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Research Paper

Osteopontin is Critical for Hyperactive mTOR-Induced Tumorigenesis in Oral Squamous Cell Carcinoma

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Abstract

Mechanistic target of rapamycin (mTOR) plays a critical role in the development of oral squamous cell carcinoma (OSCC), but the underlying mechanisms remain poorly understood. Here we have demonstrated that the expression of osteopontin (OPN) was dramatically up-regulated in OSCC tissues and cell lines. Moreover, reduction of OPN suppressed cell proliferation, colony formation, and *in vivo* tumorigenic ability of OSCC cell lines Tca8113. In addition, there was a strong positive correlation between mTORC1 activity and OPN expression in OSCC tissues and cell lines. Furthermore, mTOR complex 1 (mTORC1) enhanced OPN expression through up-regulation of ERRα. Therefore, OPN is a downstream target of mTORC1 and is crucial for OSCC development. mTORC1, ERRα, and OPN may be potential targets for treatment of OSCC with aberrant mTORC1 signaling.

Key words: mTOR; ERRα; OPN; tumorigenesis; OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) ranks among the top-ten most common cancers worldwide [1]. The etiology of OSCC is complicated and many factors are involved in its progression. Despite the advances in the treatment applied, the overall survival rate of patients with OSCC remains poor [2]. Therefore, it is of great significance to find an effective method for diagnosis and treatment of OSCC.

Mechanistic target of rapamycin (mTOR), a serine/threonine protein kinase, exerts a critical role in cell growth, proliferation, and survival [3, 4]. mTOR associates with different partners to form two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [3, 5]. mTORC1 is sensitive to rapamycin, whereas mTORC2 is rapamycin-insensitive. mTORC1 signaling pathway is frequently altered in many cancers [6]. Recently, the critical role of mTORC1 in OSCC development has been established [7-9], but the exact mechanisms remain to be further elucidated.

Osteopontin (OPN) is a secreted phosphorylated glycoprotein and is overexpressed in a variety of cancers [10, 11]. OPN plays an important role in tumor angiogenesis, apoptosis, and cancer metastasis [12]. There are few characterizations on the functional regulation of PI3K/AKT/mTOR signaling on OPN expression.

In this study, we have shown that OPN expression is elevated in OSCC and positively correlated with mTORC1 activity. Depletion of OPN suppresses tumorigenic capacity of human OSCC cells. mTORC1 positively regulates OPN expression through the activation of ERRα signaling. mTORC1, ERRα, and OPN may serve as potential targets for the treatment of OSCC with dysregulated mTORC1 signaling.

Materials and Methods

Reagents and antibodies

Rapamycin and XCT-790 were purchased from Sigma; Lipofectamine 2000 and 4-12% Bis-Tris Nu-PAGE gels were obtained from Life Technologies. OPN and β-actin antibodies were purchased from Santa Cruz; p-S6 (Ser235/236), S6, mTOR, Raptor, and Rictor antibodies were obtained from Cell Signaling. GAPDH and ERRα antibodies were purchased from Abcam.

Cell cultures

The human transformed embryonic kidney cells (HEK293T) and the human OSCC cell lines KB, HSC-2, HSC-3, Tca8113, SCC-9, and SCC-15 were obtained from the ATCC. Tsc2+/+ and Tsc2-/- mouse embryonic fibroblasts (MEFs) used in this study were kindly provided by Dr. Hongbing Zhang (Peking Union Medical College) and have been described previously [13, 14]. All cell lines were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin in 5% CO_2 at 37°C.

Clinical samples

Clinical samples of 30 OSCC and 16 paired normal mucosa tissues (adjacent normal tissues) were obtained from patients undergoing surgical resection in the affiliated hospital of stomatology, Chongqing Medical University from June 2013 to December 2013. All samples were immediately preserved in -80°C cryogenic refrigerator after surgery. All cases of OSCC were confirmed for the first time and without prior neoadjuvant therapy. All specimens were diagnosed for primary squamous cell carcinoma of the tongue by more than two pathologists. Both oral and written informed consents were taken from every patient in the study, according to a protocol approved by ethics committees of Chongqing Medical University.

Lentivirus-mediated RNA interference

The GV248 lentiviral shRNA expression vector targeting human OPN and control scrambled (shScramble) were obtained from Genechem (Shanghai, China). Lentiviruses were produced by co-transfecting the shRNA expression vector with the Vira Power lentiviral Packaging Mix (Genechem) in HEK293T cells. Culture supernatants were collected after 48 h of transfection and then used to infect Tca8113 cells.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using Trizol (Invitrogen) according to the protocol provided by the manufacturer. RNA was converted to cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa). qRT-PCR was performed using SYBR® Premix Ex Taq™ II (TaKaRa) as described previously [14]. The primer sequences were as follows: OPN forward: 5′-CTCCATTGACTCGAACGACTC-3′, reverse: 5′-CAGGTCTGCGAAACTTCTTAGAT-3′; β-actin forward: 5′-AGAAAATCTGGCACCACACC-3′, reverse: 5′-AGAGGCGTACAGGGATAGCA-3′.

