

Keywords: ROC1; MI-TCC; malignant progression; mTOR; DEPTOR

Regulator of cullins-1 expression knockdown suppresses the malignant progression of muscle-invasive transitional cell carcinoma by regulating mTOR/DEPTOR pathway

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Background: Regulator of cullins-1 (ROC1) is a key subunit in the cullin–RING ligase (CRL) protein complex. Our previous study indicated that ROC1 was essential for bladder cancer cell survival and that ROC1 knockdown inhibited CRL activity, triggering G2 phase arrest and senescence. However, the role of ROC1 in the malignant progression of bladder cancer remained unknown.

Methods: ROC1 expression in cancer cells was knocked down by siRNA silencing. The effects of ROC1 silencing were evaluated by *in vitro* assays for cell migration and by an *in vivo* mouse metastasis model. Epithelial–mesenchymal transition (EMT) induction was evaluated by immunofluorescence staining and western blotting of EMT-associated proteins. ROC1 expression in human tumours was further evaluated by immunohistochemical analysis.

Results: ROC1 knockdown suppresses bladder cancer cell migration by inhibiting EMT. ROC1 knockdown inhibited EMT by inhibiting mammalian target of rapamycin (mTOR) activity via the accumulation of the mTOR-inhibitory protein DEPTOR, a CRL substrate. DEPTOR knockdown partially rescued ROC1 knockdown-inhibited EMT and the ROC1-induced inhibition of cancer cell migration. Furthermore, *in vivo* studies using a nude mouse metastasis model confirmed the *in vitro* data. Finally, tissue microarray analysis of clinical bladder cancer specimens indicated a positive correlation between ROC1 expression and EMT.

Conclusions: ROC1 has an important role in the malignant progression of bladder cancer via the mTOR/DEPTOR pathway. ROC1 may serve as a novel therapeutic target for the treatment of muscle-invasive transitional cell carcinoma.

Bladder cancer is the fourth most common cancer and the eighth leading cause of cancer death among men worldwide (Siegel *et al*, 2015). Histologically, bladder transitional cell carcinoma (BTCC) is the most common bladder cancer subtype, accounting for ~90% of all bladder cancers (Noon and Catto, 2013). Clinically, 20–30%

of all newly diagnosed bladder cancers exhibit a type of muscle layer invasion known as muscle-invasive transitional cell carcinoma (MI-TCC). In addition, 10–30% of non-muscle-invasive bladder cancers will eventually progress to MI-TCC (Mitra and Cote, 2009). For patients with MI-TCC, cisplatin-based

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Received 17 September 2015; revised 23 November 2015; accepted 30 November 2015; published online 7 January 2016

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chemotherapy is usually recommended after surgery (Kaplan *et al*, 2014). However, the severe toxicity and relatively low anticancer efficiency of this chemotherapy regimen limits its wide application in the clinic (Noon and Catto, 2013; Kaplan *et al*, 2014). Furthermore, even with chemotherapy, progression and recurrence occur in the majority of MI-TCC patients, leading to poor prognosis. Therefore, improved understanding of the molecular mechanisms underlying bladder cancer progression and identification of novel anticancer targets for bladder cancer therapy are of great clinical significance and urgently needed.

There is increasing interest in the role of cullin–RING ligases (CRL) in the development and progression of bladder cancer. CRL, also known as Skp1, Cullin and F-box protein, belongs to the largest family of E3 ubiquitin ligases (Skaar *et al*, 2014). It has been demonstrated that CRL mediates the proteasome-targeted degradation of ~20% of ubiquitinated protein substrates (Skaar *et al*, 2014). Consequently, CRL has an important role in the ubiquitination of many proteins including cell cycle-related proteins, DNA replication proteins, signal transduction proteins and gene transcription factors (Lydeard *et al*, 2013; Skaar *et al*, 2014). CRL dysfunction has been linked to tumour development and progression (Lydeard *et al*, 2013), suggesting that it is a potential anticancer target. Indeed, the small molecule inhibitor MLN4924 inactivates CRL by inhibiting cullin activity, and effectively inhibits the growth of various cancer cells (Soucy *et al*, 2009; Gu *et al*, 2014).

The regulator of cullins-1 (ROC1), also known as RING box protein-1 (RBX1), is a key CRL subunit. ROC1 heterodimerises with distinct cullins to constitute CRL catalytic cores, but its function in cancer development and progression is poorly understood (Petroski and Deshaies, 2005; Skaar *et al*, 2014). ROC1 contains a small zinc-binding domain called the RING finger, is evolutionarily conserved from yeast to human and has an essential role in embryonic development (Petroski and Deshaies, 2005). Aberrant ROC1 expression leads to CRL dysfunction and embryonic lethality (Tan *et al*, 2009). In addition, ROC1 is essential for the maintenance of genome integrity, and several recent studies have emphasised its role in human cancers (Jia *et al*, 2011). Our previous studies indicate that ROC1 protein is overexpressed in bladder cancer and that ROC1 knockdown suppresses the growth of bladder cancer cells (Wang *et al*, 2013a, b). Our initial mechanistic study indicated that ROC1 knockdown inhibits CRL activity and triggers the accumulation of specific substrates (such as p21 and p27), leading to G2 phase arrest and senescence (Wang *et al*, 2013a). However, the roles and underlying mechanisms of ROC1 in the malignant progression of bladder cancer remain largely unknown. Therefore, the present study was designed to investigate the effect of ROC1 knockdown on malignant bladder cancer progression and identify the signalling pathways involved. The study design included *in vitro* and *in vivo* experiments and tissue microarray analysis (TMA) of clinical cancer tissue samples. The results of this study may provide a basis for the future development of novel ROC1-based targeted therapies for bladder cancer.

