB restored DEPTOR expression to inhibit mTOR kinase activity through decreasing βTrCP1. (A, B, C and D) Immunoblotting analysis of DEPTOR in GH3 and MMQ cells with different doses of CAB treatment at different time points. (E) GH3 and MMQ cells were treated with CAB (100 and 50 μM, respectively) for 24 h, DMSO used as a solvent control. Then, cell lysates were subjected to western blotting analysis with indicated antibodies. (F) MMQ cells were treated with CAB (50 μM) or DPD (1 μM) for 24 h, DMSO used as a solvent control. Western blotting analysis was performed with indicated antibodies. EV, empty vector; OE, over expression; NC, negative control. $^{*}P < 0.05$, $^{**}P < 0.01$.

2.8. Establishment of transiently and stably transfected cells

The siRNAs against rat DEPTOR, ATG7, and Beclin1 were synthesized at Gene Pharma (Shanghai, China) with the following sequences: SiDEPTOR (Si-1) 5′-GCTGTGGCAGCAGTGGCTA-3′, (Si-2) 5′- GCAAGG AAGACATTCACGATT-3′ [22]; SiATG7 5′-CAGCCUGGCAUUUGAUAAA-3′; SiBeclin1 5′-CUCAGGAGAGGAGCCAUUU-3′; siControl: 5′-UUCUCC GAACGUGUCACGU-3′. A DEPTOR cDNA clone plasmid was purchased from Tongyong Biotechnologies (Anhui, China). Transient transfections of siRNA and plasmids were conducted with Lipofectamine RNAiMAX reagent (Invitrogen) and X-treme GENE HP DNA Transfection Reagent (Roche Applied Science, Basel, Switzerland), respectively, following the manufacturer's instructions. The recombinant plasmids (pLKO.1-puro-shRNA DEPTOR, pCDH-puro-DEPTOR) were constructed, and sequenced by Boshang Biotechnologies (Shanghai, China). GH3 cell lines stably expressing DEPTOR, DEPTOR-specific shRNA or empty vectors, were constructed using the lentiviral technique. After 48 h transduction with lentiviral supernatant, cells were selected with 2 μg/mL puromycin for 1–2 weeks for stable transfectants. The stably transfected cells were used in the colony formation and tumor xenograft experiments.

2.9. Cell proliferation assay

Cell proliferation assay was performed with a MTS-based Cell Titer 96® AQueous One solution cell proliferation assay (Promega, Madison, WI, USA). GH3 and MMQ cells were plated in 96-well plates at approximately 2×10^3 and 5×10^3 cells per well, respectively. Upon addition of MTS solution, the plate was incubated at 37 °C for 2 h, and the absorbance was measured at 490 nm with a plate reader (TECAN, Mannedorf, Switzerland).

2.10. Colony formation assay

A total of 5×10^2 GH3 cells were plated in 6-well plates for 1 week. Cells were rinsed twice with PBS and fixed with methanol for 10 min,