Western blotting analysis

Western blotting analyses were performed as described previously [13, 15] using primary antibodies against OPN, mTOR, Raptor, Rictor, p-S6, S6, ERRα, GAPDH, and β-actin.

RNA interference

Cells were seeded in 12-well plates and transfected with siRNAs using Lipofectamine 2000 following the manufacturer's instructions. All siRNA were synthesized by GenePharma (Shanghai, China). siRNA targeted sequences were as follows: mTOR: 5′-CCCTGCTTCTGTCATGCCT-3′; Raptor: 5′-GGAC AACGGCCACAAGTAC-3′; Rictor: 5′-ACUTGTGA AGAATCGTATC-3′; ERRα: 5′-GGACCTATGAGAC CTTCAA-3′; negative control: 5′-TTCTCCGAACGT GTCACGT-3′.

Reporter constructs and luciferase reporter assay

A 834-bp human OPN promoter fragment was cloned into the *kpn* I / *xho* I sites of the luciferase reporter plasmid pGL3-basic (Promega) and named as OPN-luc. The primer sequences were as follows: forward, 5′-CGGGGTACCCATGGATGAGGGAACA AGG-3′; reverse, 5′-CCGCTCGAGTACCTTGGTCGG CGTTTGG-3′. The putative ERRα binding sites in the OPN promoter were mutated using the Quick Change site-directed mutagenesis kit (Promega). The primer sequences were as follows: Mut1, forward 5′-GCCCAAGGTTGCACATATTTGCAGTGACACA GCGGA-3′; reverse 5′-TCCGCTGTGTCACTGCAAA TATGTGCAACCTTGGGC-3′. Mut2, forward 5′-AAAGCTAAGCTTGAGTAGTAGACCAGTGAGG CAAGTTTTCTG-3′; reverse 5′-CAGAAAACTTGCCT CCATGGTCTACTACTCAAGCTTAGCTTT-3′. Cells were incubated in triplicate in 24-well plates and transfected with the promoter constructs (200 ng) in combination with the plasmid pRL-TK (20 ng) as an internal control. Luciferase activity was examined with the Dual-Luciferase Reporter Assay System (Promega).

Chromatin immunoprecipitation assay

Immunoprecipitation assay with an anti-ERRα antibody was performed with a SimpleChIP® Enzymatic Chromatin IP kit (Cell Signaling)

according to the manufacturer's protocol. The immunoprecipitated DNA was purified for the analysis by qRT-PCR. The primer sequences used were as follows: the putative ERRα-binding site region 1 (PBR1) of human OPN forward, 5′-TGG ATGAGGGAACAAGGATAGGTAGG-3′, reverse, 5′-GACCGTGGTTCTGAATTCCGCTGT-3′; the putative ERRα-binding site region 2 (PBR2) of human OPN forward, 5′-TGCATACTCGAAATCACAAAG CTAAG-3′, reverse, 5′-AATGGATTTTTGTTTCT-TTCGGTTTA-3′; a nonspecific ERRα-binding region (NBR) of human OPN forward, 5′-AAGAAA TAGCAATCACCTGGGCAGTC-3′, reverse, 5′-CTCC CGGCCTCCTGAATTAAACGTCT-3′.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 2×10^3 per well and the cell proliferation was evaluated with MTT assay as described previously [16].

Soft agar assay

A 0.6% (w/v %) bottom layer of low melting point agarose in common culture medium was placed in 6-well plates. On top, a layer of 0.3% agarose containing 1×10^3 cells was placed, and the colony number in the soft agar was counted after three weeks [14].

Induction of subcutaneous tumors in nude mice

Subcutaneous tumors were established in nude mice (BALB/c, 5 week old) as described previously [17, 18]. Six male mice were used in each cohort. 2×10^6 Tca8113 cells expressing shOPN[1] or shScramble in 100 μl of DMEM were s.c. inoculated into the right posterior back region. Mice were sacrificed when tumor size was greater than 1,000 mm^3, there was ulceration over the tumor, or weight loss of more than 10% occurred. All animals were maintained and used in accordance with the guidelines of the Animal Center of Chongqing Medical University.

Enzyme-linked immunosorbent assay (ELISA)

The presence of the OPN released by the OSCC cell lines into the culture medium was measured using a human OPN Quantikine ELISA kit (R&D Systems) according to the manufacturer's instructions. In brief, Cells (6×10^5 cells/well) were plated into 6-well culture plates. After 12 h, the culture medium was replaced with an equivalent volume of fresh serum-free medium with or without rapamycin (50 nM). Cell culture supernatants were collected 24 h later and the OPN levels were determined using an ELISA kit.