MATERIALS AND METHODS

Cell culture and reagents. Human bladder cancer 253J and EJ cell lines were purchased from the Chinese Academy of Science (Shanghai, China) and cultured in RPMI 1640 (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin. Cells were grown at 37 °C in a humidified 5% CO₂ environment. Rapamycin and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). Rapamycin was dissolved in DMSO and stored at –20 °C.

siRNAs and transfection. siRNA oligonucleotides for silencing various genes (such as ROC1 and DEPTOR) were obtained from Invitrogen (Shanghai, China), and the transfection procedure was performed according to the manufacturer's instructions. siRNA sequences were as follows: ROC1, 5'-GACTTTCCTGCTGT-TACCTAA-3'; DEPTOR, 5'-GCCATGACAATCGGAAATCTA-3'; and scrambled control, 5'-ACGUGACACGUUCGGAGAA-3'.

Cell viability assay. Cell proliferation was assessed using the Cell Counting Kit-8 kit (Beyotime, Shanghai, China), which was carried out as previously described (Wang *et al*, 2013a).

Wound-healing assay. Cells were grown to complete confluence, and then a wound was made in the cell monolayer using a sterile 1-ml pipette tip. To minimise the potential proliferative differences in cells treated with siRNA or control, this assay was performed using serum-free medium. The wound was photographed under a microscope at 0 and 24 h, and the gap between the cells was measured. The rate of wound healing was calculated as follows: (rate of wound healing) = ((gap width: 0 h) – (gap width: 24 h)) / (gap width: 0 h). Mean values were obtained from at least three separate experiments.

Transwell migration assay. Cell migration activity was evaluated using the transwell (8-µm pore) migration chambers. After pretreated, cells (5 × 10⁴ 253 J cells or 1 × 10⁴ EJ cells) suspended in 200 µl of serum-free medium were added to the upper chamber. The lower chamber was filled with 600 µl medium containing 20% FBS. After 24-h incubation, cell staining was performed using 0.1% crystal violet for 30 min. Non-migrated cells were carefully removed from the upper chamber filter using a cotton swab, and the cells that infiltrated the filter were counted in four random fields.

Immunofluorescence staining. Immunofluorescence staining was carried out as previously described (Wang *et al*, 2013a). Briefly, after fixation and permeation, cells were incubated with primary antibodies against E-cadherin (Cell Signaling Technology, Danvers, MA, USA) or vimentin (Santa Cruz, CA, USA), and then incubated with Alexa 548-conjugated anti-rabbit and FITC-conjugated anti-mouse IgG antibodies (Invitrogen, Carlsbad, CA, USA). Next, the cells were counterstained using 4,6-diamidino-2-phenylindole and analysed under a Zeiss LSM500 confocal microscope (Zeiss International, Oberkochen, Germany).

Western blotting analysis. Following gene transfection or drug treatments, cell lysates were prepared and quantified before performing further experiments. Western blotting analysis was carried out as previously described (Wang *et al*, 2013a). The following antibodies were used: anti-ROC1 (Abcam, Cambridge, MA, USA); anti-Phospho-4E-BP1 (Thr37/46) and anti-E-cadherin (Cell Signaling); anti-non-4E-BP1 and anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH; Epitomics, Hangzhou, China); anti-vimentin and anti-N-cadherin (Santa Cruz); and anti-DEPTOR (Sigma).

Establishment of the EJ-EGFP-luciferase cell line and lentiviral transduction. EJ cells stably expressing the EGFP–luciferase fusion protein were generated using pLEX lentivirus vectors (Thermo Scientific Inc., Shanghai, China). Briefly, firefly luciferase and green fluorescent protein fusion genes (Fluc) driven by CMV promoter were cloned into the lentivirus vector. Stably transduced EJ cells were obtained via lentivirus infection and 2 weeks of 2 µg ml⁻¹ puromycin selection.

In vivo metastasis assay. A systematic metastasis model of bladder cancer was established for the *in vivo* metastasis assay. Lenti-shROC1 containing short hairpin RNAs directed against human ROC1 and control Lenti-shCONT were used as previously described (Wang *et al*, 2013a). Briefly, EJ-EGFP-luciferase cells transfected for 120 h with Lenti-shROC1, or Lenti-shCONT were trypsinised, resuspended in PBS and then intravenously (i.v.)

injected into the tail veins of nude mice (6 weeks old, male, athymic, BALB/C nu/nu; $n = 10$ per group). Metastasis formation and growth were monitored via luciferase activity. The Luciferase Reporter Assay System E1500 (Promega, Madison, WI, USA) was used according to the manufacturer's instructions to measure firefly luciferase activity. Three months after nude mice were inoculated with the Lenti-shROC1- or Lenti-shCONT-transfected cells, the mice were killed and metastatic tissues were examined. The animal study protocol (including animal handling and experimental procedures) was reviewed and approved by the Animal Care and Use Committee of Shanghai First people's Hospital of Shanghai Jiao Tong University, Shanghai, China. The methods were carried out in accordance with the approved guidelines.

Tissue samples and immunohistochemistry. Bladder cancer tissue specimens were obtained from 45 patients who underwent surgery in the Shanghai Jiao Tong University Affiliated

First People's Hospital between January 2004 and May 2006. The patients included 31 males and 14 females between 45 and 86 years of age (median age of 62 years) who were diagnosed with primary BTCC. Twenty-six of these patients underwent transurethral resection, 10 underwent partial cystectomy and 9 underwent radical cystectomy. Tumour grade and stage were assessed according to the World Health Organization 1973 criteria and the American Joint Committee on Cancer 2002 TNM system. The protocol for the use of human surgical samples was reviewed and approved by the Medical Ethics Committee of Shanghai First People's Hospital of Shanghai Jiao Tong University (Permit Number: 2013KY004). Tissue sample collection and processing were carried out in compliance with approved guidelines. Informed consent was obtained from each patient.