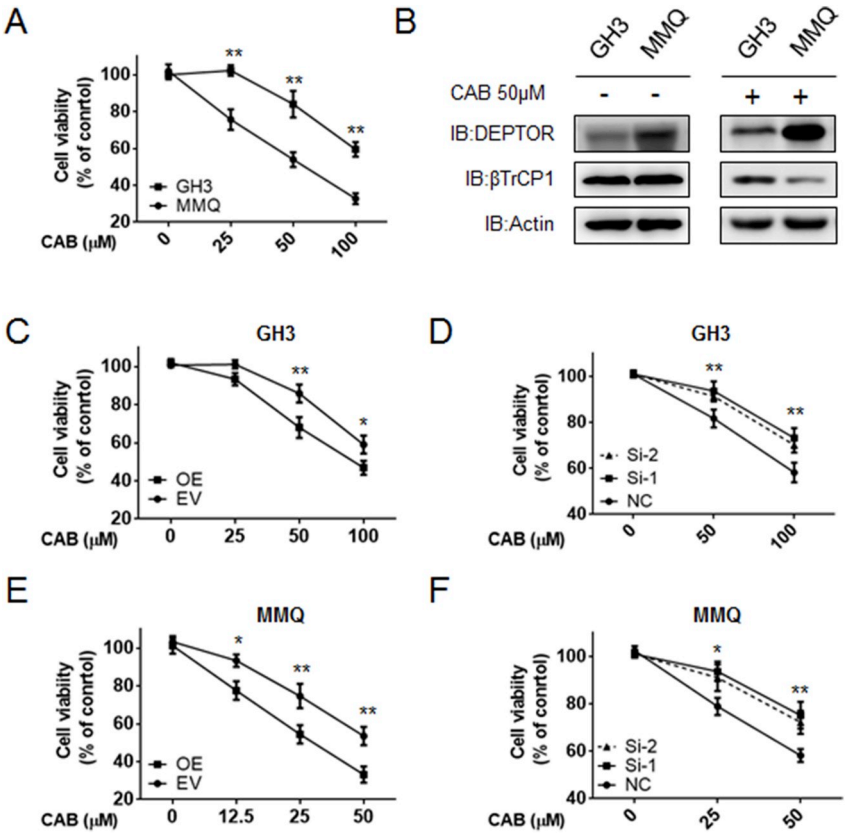

Fig. 6. DEPTOR overexpression conferred PA cell lines sensitivity to CAB. (A) Cell viability of GH3 or MMQ was determined by the MTS assay with the indicated concentrations of CAB for 24 h. (B) GH3 and MMQ cells were treated with or without CAB (50 μM). Immunoblotting analysis of DEPTOR and βTrCP1 was carried out. (C, D, E and F) GH3 or MMQ cells were transfected with the indicated plasmids or siRNA. Then, 36 h after transfection, the cells were treated with the indicated concentrations of CAB. After another 24 h, cell viability was measured by a MTS assay. $^{*}P < 0.05$, $^{**}P < 0.01$. EV, empty vector; OE, over expression; NC, negative control.

followed by staining with 0.5% crystal violet dye (in methanol/deionized water, 1:5; Beyotime) for 10 min. Excess crystal violet dye was washed away with deionized water on a shaker for five times (10 min per wash). Microscopy was obtained by using an Axiovert 200 microscope (Carl Zeiss, Oberkochen, Germany) and cell colonies were counted using Image J software (Rawak Software Inc., Stuttgart, Germany).

2.11. Xenograft mouse model

The study was approved by the Ethical Review Board in Ruijin Hospital of Shanghai Jiao Tong University School of Medicine. All animals were handled according to the Guide for the Care and Use of Laboratory Animals' and the Principles for the Utilization and Care of Vertebrate Animals. Four-week-old female nude mice were purchased from the Shanghai Slack Laboratory Animal Co., Ltd. (Shanghai, China) and were kept under specific pathogen-free conditions at the Animal Research Facility of Ruijin Hospital. Parental GH3 cells or DEPTOR overexpressing GH3 cells (2×10^6) were injected subcutaneously within a volume of 100 μL in the flank. Tumor size was measured by caliper ruler every two days after injection. Mice were sacrificed in the ninth day, and tumors were dissected and measured followed by photography and immunohistochemistry.

2.12. Statistical analysis

All data are presented as mean ± SD and analyzed by using Graphpad Prism, version 5 (GraphPad Software, La Jolla, CA, USA). Differences among categorical variables were analyzed by using one-way ANOVA/SNK test or independent-sample Student's *t*-test. The immunoreactive scores of DEPTOR were analyzed using non-parametric Kruskal-Wallis *H* test. Statistical significance was concluded as $P < 0.05$, and denoted in figures with one asterisk ($P < 0.05$), two asterisks ($P < 0.01$), or three asterisks ($P < 0.001$).

