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VEGF Stimulation Enhances Livin Protein Synthesis Through mTOR Signaling

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ABSTRACT

Livin is a member of inhibitors of apoptosis proteins (IAPs) and overexpressed in transformed cells and several cancers. Although strategies to decrease Livin levels have been conducted for rational cancer therapy, the molecular mechanism controlling Livin expression in tumors has not been completely elucidated. Here, we show that vascular endothelial growth factor (VEGF) stimulation can increase Livin expression in HeLa cells or SK-MEL-28 cells. This response is independent of de novo gene transcription or changes in mRNA expression but occurs at protein expression levels. VEGF stimulation results in mTOR signaling activation which changes the phosphorylation status of 4E-BP1, the downstream of mTOR signaling, and ultimately contributes to the translation initiation of Livin protein. Livin silencing, Rapamycin alone or in combination with cytotoxic agent can reduce Livin protein levels, and decrease cells viability. Thus, ablation of Livin translation contributes to remove an anti-apoptotic mechanism potentially contributing to aggressive tumor behavior. Pharmacologic inhibition of VEGF/mTOR/Livin signaling may provide a novel strategy for cancer treatment. J. Cell. Biochem. 111: 1114–1124, 2010.

KEY WORDS: VEGF; LIVIN; 4E-BP1; mTOR; RAPAMYCIN

The inhibitors of apoptosis proteins (IAPs) are a family of highly conserved cell apoptosis inhibitors that have been found in yeast, invertebrates, and vertebrates [Salvesen and Duckett, 2002]. Their expressions are regulated by multiple mechanisms including transcriptional and translational control. IAPs play important roles in multiple cellular functions including direct inhibition of caspases, E3 ubiquitin ligase activity, modulation of receptor-mediated signal transduction, and regulation of mitotic spindle formation [Rumble and Duckett, 2008]. More importantly, IAPs are suggested to play a critical role in the establishment and maintenance of tumors. Modulation of IAPs level or their activity thus offers huge therapeutic potential for caner treatment [Hunter et al., 2007].

Livin is a member of IAPs family, containing a single BIR domain and a RING finger domain [Kasof and Gomes, 2001]. Livin is not detectable in most normal differentiated tissues with the exception of the placenta, normal testes, spinal cord, and lymph node, but is present in transformed cells and several cancers, including carcinomas of the breast, cervix, and prostate, as well as melanomas and lymphoma cells [Lin et al., 2000; Vucic et al., 2000; Liu et al., 2007]. Like other IAP family proteins, Livin can interact with downstream caspases, such as caspase-3, caspase-7, and caspase-9, leading to their inactivation and degradation [Boaz et al., 2003; Chang and Schimmer, 2007]. Previous studies demonstrated that transfection of Livin in HeLa cells results in protection from apoptosis induced by expression of FADD, Bax, RIP, RIP3, and DR6 [Kasof and Gomes, 2001]. Livin overexpression also renders malignant cells resistant to chemotherapy [Chang and Schimmer, 2007]. In contrast, treatment of cancer cells with Livin antisense oligo-DNA causes apoptotic cell death, indicating that Livin expression is essential for survival of certain cancer cells [Crnković-Mertens et al., 2006; Hao et al., 2007; Liu et al., 2007]. Given the role of Livin in controlling apoptosis

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and its selective up-regulation in malignancies, this IAP may be potential molecular target for anticancer strategies.

Vascular endothelial growth factor (VEGF) is a potent mitogen for cells, and its expression is correlated with increased tumor angiogenesis [Hicklin and Ellis, 2005; Ylä-Herttuala et al., 2007]. VEGF plays a crucial role in tumor expansion by initiating permeabilization of blood vessels, by extravasation of plasma proteins, by invasion of stromal cells, and by causing the sprouting of new blood vessels that supply the tumor with oxygen and nutrients [Tammela et al., 2005; Zhang et al., 2007]. Previous study revealed that overexpression of Livin and VEGF contributes to tumor progression. Livin expression is significantly correlated with tumor stages, and positively correlated with VEGF levels, implying a potential link between Livin expression and VEGF [Chen et al., 2008].

Here, we demonstrate that the expression levels of Livin are obviously induced in HeLa cells and melanoma cell lines SK-MEL-28 in response to VEGF stimulation. The induction occurs at Livin translation level through the activation of mammalian target of rapamycin (mTOR) signaling.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Antibodies to 4E-BP1, phospho-4E-BP1 (Thr37/46, Ser-65, and Thr-70), p70S6K1, phospho-p70S6K (Ser371), eIF2α, phospho-eIF2α (Ser51) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to XIAP and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Livin antibody was from Abcam (Cambridge, UK). Cycloheximide, rapamycin, doxorubicin, and LY294002 were from Calbiochem (San Diego, CA). VEGF, platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF) were purchased from Sigma (St. Louis, MO).