Immunohistochemistry was performed according to a previously described protocol (Wang *et al*, 2013a). Primary antibodies against ROC1 (Abcam), DEPTOR (Sigma) and E-cadherin

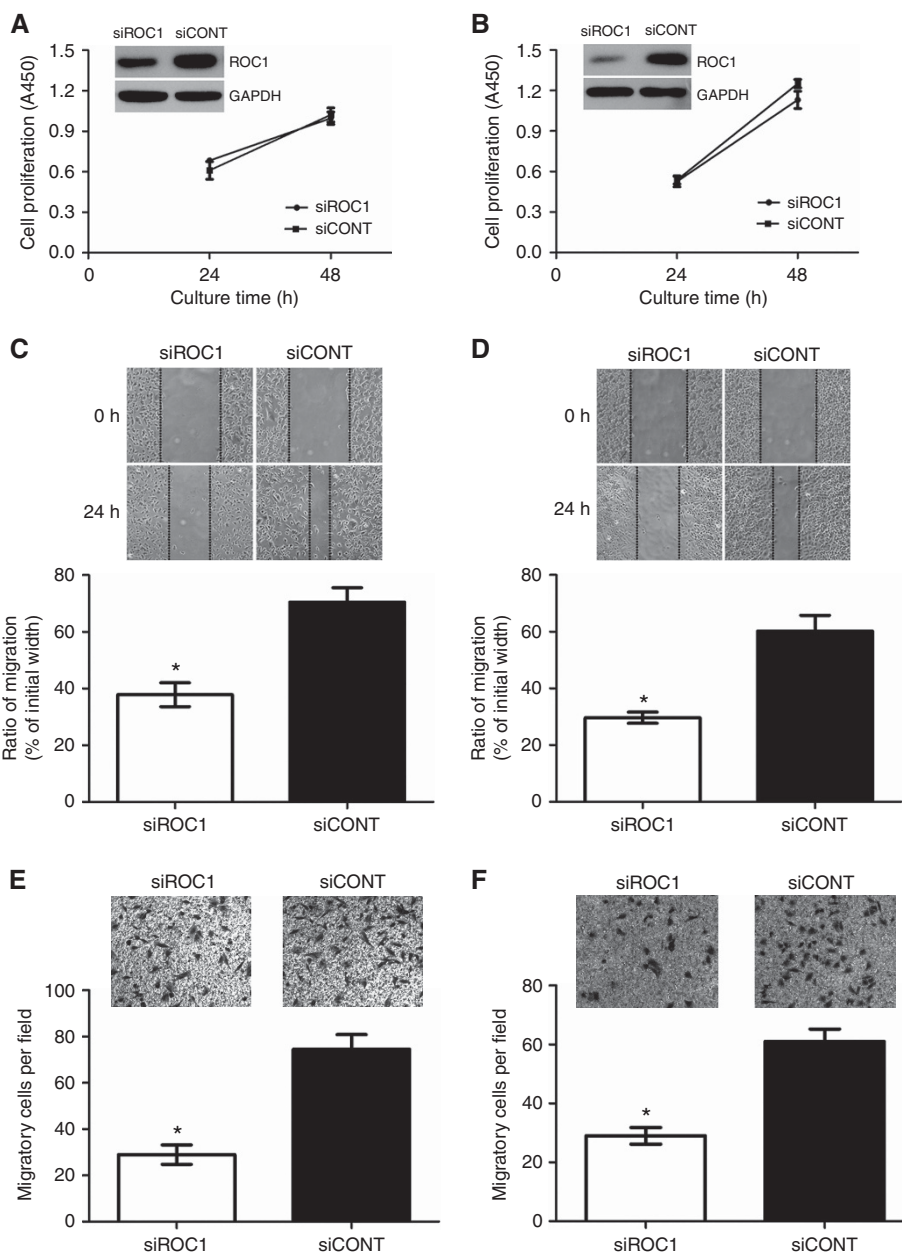


Figure 1. Inhibition of MI-TCC cell migration after ROC1 knockdown. (A, C and E) 253J cells; (B, D and F) EJ cells. Cells were transiently transfected with siROC1 or siCONT for 24 h and then subjected to cell viability CCK8 (A, B), wound-healing (C, D) or transwell migration assay (E, F). Representative results of three independent experiments are shown as means \pm s.e.m. * $P < 0.05$.

(Boster, Wuhan, China) were used according to the manufacturers' instructions.

Statistical analysis. Data were expressed as means \pm s.e.m. Statistical analyses were performed using the Bonferroni *t*-test after one-way analysis of variance for multi-group comparisons. Two-group comparisons were analysed using Student's *t*-tests. The correlations between ROC1, DEPTOR and E-cadherin expression were analysed by Pearson's χ^2 -tests. $P < 0.05$ was considered statistically significant. All statistical evaluations were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

ROC1 knockdown inhibits MI-TCC cell migration. To assess the role of ROC1 in MI-TCC cell migration, we knocked ROC1 expression down in two invasive bladder cancer cell lines (253J and EJ cells) using ROC1 siRNA and a scrambled siRNA control (siCONT) (Wang *et al*, 2013a). To minimise the potential influence of proliferation, our wound-healing and transwell migration assays were done after pretreated 24 h, when cell viability were not changed significantly (Figure 1A and B; Supplementary Figure S1A and S1B). Wound-healing assays using the transfected cells established that siROC1-treated cells migrated remarkably slower than siCONT-treated cells (Figure 1C and D). Transwell migration assays further demonstrated that cell motility was significantly impaired in siROC1-treated cells when compared with siCONT-treated cells (Figure 1E and F).