3. Results

3.1. DEPTOR was downregulated in human primary pituitary tumors

To identify abnormally expressed genes potentially involved in pituitary tumor pathogenesis, we conducted microarray analysis on normal human pituitary glands and prolactinomas, to compare their gene expression profiles. Hierarchical clustering identified systematic variations in the gene expressions between normal pituitary glands and prolactinomas (Fig. 1A). Microarray data have been archived in NCBI Gene Expression Omnibus (accession number GSE119063). In particular, we found that the DEPTOR transcripts were remarkably decreased by approximately 14.9-fold in prolactinoma tissues (Fig. 1B). Moreover, analysis of previously microarray datasets from the Gene Expression Omnibus repository database showed that DEPTOR expression was also decreased in gonadotroph and nonfunctioning tumors compared to normal pituitary tissues (GSE26966 and GSE51618) (Supplementary Fig. 1A and B). These data suggested that DEPTOR downregulation may be associated with the initiation or development of PAs.

We then detected the expression of DEPTOR in PA tissues and human normal pituitary glands by immunohistochemistry. Consistent with the profile data, immunohistochemistry confirmed that DEPTOR protein expression was lower in 82 PA specimens with all subtypes compared to that in 12 normal pituitary tissues (Fig. 1C, Table 1). Correlation analysis of the expression of DEPTOR with clinicopathological features of 82 patients was shown in Table 2, while the tumor characteristics and clinical features of 82 patients were summarized in Supplementary Table 1. It showed that DEPTOR expression was correlated with tumor size, but, did not seem to be associated with the sex, age or cavernous sinus invasion staging of the PA patients (Table 2). Additionally, we examined the expression of DEPTOR in PA tissues by quantitative real-time PCR and analyzed the correlation between the expression level of DEPTOR and progression of pituitary