CELL CULTURE AND siRNA TRANSFECTION

HeLa cell line and human malignant melanoma cell line, SK-MEL-28, were obtained from American Type Culture Collection (Manassas, VA), and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U of penicillin, and 100 mg/ml streptomycin in 5% CO_2 at 37°C. siRNA transfection were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The siRNA sequences were as follows: 4E-BP1 oligo1 sense: 5′-CUCACCUGUGACCAA-AACAUU-3′, antisense: 5′-UGUUUUGGUCACAGG UGAGUU-3′; 4E-BP1 oligo2 sense: 5′-GAGUCACAGUUUGAGAUGGUU-3′, anti-sense: 5′-CCAUCUCAAACUGUGACUCUU-3′.

QUANTITATIVE RT-PCR ANALYSIS

Total RNAs were extracted using TRIzol reagent (Invitrogen). Equal amounts of RNA were converted into cDNA through SuperScript II Reverse Transcriptase (Invitrogen). To verify RNA integrity and equal input levels, β-actin mRNA was determined, and the data were expressed as ratios relative to β-actin levels. Real-time PCRs were performed using the ABI Prism 7000 sequence detection system. The real-time PCR primers were as follows: Livin sense: 5′-CCA GGGTGGGCCCGGG GGTCA-3′, antisense: 5′-GCGGGGT CCA-CAGCGC T CCTGC-3′; β-actin sense: 5′-GACGGCCAGGTCATCAC-TATTG-3′, antisense: 5′-AGGAAGGCTGG AAA AGAGCC-3′.

WESTERN BLOTS

Treated cells were harvested, washed with ice-cold PBS and resuspended in buffer consisting of 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 μg/ml aprotinin for 20 min on ice. The extract was obtained by centrifugation at 12,000*g* at 4°C for 15 min. The concentration of protein was measured through Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of proteins were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto PVDF membrane. The membrane was immunoprobed with corresponding antibody, and visualized using ECL plus reagent (Amersham).

LIVIN PROMOTER ACTIVITY

Livin promoter (Livin p-LUC) plasmid was constructed as previously described [Yuan et al., 2007]. Briefly, Livin p-LUC fragment was amplified by PCR from human genome DNA and inserted into the *Sma*I site of a promoterless luciferase reporter vector, pGL3-Basic (Promega, Madison, WI). The forward primer was 5′-ATGGGTTTTCCCAGCTTTTAT-3′ and the reverse primer was 5′-GGAGGGAAC ACTGGCTCTGACC-3′. Cells were transfected with Livin promoter plus PRL-CMV plasmid. After 36 h transfection, cells were cultured in serum-deprived conditions for 16 h, then stimulated with VEGF or 10% FBS for indicated time. Renilla luciferase-normalized luciferase activity was analyzed using a microplate reader (Synergy 4 Hybrid Multi-Mode; BioTek Instruments).

ELISA-BASED MTOR KINASE ACTIVITY ASSAY

mTOR kinase activity was measured using the K-LISA kit (EMD, San Diego, CA) as described previously [Jiang et al., 2008]. Two steps were preceded as following.

Immunoprecipitation and kinase activity. Treated cells were homogenized, and the supernatant was precleared by adding Protein G-Plus agarose beads, then 10 μl of mTOR antibody was added to the precleared lysates and rotated for 1 h at 4°C. Next, 50 μl of protein G-Plus agarose beads was added in the tube and rotated for 3 h at 4°C. The agarose beads were washed, gently mixed with 2× Kinase Assay Buffer working solution and recombinant p70S6K-GST fusion protein (EMD), and incubated at 30°C for 30 min. The reaction was stopped by adding stop solution, and the supernatant was stored at −80°C until the assay can be completed.

ELISA. Hundred microliters of supernatant obtained from the kinase activity was added to each well of glutathione-coated 96-well plates (EMD) and incubated for 60 min at 30°C, followed by three times wash with Plate Wash Buffer. Then, 100 μl of anti-p70S6K-T389 Working Solution was added to each well and incubated for 1 h at room temperature. After three washes with the Plate Wash Buffer, 100 μl of HRP antibody-conjugate was added and incubated for 1 h at room temperature. After washes, 100 μl of TMB substrate was

added and incubated for 20 min at room temperature followed by adding 100 μl of ELISA Stop Solution to each well. The plate was read at 450–595 nm. A serially diluted standards (enriched rat brain fraction; EMD), which were 50, 16.67, 5.56, 1.85, 0.62, and 0.21 μl, was performed. Since the activity unit of the mTOR standard was not provided by the company EMD, the data were expressed as arbitrary unit, that is, the microliter.