ROC1 knockdown inhibits EMT in MI-TCC cells. EMT strongly influences the migratory ability of cancer cells (Lamouille *et al*,

2014). EMT is characterised by reduced expression of epithelial markers (such as E-cadherin) and upregulation of mesenchymal markers (such as N-cadherin and vimentin) (Lamouille *et al*, 2014). We investigated whether ROC1 knockdown influenced EMT by examining the expression levels of specific markers. Immunofluorescence microscopy analysis of ROC1-knockdown cells showed increased E-cadherin expression and decreased vimentin expression when compared with control cells (Figure 2A and B). Consistent with these observations, western blot analysis indicated that E-cadherin expression was increased by ROC1 knockdown, whereas the expression of N-cadherin and vimentin were decreased (Figure 2C and D). These data indicated that ROC1 knockdown inhibits EMT in 253J and EJ cells.

ROC1 knockdown inhibits the mTOR pathway via DEPTOR protein accumulation. To investigate the mechanisms underlying ROC1 knockdown-inhibited EMT in bladder cancer cells, we explored the possible involvement of the mTOR pathway. As evidenced by reduced 4E-BP1 phosphorylation (p-4E-BP1), ROC1 knockdown resulted in mTOR inactivation in both 253J and EJ cells (Figure 3A and B). mTOR-induced phosphorylation of 4E-BP1 has previously been reported and this could serve as a classical marker of mTOR activation (Cao *et al*, 2015). To investigate which molecule was primarily responsible for suppressing mTOR activation, we analysed the expression of DEPTOR protein in ROC1-knockdown cells and controls. DEPTOR is a CRL ^{β -TrCP} substrate and a naturally occurring mTOR inhibitor. DEPTOR protein levels in the ROC1-knockdown tumour cells were significantly elevated when compared with control cells (Figure 3A and B). To investigate DEPTOR's contribution to ROC1 knockdown-inhibited EMT, we performed a simultaneous siRNA knockdown of DEPTOR and ROC1 expression in EJ cells

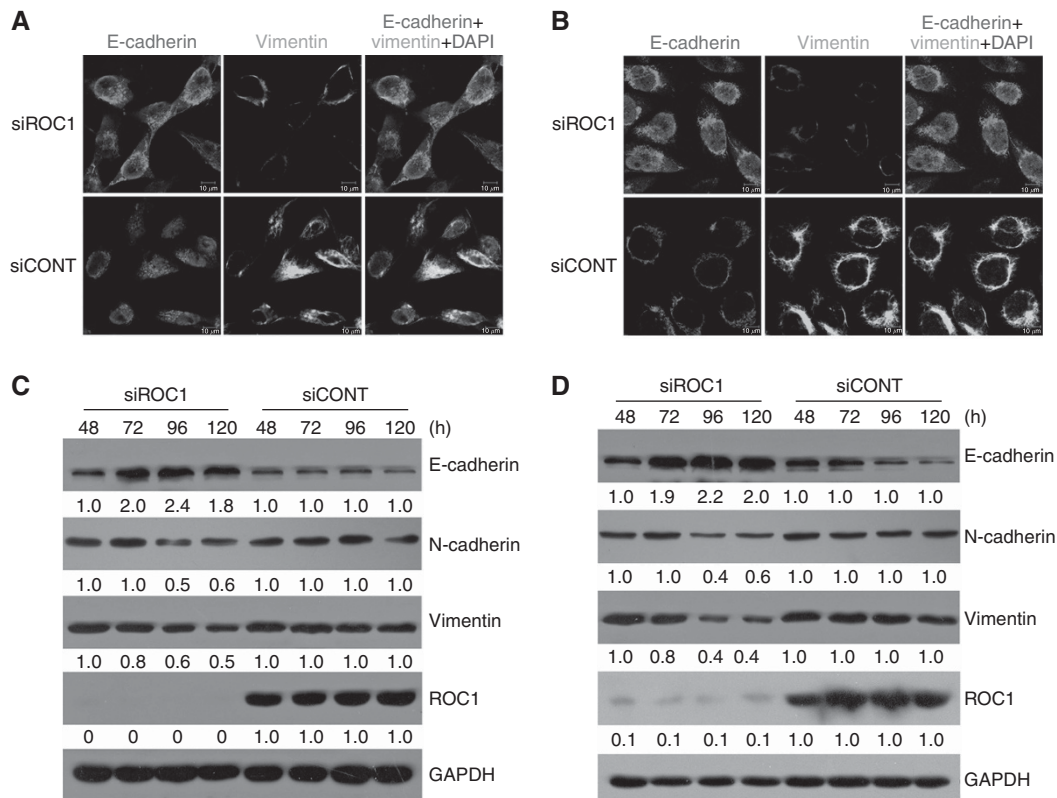


Figure 2. ROC1 knockdown induction of MI-TCC cell MET. ROC1 knockdown-induced MET in 253J (A) and EJ (B) cells. Cells were transfected with siROC1 or siCONT for 96 h, stained for E-cadherin (red) and vimentin (green), and then examined under a confocal microscope. Nuclear DNA was stained with DAPI (blue). Scale bar = 10 μ m. The expression of EMT-associated markers in 253J (C) and EJ (D) cells were examined using western blots; the quantification relative to GAPDH was accomplished with densitometric analysis by Image J software. Representative results of three independent experiments are shown.