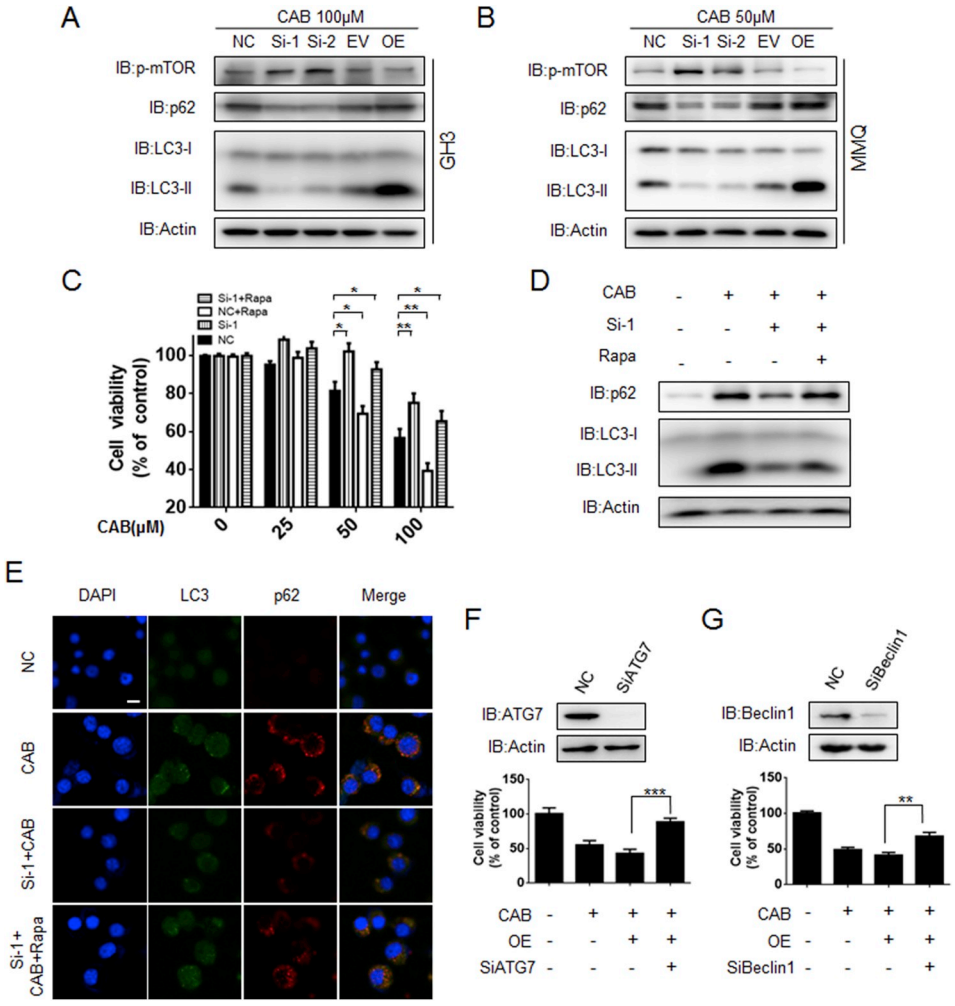

Fig. 7. Overexpression of DEPTOR enhanced autophagic cell death induced by CAB. (A) GH3 or (B) MMQ cells were transfected with the indicated plasmids or siRNA. After transfected for 36 h, the cells were treated with the indicated concentrations of CAB for 24 h. Then, cell lysates were subjected to western blotting with indicated antibodies. (C) GH3 cells were transfected with the siRNA or siControl (NC) as before, planted in 96 well plates treated with or without 100 nM rapamycin (Rapa) and with the indicated concentrations of CAB for 24 h. Cell viability was measured by MTS assay. (D and E) The treatment of D and E was the same as in C, followed by western blotting assay or immunofluorescence staining with indicated antibodies. (F and G) DEPTOR-overexpression GH3 cells were transfected with ATG7 or Beclin1 siRNA for 48 h before treating with CAB for 24 h. Cell viability was measured by MTS assay. $^{*}P < 0.05$, $^{**}P < 0.01$. EV, empty vector; OE, over expression; NC, negative control.

tumors. DEPTOR expression was decreased by 2.34-fold in large pituitary tumors (> 3 cm) compared to the small tumors (< 3 cm) (Supplementary Fig. 2).

3.2. DEPTOR suppressed PA cells proliferation in vitro and in vivo

Contradictory data have been reported suggesting DEPTOR functioning either as tumor promoter or a tumor suppressor [23,24]. To clarify the role of DEPTOR in PA development, we manipulated its expression in two PA cell lines, GH3 and MMQ. Overexpression and knockdown of DEPTOR, respectively, were confirmed by western blotting analysis (Fig. 2A and B). The MTS assay showed that overexpression of DEPTOR strongly inhibited cell proliferation in both GH3 and MMQ cells, while the knockdown increased cell proliferation (Fig. 2C and D). During the period of time, the efficiency of silencing in MMQ cells was detected by western blotting in Supplementary Fig. 3. In colony formation assays, GH3 cells were infected with lentiviral DEPTOR overexpressing or shRNA construct. In accordance with proliferation, DEPTOR overexpression inhibited GH3 cell colony formation (Fig. 2E), and DEPTOR knockdown in GH3 cells resulted in an increase in colony formation (Fig. 2F). In addition, we also tested the production of PRL and GH in GH3 cells, and PRL in MMQ cells. However there was no difference in hormone production with overexpression or knockdown of DEPTOR in these cells (Supplementary Fig. 4). To evaluate the suppressive function of DEPTOR in tumorigenesis *in vivo*, we injected DEPTOR overexpressing GH3 cells (OE group) and control GH3 cells (EV group) subcutaneously into female nude mice, respectively. As shown in Fig. 2G, DEPTOR overexpression significantly reduced xenograft tumor sizes as compared to control group (2.83-fold, $p < 0.001$). These results demonstrated that DEPTOR overexpression could inhibit pituitary tumor cells proliferation *in vitro* and *in vivo*.