Statistical analysis

The tumor development and survival data were analyzed with Kaplan–Meier log-rank test and other data were analyzed using a 2-tailed paired Student's t-test as described previously [13, 14]. It is statistically significant when $P < 0.05$.

Results

The expression of OPN is significantly up-regulated in OSCC and positively correlated with mTORC1 signaling

OPN plays a critical role in tumorigenesis and is frequently overexpressed in various types of cancers [11, 19, 20]. However, the role of OPN in the development of OSCC remains largely unknown. To investigate the expression abundance of OPN in OSCC, we performed qRT-PCR with cDNA from 30 OSCC tissues and 16 normal mucosa tissues. As shown in Figure 1A, the mRNA level of OPN was significantly higher in OSCC tissues compared with that of normal tissues. To determine whether the protein levels of OPN were also elevated in OSCC tissues, we measured the expression level of OPN in OSCC tissues by western blotting. The result revealed that OPN was markedly up-regulated in OSCC tissues compared to adjacent normal tissues. Interestingly, there were consistent increase of p-S6 level with OPN expression in the tumor tissues (p-S6 is an indicator of mTORC1 activity) [21] (Figure 1B). To further explore the relationship between mTORC1 and OPN, we used six human OSCC cell lines (KB, HSC-2, HSC-3, Tca8113, SCC-9, and SCC-15) to analyze the expression levels of p-S6 and OPN. As shown in Figure 1C, the expression level of p-S6 was positively correlated with OPN in these cell lines. Taken together, these data indicate that OPN is up-regulated in OSCC and the expression of OPN is positively correlated with mTORC1 activity in OSCC.

mTORC1 positively regulates OPN in OSCC cell lines

mTOR exists in two multi-protein complexes (rapamycin-sensitive mTORC1 and rapamycin-insensitive mTORC2). To determine whether OPN is controlled by mTORC1, we evaluated the effect of rapamycin, specific inhibitor of mTORC1 on OPN expression. As depicted in Figure 2A, the expression and secretion of OPN were decreased in response to rapamycin treatment in Tca8113 cells and KB cells. In order to confirm the regulation effect of mTORC1 on OPN expression, we examined the expression level of OPN in mTOR-, Raptor (a specific component of mTORC1)-, or Rictor (a specific component of mTORC2)-knockdown cells. Knockdown of mTOR or

Raptor with siRNAs dramatically decreased the expression level of OPN in Tca8113 cells, whereas the level of OPN in cells transfected with Rictor siRNAs was no change with that in the control (Figure 2B). Collectively, these data suggest that it is mTORC1, but not mTORC2, that up-regulates OPN expression.

mTORC1 up-regulates OPN through activation of ERRa signaling

Estrogen-related receptor α (ERRα) is an orphan nuclear receptor that plays a crucial role in the transcriptional regulation of mitochondrial function and energy metabolism [22, 23]. Next we investigated whether there was a contribution of ERRα in mTOR-mediated up-regulation of OPN. We first determined whether ERRα is controlled by mTORC1. As shown in Figure 3A, the protein levels of ERRα were dramatically decreased after treatment with rapamycin in KB and Tca8113 cells. Moreover, knockdown of Raptor remarkably reduced ERRα expression in Tca8113 cells and KB cells (Figure 3B). Given that TSC2 is the key negative regulator of mTORC1, Tsc2-deficient MEFs have been widely used as cell models for the study on mTOR-related diseases [24-26]. The ERRα expression was markedly increased in Tsc2-/- MEFs compared to WT MEFs, and impaired by the treatment with rapamycin (Figure 3C). Therefore, these results suggested that ERRα is a downstream effector of mTORC1.

To investigate whether mTORC1 up-regulates OPN via ERRα, Tca8113 and KB cells were treated with XCT-790, a specific inhibitor of ERRα. As shown in Figure 3D, the levels of OPN were markedly decreased in the presence of XCT-790. Moreover, knockdown of ERRα with siRNAs led to decrease of OPN expression in Tca8113 and KB cells (Figure 3E). Taken together, these data indicated that mTORC1 up-regulates OPN through activation of ERRα signaling.