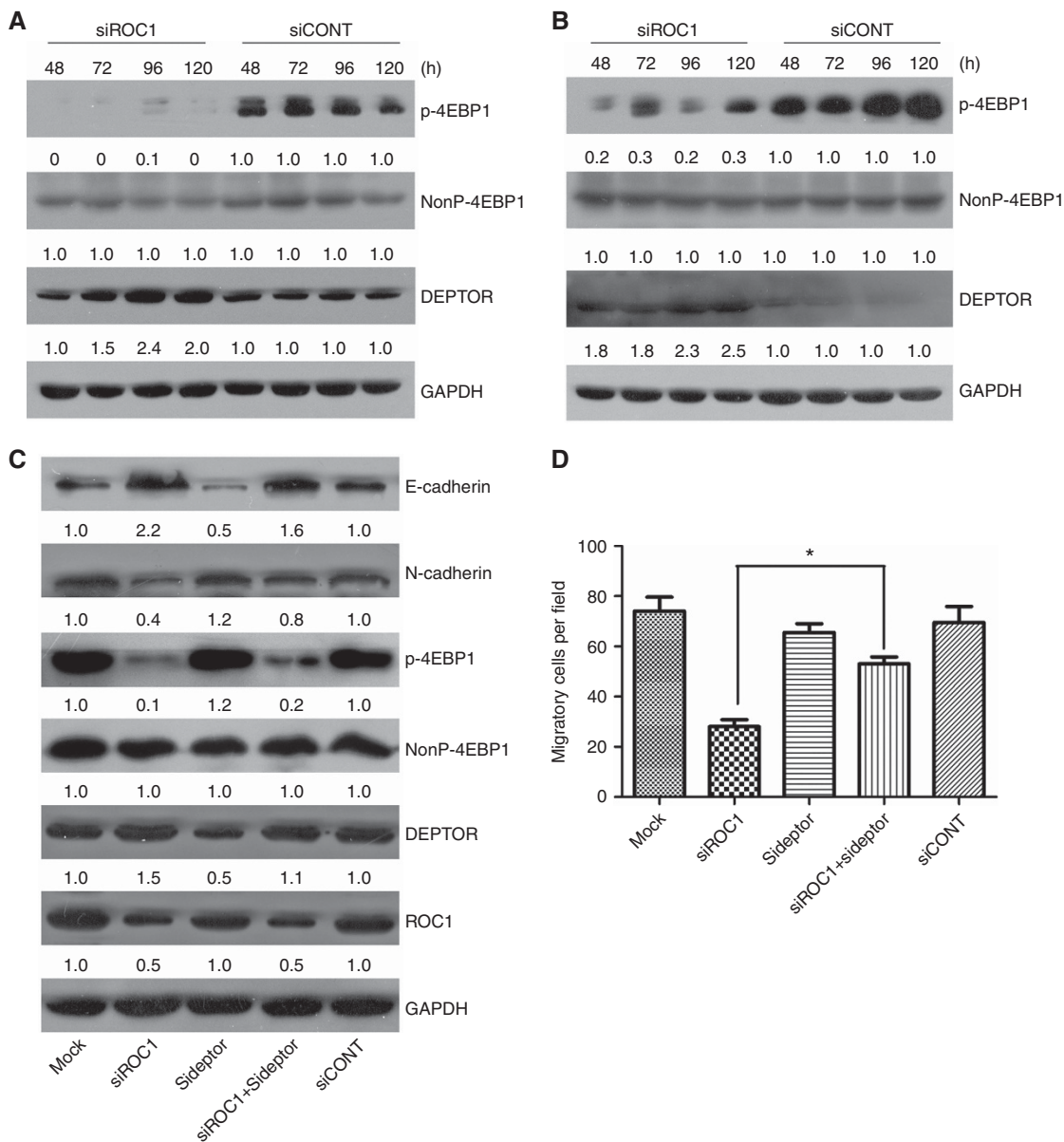


Figure 3. Effects of ROC1 knockdown on the mTOR pathway. mTOR pathway-associated protein expression in 253J (A) and EJ (B) cells was examined via western blot. EJ cells were transfected with siRNA for 96 h and then subjected to western blotting analysis (C) and transwell migration assays (D). Representative results of three independent experiments are shown. Columns, means of three independent experiments; bars, s.e.m. *P<0.05. Treated cells were subject to western blot analysis with GAPDH as a loading control, and the quantification relative to GAPDH was conducted by densitometric analysis using Image J software.

and found that the DEPTOR knockdown markedly attenuated ROC1 knockdown-inhibited EMT. This was demonstrated by a reduction in E-cadherin expression and increasing N-cadherin expression (Figure 3C). Transwell migration assays demonstrated that DEPTOR knockdown partially restored migratory ability following ROC1 knockdown in EJ cells (Figure 3D). In addition, DEPTOR knockdown alone did not significantly impair cell motility when compared with siCONT-treated cells (Figure 3D). These results indicated that DEPTOR was essential for ROC1 knockdown-inhibited EMT and suggested the involvement of the mTOR pathway.

Rapamycin-induced mTOR pathway inhibition suppresses EMT in bladder cancer cells. To investigate the role of mTOR kinase in EMT regulation, we investigated rapamycin's effects on EMT in bladder cancer cells. Cells were treated with various concentrations of rapamycin, and EMT marker expression was examined. After

72 h rapamycin treatment, mTOR kinase activity was significantly inhibited. This inhibition was reflected by a reduction in p-4E-BP1. EMT was also notably inhibited in rapamycin-treated cells. EMT inhibition was demonstrated by increased E-cadherin expression and decreased N-cadherin and vimentin expression. Rapamycin treatment also significantly suppressed 253J and EJ cell migration (Figure 4C and D). These results suggested that mTOR inhibition could effectively suppress EMT in bladder cancer cells.