3.3. DEPTOR suppressed mTORC1 and mTORC2 activity

Because DEPTOR is a naturally negative regulator of mTOR [14], GH3 and MMQ cells was used to assess the effect of DEPTOR expression on the mTOR signaling pathway. We found that overexpression of DEPTOR in GH3 and MMQ cells decreased phosphorylation of mTOR and AKT, a critical downstream effector of the mTORC2, as well as decreased phosphorylation of S6K1 and 4EBP1, two key downstream effectors of the mTORC1, indicating downregulation of mTORC1 and mTORC2 activity (Fig. 3A and B). In contrast, knockdown of DEPTOR in these cells increased phosphorylation of those proteins (Fig. 3A and B). Immunohistochemistry showed that the xenograft tumors from DEPTOR overexpressing GH3 cells implicated a decrease in phosphorylation of 4EBP1 (Supplementary Fig. 5). These data indicated that DEPTOR inhibited the mTOR signaling pathway in pituitary tumor cells.

3.4. DEPTOR expression was decreased in dopamine-resistant prolactinomas

Dopamine agonists (DA) are effective in the treatment of prolactinomas [25]. However, some tumors develop resistance, thus limiting its efficacy. To understand the role of DEPTOR in DA actions, we compared DEPTOR mRNA expression in BRC-sensitive and -resistant

prolactinomas by quantitative real-time PCR. DEPTOR mRNA expression in five BRC-resistant prolactinomas was decreased by 2.28-fold compared to that in four sensitive tumors (Fig. 4A). Western blotting also showed that DEPTOR protein expression was lower in the five BRC-resistant prolactinomas than that in the four sensitive tumors (Fig. 4B). Consistently, immunohistochemical staining showed that DEPTOR expression was lower in thirteen BRC-resistant prolactinomas than in twenty-one BRC-sensitive tumors (Fig. 4C, Table 3). The clinical information and laboratory test results for these 34 prolactinomas are listed in Supplementary Table 1. These data suggest that the decreased DEPTOR expression may be involved in DA resistance in prolactinomas.

3.5. CAB restored DEPTOR expression to inhibit mTOR kinase activity via decreasing βTrCP1 expression

Our previous study showed that AKT/mTOR signaling was significantly inhibited in the GH3 and MMQ cells by CAB [26]. In addition, βTrCP1–containing E3 ubiquitin ligase was found to regulate the stability of DEPTOR [27–29], and its level was reduced by a PI3K/mTOR inhibitor in a proteasome-dependent manner [30]. We thus tested whether CAB could restore DEPTOR expression by reducing βTrCP1 expression. We found that DEPTOR protein expression was increased in GH3 and MMQ cells by CAB in a dose- and time-dependent manner (Fig. 5A–D). The increased DEPTOR protein expression in CAB-treating PA cells was not due to gene transcription enhancement (Supplementary Fig. 6). We examined the expression of βTrCP1 and phosphorylation of mTOR in GH3 and MMQ cells with or without CAB treatment. CAB inhibited the expression of βTrCP1 and the phosphorylation of mTOR (Fig. 5E). Considering that MMQ cells have a high expression of DRD2 but GH3 cells are lack of DRD2 expression [31], MMQ cells were treated with DPD, a DRD2 antagonist. Increased βTrCP1 protein expression and phosphorylation of mTOR, and decreased DEPTOR protein expression were observed (Fig. 5F). Taken together, our data suggest that CAB inhibited mTOR kinase activity by stabilizing DEPTOR expression through reducing E3 ubiquitin ligase βTrCP1 protein.