To explore the mechanism by which ERRα up-regulates OPN expression, we analyzed the promoter region of OPN gene and found that there were 2 putative ERRα-binding sequence (-400/-391 TAAAGGACA, -630/-621 CACAGGTCA) in the promoter of OPN gene (Figure 3F). We inserted a 834-bp fragment of the promoter sequence of human OPN gene containing the putative ERRα-binding sequences, into the luciferase reporter plasmid to evaluate the transcriptional activity of ERRα on this region. The transcriptional activity was significantly impaired when one of the putative ERRα-binding sites was mutated (Figure 3G). ChIP assay revealed that the direct binding of ERRα to one of the putative ERRα-binding sites within the promoter of OPN gene were significantly attenuated by the treatment of rapamycin or XCT790 (Figure 3H). Taken together, ERRα transcriptionally regulates OPN expression downstream of mTORC1 signaling.

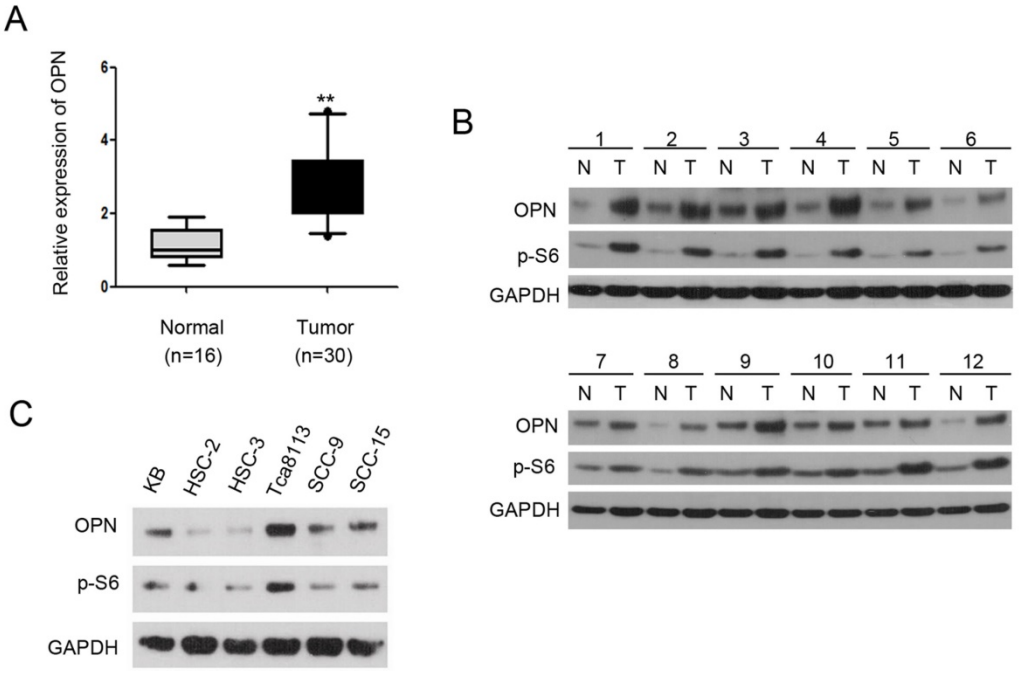

Figure 1. OPN expression is elevated in OSCC and positively correlated with mTORC1 activity. (A) qRT-PCR analysis of OPN expression in 30 OSCC tissues compared to 16 normal mucosa tissues. $^{**}P<0.01$, compared with the respective controls. Box plots display the median, 25th and 75th percentiles. Whiskers represent 5-95 percentiles and dots the outliers. (B) Western blotting analysis of OPN and p-S6 levels in 12 paired of OSCC tissues and adjacent normal/pre-cancerous tissues and GAPDH was used as a control. (C) Cell lysates from KB, HSC-2, HSC-3, Tca8113, SCC-9, and SCC-15 cells were subjected to immunoblotting with the indicated antibodies.