ROC1 knockdown suppresses MI-TCC metastasis *in vivo*. For *in vivo* confirmation of our *in vitro* findings, we examined the effects of ROC1 knockdown using an athymic nude mouse metastasis model. EGFP-luciferase-labelled EJ cells were *i.v.* injected into athymic nude mice following ROC1 knockdown and systemic metastasis was assessed by detecting luciferase activity. All shCONT-cell-inoculated mice (10/10) exhibited pulmonary metastases. In contrast, only 60% (6/10)

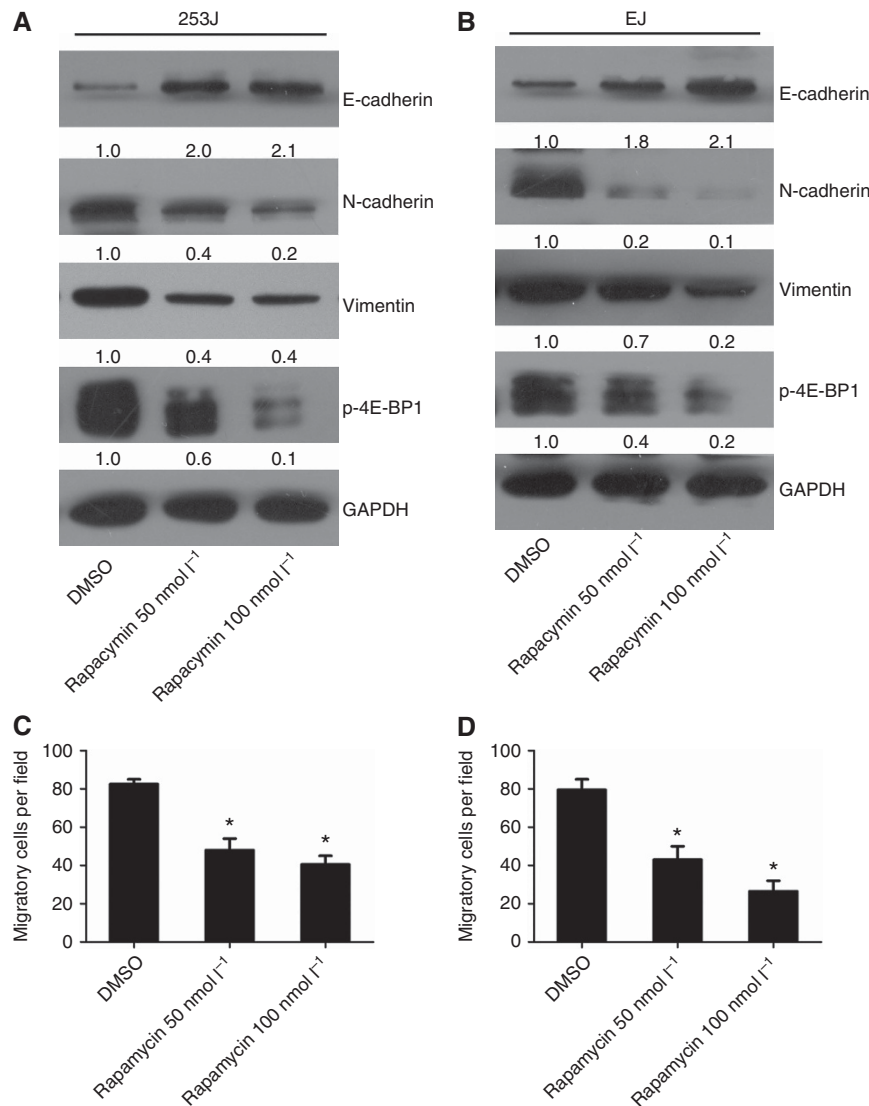


Figure 4. The effects of rapamycin-induced inhibition of the mTOR pathway on EMT. (**A, C**) 253J cells. (**B, D**) EJ cells. Cells were treated with different concentrations of rapamycin (0, 50 and 100 nmol l⁻¹) for 72 h and analysed via western blotting (**A, B**) or transwell migration assays (**C, D**). Representative results of three independent experiments are shown. Columns, means of three independent experiments; bars, s.e.m. **P*<0.05. Treated cells were subject to western blot analysis with GAPDH as a loading control, and the quantification relative to GAPDH was conducted by densitometric analysis using Image J software.

ROC1-knockdown mice exhibited lung metastasis nodules. ShROC1 cell injection resulted in a remarkable decrease in metastasis when compared with the control group (Figure 5A). We also detected the expression levels of DEPTOR and E-cadherin proteins in ROC1-knockdown tumour tissues and found that they were increased in comparison with the control group (Figure 5B). These data demonstrated that ROC1 knockdown suppressed EMT and MI-TCC metastasis *in vivo*.

ROC1 expression is positively associated with EMT in human BTCC tissues. Using bladder cancer TMA analysis, we evaluated the relationships between the pathological stages of bladder cancer and the expression levels of ROC1, DEPTOR and E-cadherin. Using the immunoreactivity score system, expression levels were divided into two categories (low or high). The TMA analysis indicated that ROC1 expression was increased, but that DEPTOR and E-cadherin expression were reduced in invasive bladder cancer tissues when compared with non-muscle-invasive bladder cancer tissues (Figure 6A). Furthermore, the TMA analysis indicated that ROC1 and DEPTOR expression levels were related to the tumour stage (Figure 6B). ROC1 expression exhibited significant negative

correlations with DEPTOR (*P*=0.014, *R*=−0.378) and E-cadherin (*P*=0.001, *R*=−0.7756; Table 1). Collectively, cancer tissues with high ROC1 expression had lower DEPTOR and E-cadherin expression.