3.6. DEPTOR sensitized PA cell lines to CAB

We found that CAB was more effective in suppressing cell proliferation in MMQ cells than in GH3 cells (Fig. 6A), consistent with the previous reports [26,32]. Similarly, DEPTOR protein level was higher in MMQ cells than that in GH3 cells, and CAB treatment resulted in a faster clearance of βTrCP1 in MMQ cells than that in GH3 cells (Fig. 6B). Overexpression of DEPTOR was found to enhance the anti-proliferative activity of CAB treatment in both GH3 and MMQ cells (Fig. 6C and E). In contrast, knockdown of DEPTOR decreased the anti-proliferative activity of CAB treatment in these cells (Fig. 6D and F). These results implicate that the DEPTOR levels in PA cells promote CAB sensitivity.

3.7. DEPTOR enhanced CAB-induced autophagy-dependent cell death

Autophagic cell death is considered one of the three major forms of cell death [33]. Our previous study had shown that CAB induced autophagy and inhibited the autophagic flux, leading to autophagy-dependent cell death [26]. To test whether DEPTOR enhanced autophagy to contribute to CAB-induced cell death in PA cells, GH3 and MMQ cells were treated with CAB following transfection with DEPTOR siRNA or overexpression plasmids, and phosphorylation of mTOR, as well as expression of LC3-II and p62 were examined by western blotting. We found that knockdown of DEPTOR increased phosphorylation of mTOR and decreased p62 and LC3-II expression in both cell lines, while DEPTOR overexpression had the opposite effects (Fig. 7A and B). These data showed DEPTOR enhanced disruption of autophagic flux induced by CAB. In consideration of autophagy induction by inactivation of mTOR, rapamycin, an inhibitor of mTOR would have the opposite effect with DEPTOR knockdown. Then, DEPTOR knockdown was found to impair the anti-proliferative activity of CAB in GH3 cells; however, the anti-proliferative activity was partially reversed by rapamycin (Fig. 7C). Western blotting showed that DEPTOR knockdown inhibited p62 and LC3-II expression induced by CAB in GH3 cells, while, rapamycin reversed the suppression effect on p62 and LC3-II level (Fig. 7D). This result was further confirmed by immunofluorescence staining and confocal microscopy: CAB induced p62 and LC3-II in GH3 cells, but these proteins were decreased with DEPTOR knockdown. However, when rapamycin was used with CAB, p62 and LC3-II were still visible in DEPTOR-knockdown GH3 cells (Fig. 7E).

In our previous study, we found that CAB augmented lysosome acidification, leading to impaired lysosomal degradation and accumulation of p62 [26]. BafA1, a specific inhibitor of vacuolar-type H(+)-ATPases, can increase the lysosomal pH. BafA1 was used to treat with DEPTOR-knockdown or DEPTOR-overexpression GH3 cells along with CAB treatment. As shown in Supplementary Fig. 7A, the expression of LC3-II and p62 was increased upon overexpression of DEPTOR, but the effect was reversed by BafA1. In contrast, the expression of LC3-II and p62 was decreased after DEPTOR knockdown, and further decreased with BafA1 treatment (Supplementary Fig. 7B). To test whether enhanced autophagy by DEPTOR modulation with CAB treatment was responsible for cell death, siRNA was used to effectively knockdown expression of ATG7 and Beclin1, which are required for autophagy initiation, in GH3 cells. After ATG7 and Beclin1 knockdown, the enhanced anti-proliferative effect by CAB with DEPTOR overexpression was diminished (Fig. 7F and G).

Taken together, these results suggest that DEPTOR enhances autophagy-dependent cell death induced by CAB.