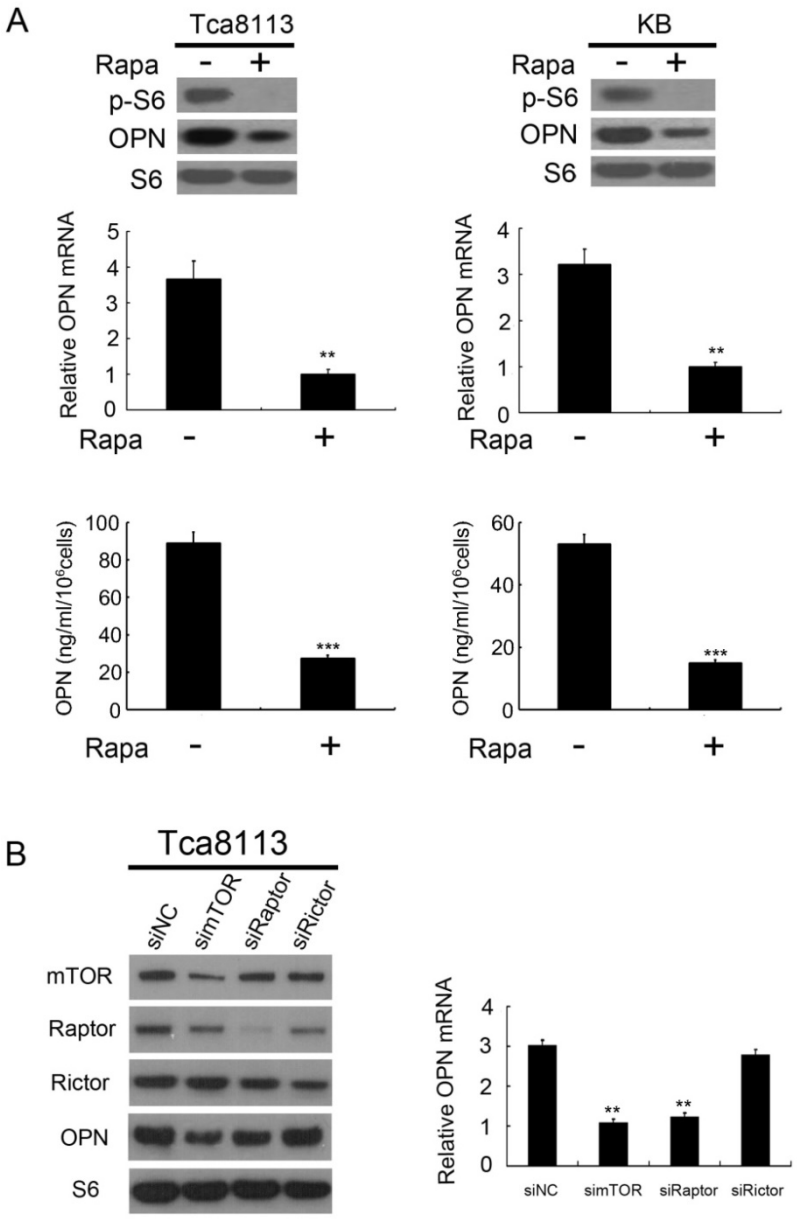

Figure 2. mTORC1 positively regulates OPN expression. (A) Tca8113 and KB cells were treated with or without 50 nM rapamycin (Rapa) for 24 h. Expression levels of OPN, p-S6 and S6 were detected by western blot analysis (top). The mRNA levels of OPN were analyzed by qRT-PCR (middle). OPN levels in the cell supernatants were detected by an ELISA (bottom). Data indicate mean ± SD of triplicate samples. ***P*<0.01; ****P*<0.001, compared with the respective controls. (B) Tca8113 cells were transfected with mTOR, Raptor or Rictor siRNAs for 48 h. Cell lysates were subjected to immunoblotting with the indicated antibodies (right). The mRNA levels of OPN were analyzed by qRT-PCR (left). Data indicate mean ± SD of triplicate samples. ***P*<0.01, compared with the respective controls.

Depletion of OPN suppresses tumorigenic capacity of human OSCC cells

To investigate the role of OPN in the tumorigenesis of human OSCC cells, we knocked down OPN expression with two different shRNAs for OPN (shOPN[1] and shOPN[2]) in Tca8113 cells. Both shOPN[1] and shOPN[2] dramatically decreased the OPN expression (Figure 4A). Reduction of OPN expression led to a decrease of cell proliferation and suppression of colony formation in Tca8113 cells (Figure 4B and C). To determine whether depletion of OPN influences the tumorigenic ability of Tca8113 cells *in vivo*, we inoculated Tca8113 cells expressing shOPN[1] or shScramble subcutaneously into nude mice. Depletion of OPN substantially inhibited the tumorigenesis of Tca8113 cells in nude mice (Figure 4D). Impaired OPN expression in the tumor tissues derived from these nude mice was confirmed by western blotting (Figure 4E). Moreover, IHC analysis showed that reduction of OPN expression led to a decrease of proliferation marker Ki-67 expression *in vivo* (Figure 4F). Taken together, these data indicate that OPN is critical for the proliferation, colony formation, and tumor growth of OSCC cells.