DISCUSSION

This investigation made several discoveries concerning the role of ROC1 in the malignant progression of MI-TCC. First, we demonstrated that ROC1 knockdown inhibited the migration of bladder cancer cells by inhibiting EMT. Second, we showed that ROC1 knockdown inhibited mTOR activity via the accumulation of the mTOR-inhibitory protein DEPTOR. Third, we demonstrated that *in vivo* metastasis was suppressed by ROC1 knockdown. Finally, TMA analysis of clinical samples indicated that there was a positive correlation between ROC1 expression and EMT induction. These data suggest that ROC1 has an important role in bladder cancer progression. ROC1 protein could be an attractive anticancer target, and a treatment strategy targeting

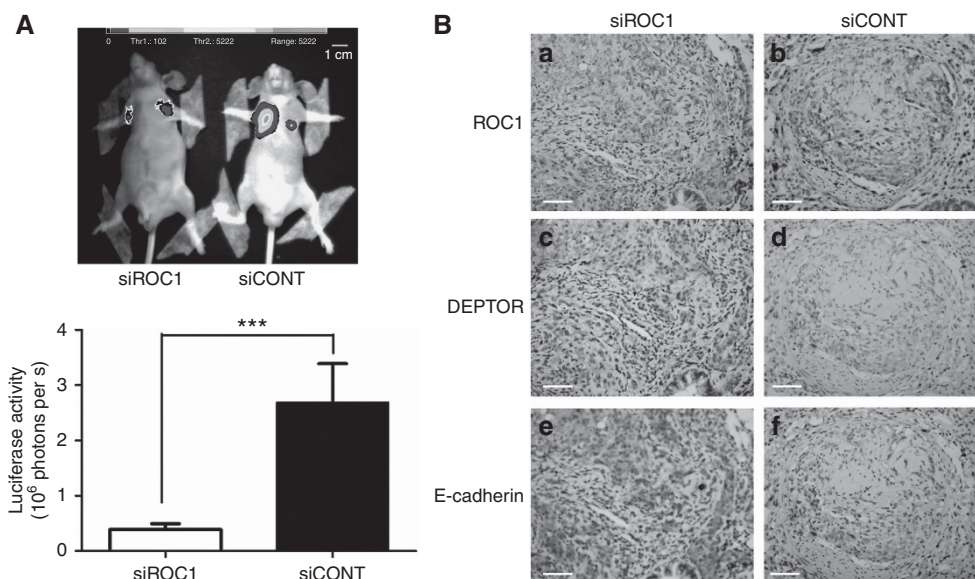


Figure 5. Effects of ROC1 knockdown on metastasis in vivo. **(A)** Representative photographs of systematic metastasis detection in nude mice 12 weeks after the injection of shROC1 or shCONT cells. **(B)** IHC of lung metastasis nodules with the indicated antibodies in both groups. Columns, the mean luciferase activity of 10 mice; bars, s.e.m. ****P*<0.01. Scale bar = 50 μm.

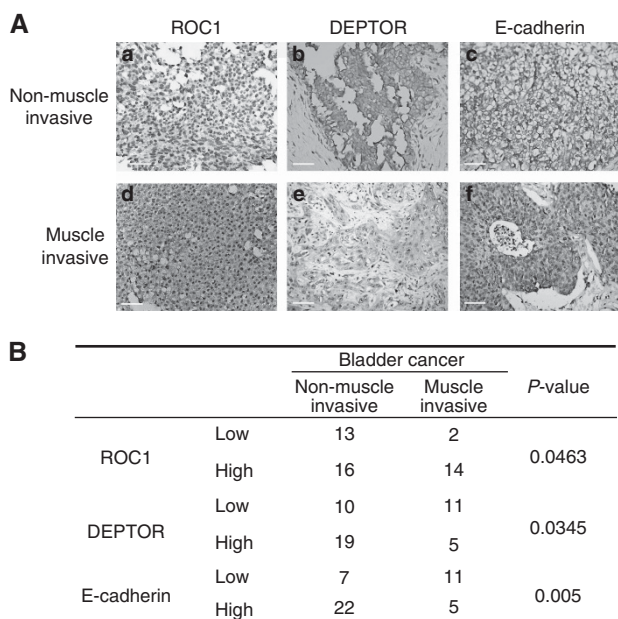


Figure 6. ROC1 expression in human bladder cancer tissues. **(A)** Immunohistochemical staining of ROC1, DEPTOR and E-cadherin in human bladder cancer. Representative immunohistochemistry images of non-muscle-invasive or muscle-invasive cancer are shown. **(B)** Correlation of the immunostaining intensities of ROC1, DEPTOR and E-cadherin with cancer invasiveness. Immunostaining intensity was divided into two groups (high and low), and the association of intensity with cancer invasiveness (non-muscle-invasive cancer or muscle-invasive cancer) was analysed by a χ^2 -test for each protein. The *P*-value for each association is shown. Scale bar = 50 μm.

ROC1 may be capable of hindering the metastasis of bladder cancer.

Cancer progression is a complex, multistep process, and the acquisition of migratory ability is the prerequisite of metastasis (Hanahan and Weinberg, 2011). Studies have demonstrated that EMT induction is a pivotal cellular process that promotes the mobility of cancer cells and leads to the metastasis of epithelial

Table 1. ROC1, DEPTOR and E-cadherin expression and correlations

	ROC1 expression (n = 45)		
	Low	High	<i>P</i> -value
DEPTOR			
Low	3	18	0.014
High	12	12	
E-cadherin			
Low	1	17	0.001
High	14	13	

Correlations between ROC1, DEPTOR and E-cadherin expression were analysed by Pearson's χ^2 -tests. ROC1 expression exhibited negative correlations with both DEPTOR (*P*=0.014, *R* = -0.378) and E-cadherin (*P*=0.001, *R* = -0.7756).

cancers, including bladder cancer. EMT is characterised by the loss of epithelial and the acquisition of mesenchymal characteristics. This transition grants cancer cells the ability to migrate and invade distant sites (Lamouille *et al*, 2014). E-cadherin is the key mediator of cell-cell adhesion in epithelial tissues. In this study, we demonstrated that ROC1 knockdown suppressed cancer cell migration through EMT inhibition that was characterised by increased E-cadherin expression. Furthermore, using the EJ mouse metastasis model, we found that ROC1 played an important role in promoting cancer metastasis via similar mechanisms. Consistent with our findings, and further supporting the importance of CRL in the EMT process of cancer cells, the suppression of CRL activity by cullin4A silencing reversed EMT by regulating the transcriptional downregulation of ZEB1 (Wang *et al*, 2014). Notably, ROC1 knockdown was recently reported to inhibit cell migration via induction of RhoA accumulation in human vascular endothelium (Yao *et al*, 2014). These results suggest that inactivation of CRL could inhibit cell migration via different pathways.