4. Discussion

The results of this study suggested that DEPTOR was possibly a novel tumor suppressor that was important for PA cells progression and dopamine-resistance. Although accumulated evidence has implicated that DEPTOR plays a pivotal role in the development and progression of human malignances, it is surprising that DEPTOR can behave either as an oncogene or oncosuppressor, depending on the cell- or tissue-contexts [23,24,34]. Even, opposite results were obtained in the same myeloma cells [14,35]. DEPTOR, an inhibitory subunit, was found to bind to both mTORC1 and mTORC2 [14,24]. In vitro kinase assays showed that DEPTOR depletion increases the activity of mTORC1 and mTORC2 [14]. DEPTOR has been suggested to play an important role in multiple fundamental cell processes such as mRNA translation, ribosome biogenesis, cell growth and proliferation, autophagy as well as inflammation [26]. In several tumor models, such as lung adenocarcinoma, pancreatic cancer, colorectal cancer and liver cancer, DEPTOR expression is much lower than in corresponding normal tissue. As its loss promotes growth and survival by activating AKT/mTOR signaling, DEPTOR mainly acts as a tumor suppressor in these tumors [8,15–17]. However, in multiple myeloma, cervical squamous cell carcinoma and differentiated thyroid carcinoma, DEPTOR overexpression inhibited mTORC1 and blocked the negative feedback loops from mTORC1 to PI3K and affected PI3K's action toward mTORC2, resulting in that DEPTOR stimulated cell proliferation [14,36–38]. In our study, we found DEPTOR overexpression suppressed both mTORC1 and mTORC2 activity in PA cells, resulting in proliferation inhibition *in vitro* and growth suppression *in vivo*. Of course, it would be even better if these results could be further confirmed in primary pituitary tumor cells.

Several publications have shown regulation of DEPTOR by cancer treatment drugs. For example, treatment of liver cancer cells with metformin led to an increase in DEPTOR protein levels by inhibition of proteasome activities, thus suppressing cell proliferation through inhibiting mTOR signaling pathway [16]. Bortezomib, one of the major drugs used for the treatment of multiple myeloma, was found to

increase DEPTOR level by upregulating expressionist transcription in multiple myeloma cell lines [39]. A recent study reported DEPTOR upregulation in response to gefitnib, a specific EGFR inhibitor, by inhibiting the degradation of DEPTOR via downregulating the function of mTOR autoamplification loop in lung adenocarcinoma cell lines [8]. Consistent with these studies, we found that DEPTOR expression was induced in GH3 and MMQ cells upon treating with CAB. Our data indicated that CAB inhibited mTOR kinase activity by stabilizing DEPTOR expression via decreasing βTrCP1 expression.

For pituitary tumor treatment, DA is primarily used to treat with prolactinomas, and is effective in inhibiting prolactin hypersecretion, reducing tumor size, and restoring gonadal function [25]. However, tumor resistance occurs in some patients, becoming the main reasons why treatment fails and tumor increases again [40–44]. We previously found that CAB, mainly known as DRD2 agonist, suppressed mTOR signaling pathway and induced autophagy-dependent cell death in GH3 and MMQ cells [26]. Additionally, our another study further demonstrated that activation of DRD5 suppressed tumor growth *in vitro* and *in vivo* in different cell types including PA cells, by inhibiting the mTOR pathway, inducing autophagy, and causing autophagic cell death [31]. These studies all have shown that dopamine agonists and the mTOR signaling pathway played an important role in PA therapy. In this study, we found that CAB treatment enhanced DEPTOR expression to inhibit mTOR kinase activity via inducing a clearance of βTrCP1 in GH3 and MMQ cells. Overexpression of DEPTOR significantly enhanced the anti-proliferative activity of CAB treatment by inactivation of mTOR signaling pathway. We further demonstrated that DEPTOR enhanced autophagy-dependent cell death induced by CAB.

In summary, our study revealed that DEPTOR was downregulated in PAs and that overexpression of DEPTOR impaired the proliferation of pituitary tumor cell lines *in vitro* and *in vivo*. In addition, DEPTOR expression could be restored by CAB via inhibiting βTrCP1 expression. Moreover, DEPTOR overexpression sensitized pituitary tumor cell lines to CAB, increasing its anti-proliferative functions by autophagy promotion through inactivation of the mTOR signaling pathway. Therefore, our results implicate that DEPTOR may be a therapeutic target for pituitary tumors.

Declaration of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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