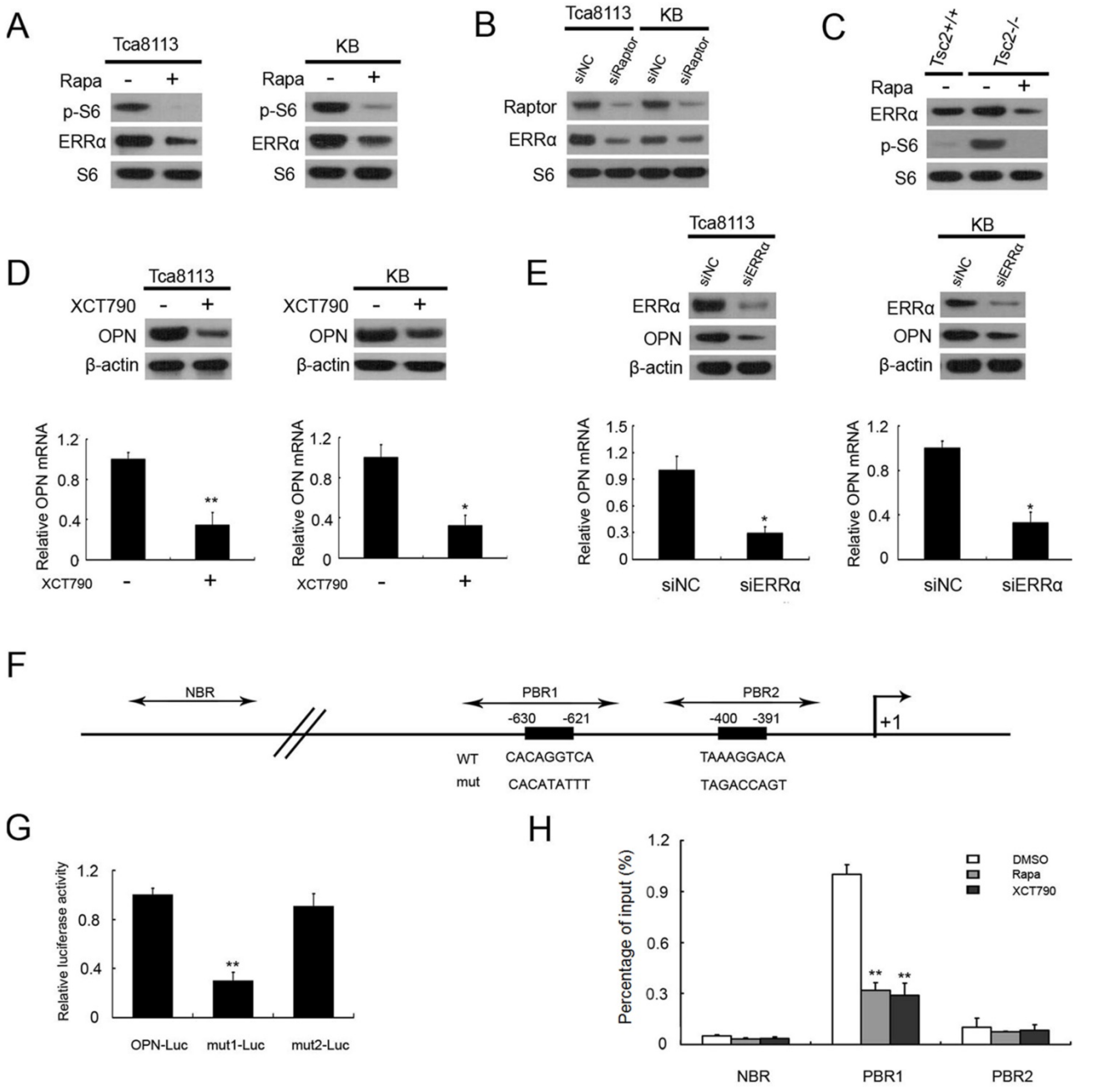

Figure 3. mTORC1 up-regulates OPN through activation of ERRα signaling.(A) Cell lysates from Tca8113 and KB cells treated with or without 50 nM Rapa for 24 h were subjected to immunoblotting with the indicated antibodies. (B) Cell lysates from Tca8113 or KB cells transfected with Raptor or the control (siNC) siRNAs for 48 h were subjected to immunoblotting with the indicated antibodies.(C) Cell lysates from Tsc2+/+ MEFs and Tsc2-/- MEFs treated with or without 20 nM Rapa for 24 h were subjected to immunoblotting with the indicated antibodies. (D) Tca8113 and KB cells were treated with or without 50 nM Rapa for 24 h. The proteins were detected by immunoblotting with the indicated antibodies (top). The mRNA levels of OPN were analyzed by qRT-PCR (bottom). Data indicate mean ± SD of triplicate samples. ***P*<0.01, compared with the respective controls. (E) Tca8113 and KB cells were transfected with ERRα or the control siRNAs for 48 h. The proteins were detected by immunoblotting with the indicated antibodies (top). The mRNA levels of OPN were analyzed by qRT-PCR (bottom). Data indicate mean ± SD of triplicate samples. ***P*<0.01, compared with the respective controls. (F) Schematic representation of the putative wild-type (WT) and mutated (mut) ERRα-binding sites within the promoter region of human OPN gene. Dark rectangles indicate the predicted ERRα binding sites; two-way arrows indicate the fragments amplified in ChIP real-time PCR analysis. The transcription start site is indicated by an arrow above the gene. PBR1, putative binding region 1; PBR2, putative binding region 2; NBR, nonspecific binding region. (G) Tca8113 cells were co-transfected with pOPN-Luc, pmut1-Luc, or pmut2-Luc reporter plasmid and pRL-TK plasmid. Relative luciferase activity was examined 48 h after transfection. Data indicate mean ± SD of triplicate samples. ***P*<0.01, compared with the respective controls. (H) Tca8113 cells treated with Rapamycin, XCT790, or DMSO were subjected to ChIP assay using an anti-ERRα antibody. Normal rabbit IgG antibody served as the negative control. qRT-PCR were performed to amplify a fragment surrounding PBR1, PBR2, or NBR. The data were plotted as the ratio of immunoprecipitated DNA subtracting nonspecific binding to IgG vs. total input DNA. Data indicate mean ± SD of triplicate samples. ***P*<0.01, compared with the respective controls.