When alternative E-cadherin expression regulatory pathways were examined in 253J and EJ cells, we found that mTOR activity was strongly inhibited by ROC1 knockdown. The mTOR pathway has a pivotal role in the regulation of various cellular process, including cell death, apoptosis, autophagy and EMT (Betz and Hall, 2013). The mTOR pathway is a positive regulator of EMT (Gherardi *et al*, 2012; Lamouille *et al*, 2014). In the present study,

ROC1 knockdown suppressed mTOR activity, as evidenced by p-4E-BP1 downregulation. Consistent with our findings, other recent studies have reported that mTOR activity is suppressed in cancer cells via CRL activity inhibition (Zhao *et al*, 2012; Li *et al*, 2014). However, the exact mechanisms underlying the changes in mTOR kinase regulation of EMT after ROC1 knockdown remain elusive. It has been reported that the mTOR pathway induces EMT by transactivating the RhoA and Rac1 signalling pathways (Gordon *et al*, 2014). One conceivable explanation may be that mTOR pathway inhibition after ROC1 knockdown alters cellular cytoskeletal arrangements and decreases the activation of RhoA and Rac1, consequently suppressing EMT in MI-TCC cells. However, further study is needed to establish the exact mechanism responsible for the ROC1-induced regulation of EMT in MI-TCC.

ROC1 knockdown triggers CRL inactivation and the accumulation of CRL substrates (such as p21 and p27), leading to cellular responses (such as G2 arrest and senescence) (Jia *et al*, 2011; Wang *et al*, 2013a). Soon after ROC1 knockdown (e.g., at 24 h, when cell viability has not yet been significantly altered) ROC1 knockdown-induced inhibition of mTOR in MI-TCC cells was dependent on DEPTOR accumulation. DEPTOR is a recently identified endogenous mTOR inhibitor that directly binds to mTOR and negatively regulates its activity (Peterson *et al*, 2009). DEPTOR is degraded by the CRL ^{β -TrCP} E3 ubiquitin ligase (Duan *et al*, 2011; Gao *et al*, 2011; Zhao *et al*, 2011). With the cooperation of ROC1, the F-box protein β -TrCP binds to DEPTOR and promotes DEPTOR degradation in the CRL complex. Here we demonstrated that ROC1 knockdown triggered DEPTOR accumulation and subsequent mTOR inactivation. Correspondingly, blocking DEPTOR expression restored EMT induction upon ROC1 knockdown. We observed that simultaneous abrogation of ROC1 and DEPTOR only partially restored EMT induction. This indicates that DEPTOR is necessary but not sufficient for ROC1 knockdown-induced mTOR inactivation and EMT inhibition, and that other EMT-regulatory pathways may be involved. Therefore, additional investigation of the underlying mechanisms is needed.

DEPTOR directly binds mTOR and inhibits the activities of both mTORC1 and mTORC2 (Peterson *et al*, 2009). Rapamycin is a specific mTOR inhibitor, but has low anticancer efficacy in bladder cancer treatment due to selective inhibition of mTORC1 activity, which limits its clinical effectiveness (Sun, 2013; Ghosh *et al*, 2014). In the present study, we demonstrated that ROC1 knockdown induced DEPTOR accumulation. This could have important implications for the development of new bladder cancer treatment strategies. ROC1 knockdown-induced DEPTOR accumulation may inhibit the activity of both mTORC1 and mTORC2 and, therefore, may overcome the selective inhibition of mTORC1. Furthermore, ROC1 knockdown may induce the accumulation of other mTOR blockers that are CRL substrates, including HIF-1 α , REDD1 and TSC2 (Jaakkola *et al*, 2001; Hu *et al*, 2008; Katiyar *et al*, 2009). Therefore, we speculate that RNA interference or pharmacological inhibition of ROC1 could be a novel approach for improving the responsiveness of bladder cancer patients to chemotherapy.

In our TMA analysis, ROC1 expression was associated with bladder cancer invasion. ROC1 expression exhibited a significant negative correlation with DEPTOR ($P=0.009$, $R=-0.378$) and E-cadherin ($P=0.001$, $R=-0.7756$) levels. As bladder tumours with high ROC1 expression tended to have lower DEPTOR (60%) and E-cadherin (56.7%) expression levels, low E-cadherin expression was correlated with the malignant progression of bladder cancer. This result agrees with results reported by previous studies (Lamouille *et al*, 2014; Knowles and Hurst, 2015). We speculate that ROC1 could be a promising prognostic factor for bladder cancer progression. However, the sample numbers of the current study are limited, and, therefore, the statistical significance found in this study may not be stable. Furthermore, correlation of ROC1

expression with patient prognosis was not investigated. Therefore, future studies are needed to investigate whether or not ROC1 is a promising prognostic factor for bladder cancer progression. These studies should be conducted using a greater sample size and a longer follow-up period.

In conclusion, the present study demonstrated that ROC1 has an important role in bladder cancer progression by regulating the mTOR/DEPTOR pathway. This suggests that ROC1 could be a novel anticancer target for bladder cancer therapy. Further studies clarifying the detailed mechanisms underlying the role of ROC1 in bladder cancer are needed.

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (No. 81402379), the Shanghai Municipal Natural Science Fund of Medicine (No. 134119a2801) and the Medical Scientific Research Foundation of Jiangsu Province, China (No. Z201526).

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