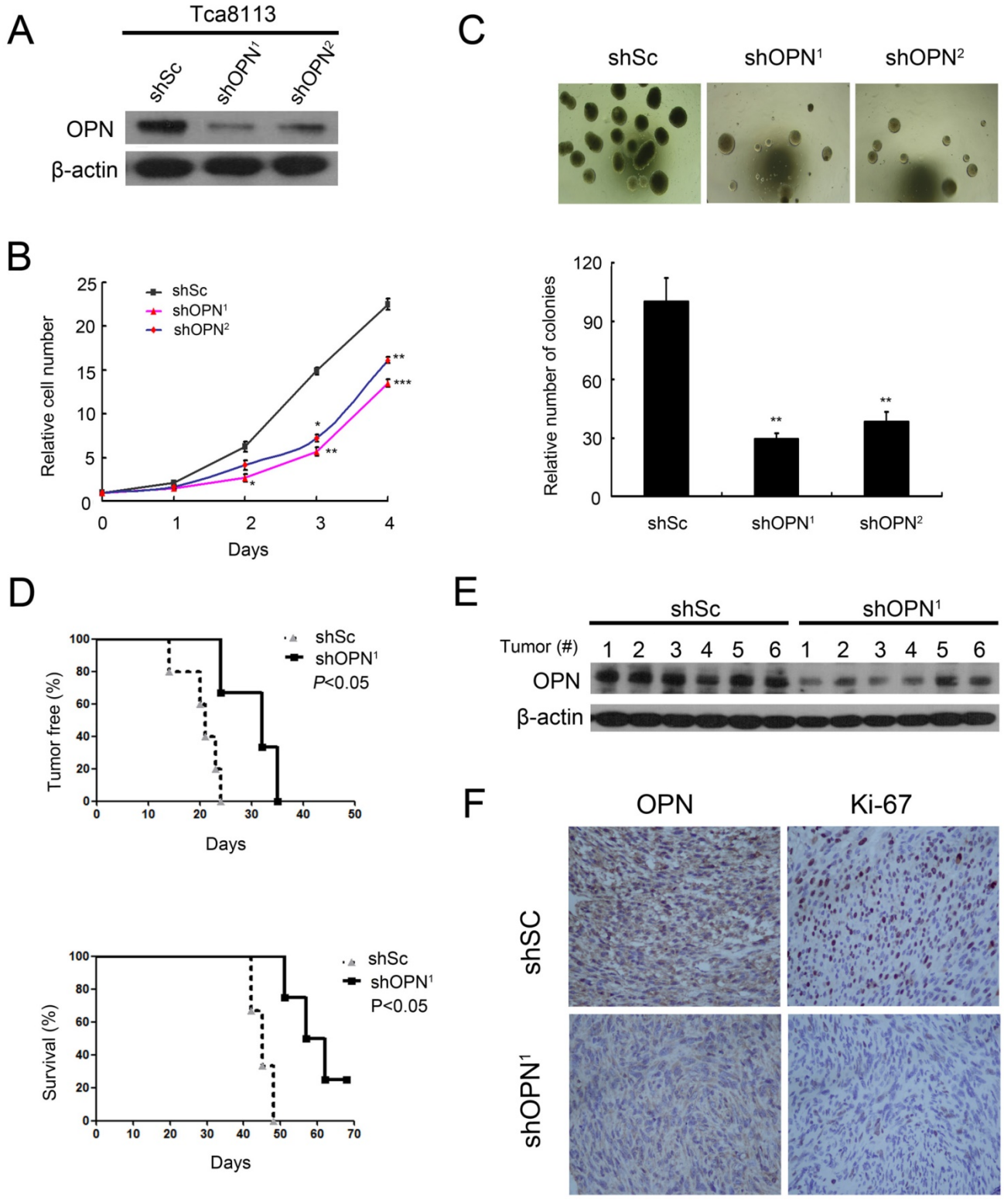

Figure 4. Depletion of OPN suppresses cell proliferation, colony formation, and tumorigenesis. (A) Two independent shRNAs that target OPN (shOPN[1] and shOPN[2]) or a control shRNA (shSc) were stably expressed in Tca8113 cells. The expression of OPN was examined by western blotting and β-actin was used as a control. (B) The proliferation of Tca8113 cells expressing shSc, shOPN[1], or shOPN[2] was examined by MTT assay. Data indicate mean ± SD of triplicate samples. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with the respective controls. (C) The number of colonies formed in soft agar by Tca8113 cells expressing shSc, shOPN[1], or shOPN[2]. Representative images were presented. Data indicate mean ± SD of triplicate samples. ***P*<0.01, compared with the respective controls. (D) Tca8113 cells expressing shSc or shOPN[1] were inoculated subcutaneously into nude mice, and followed for tumor development (top panel) and survival (bottom panel). (E) Tumor tissues from these nude mice were subjected to immunoblotting for OPN expression and β-actin was used as a control. (F) Tumor tissues were subjected to immunohistochemical staining. Representative images were presented.

Discussion

The underlying mechanisms of OSCC mediated by hyperactivated mTOR remain largely elusive. In this study, we have demonstrated that OPN expression is significantly elevated in OSCC. Moreover, depletion of OPN inhibits tumorigenic capacity of human OSCC cells. In addition, OPN levels positively correlated with mTORC1 activity in OSCC. Furthermore, mTORC1 positively regulates OPN via activation of ERRα signaling.

The level of OPN is obviously increased in the serum of patients with tumor metastasis, and it may be a diagnostic indicator of tumor metastasis [27]. It has also been reported that OPN is overexpressed in many cancers, including oral squamous cell carcinoma and early invasive tongue squamous cell carcinoma [28, 29]. Aberrant OPN expression was associated with the malignization of oral squamous epithelium [30]. In this study, we also found that OPN was overexpressed in OSCC tissues. We demonstrated that OPN was required for the growth of OSCC cells *in vitro* and *in vivo* through knockdown of OPN expression in Tca8113 cells, MTT assay, clone formation assay, and human OSCC xenografts in nude mice. Therefore, OPN may be a candidate target for the treatment against OSCC.

The aberrant PI3K/AKT/mTOR signaling pathway plays a critical role in the development of cancers [31-33]. The PI3K/AKT/mTOR signaling is reported to be activated in OSCC [8, 9]. mTOR exists in two different complexes: mTOR complex 1 (mTORCl) and mTOR complex 2 (mTORC2). mTORC1 is sensitive to rapamycin and composed of mTOR, Raptor, and mLST8/GβL. mTORC2 is insensitive to rapamycin and composed of mTOR, Rictor, mLST8/ GβL, and Sin1 [34, 35]. mTORC2 plays an important role in phosphorylation of AKT. Here we demonstrated that the mTOR signaling was activated in OSCC tissues examined, which suggest that there may be a putative link between activation of PI3K/AKT/mTOR signaling pathway and OPN overexpression in OSCC. Subsequent research revealed that OPN was positively regulated by mTORC1, but not mTORC2, in OSCC cell lines Tca8113 and KB. This work established that OPN is a novel effector downstream of mTORC1, and there is a strong link between mTORC1-OPN axis and OSCC development.

Cell- and tissue-specific OPN expression is regulated by hormones, growth factors, and oncogenes. The sequence of OPN promoter is highly conservative and can be combined with a variety of transcription factors [36-38]. The majority of studies have shown that OPN expression is frequently regulated at the level of transcription [38]. ERRα is an orphan nuclear receptor, which is a member of the nuclear receptor superfamily [39]. ERRα is able to combine with DNA to activate transcription [40, 41]. OPN is reported to be a target gene of ERRα in osteoblast differentiation [41]. Although there is no classic estrogen responsive element in the OPN promoter, estrogen can selectively bind to 7 steroid factor response elements of OPN promoter via ERα and ERRα to activate OPN transcription [42]. In this study, we demonstrated that mTORC1 is a positive regulator of ERRα, and ERRα regulate OPN transcription through directly binding to the promoter region of OPN gene. The newly identified mTORC1-ERRα-OPN signaling cascade contributes to the mechanism of OSCC.

In conclusion, hyperactive mTORC1 signaling contributes to OSCC via activation of ERRα-OPN signaling. This study provides new insight into the mechanism of OSCC mediated by hyperactive mTOR. mTORC1, ERRα, and OPN may be candidate targets for treatment of OSCC with abnormal mTORC1 signaling.

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Competing Interests

The authors have declared that no competing interest exists.

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