ANALYSIS OF CELL VIABILITY

The 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to estimate cell viability. Briefly, cells were plated at a density of 1×10^4 cells per well in 96-well plates. After exposure to specific treatment, the cells were incubated with MTT at a final concentration of 0.5 mg/ml for 3 h at 37°C. After removal of the medium, 100 μM DMSO solutions were added to dissolve the formazan crystals. The absorbance at 570 nm wavelength was detected using a microplate reader (Synergy 4 Hybrid Multi-Mode; BioTek Instruments). Caspase 3 activity was assayed by Promega kit according to the manufacturer's protocol.

DATA ANALYSIS

Data were expressed as means ± SEM. Statistical significance was assessed by one-way ANOVA followed by Bonferroni multiple comparison post-tests. Differences of $P < 0.05$ were considered statistical significance.

RESULTS

VEGF STIMULATION SPECIFICALLY MODULATES LIVIN EXPRESSION

HeLa cells exhibited low expression of endogenous Livin in serum-free medium. VEGF stimulation could obviously induce Livin expression, and the induction was in a concentration-dependent manner (Fig. 1A).VEGF induction of Livin was detectable as early as 5 h after stimulation, and persisted throughout a 10- or 20-h time interval (Fig. 1B). In contrast, other growth factors including EGF, PDGF, and IGF-1 had no effect on Livin expression (Fig. 1C). FBS did not induce Livin expression for the first 5 h, but elevated Livin expression was detected after 10 and 20 h treatment (Fig. 1B). To assess whether the observation can be extended to other cell lines, we also performed similar studies in SK-MEL-28 cells, a melanoma cell line that constitutively expresses high levels of Livin. The results demonstrate that VEGF but not EGF, PDGF or IGF-1 stimulation leads to increased expression of Livin protein (Fig. 1D). In addition, we found that VEGF treatment does not influence the expression of β-actin or XIAP, another IAP member (Fig. 1E). Taken together, these results suggest that changes in Livin expression are specifically regulated by VEGF stimulation.

VEGF STIMULATION DOES NOT CHANGE LIVIN mRNA EXPRESSION OR PROMOTER ACTIVITY

To investigate the mechanism of VEGF induction of Livin, we first analyzed potential changes in Livin mRNA levels in untreated or VEGF-treated cultures by real-time PCR. The result shows that VEGF stimulation does not significantly increase Livin mRNA expression, as compared with untreated cultures. However, addition of FBS results in increased Livin mRNA expression in HeLa cells after 10 and 20 h treatment and in SK-MEL-28 cells after 5, 10, and 20 h treatment (Fig. 2A,B).

Given the possibility that Livin is a direct target gene of VEGF signaling pathway, we also investigated whether Livin promoter activity was modulated by VEGF treatment. We constructed a luciferase reporter Livin p-LUC which bears a 2,812-bp long Livin promoter (schematically shown in Fig. S1). HeLa cells or SK-MEL-28 cells were transfected with Livin p-LUC to compare Livin gene promoter activity in the response to FBS or VEGF treatment. The result demonstrats that VEGF stimulation does not change the activity of Livin promoter, as compared with the untreated group. By contrast, addition of FBS results in a significant increase in luciferase activity of Livin p-LUC both in HeLa cells and SK-MEL-28 cells after 5, 10, or 20 h treatment (Fig. 2C,D), suggesting that the activity of Livin gene promoter is not modulated by VEGF stimulation.

VEGF STIMULATION MODULATES LIVIN EXPRESSION AT PROTEIN LEVELS THROUGH mTOR SIGNALING PATHWAY

Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms. It exerts its effect by interfering with the translocation step in protein synthesis (movement of two tRNA molecules and mRNA in relation to the ribosome), thus blocking translational elongation [Wilson et al., 2000]. Exposure to cycloheximide resulted in rapid disappearance of Livin levels, detected as early as 3 h after addition and throughout the 15-h time interval (Fig. 3A). VEGF stimulation could not restore Livin levels in the presence of cycloheximide. However, in the control experiment FBS stimulation could partially restore Livin level after 15 h treatment in the presence of cycloheximide (Fig. 3A). These results indicate that VEGF induction of Livin expression is regulated at protein expression levels.

mTOR is an evolutionarily conserved protein kinase that is a central component of the signaling mechanism regulating cell growth and proliferation by modulating protein synthesis [Hay and Sonenberg, 2004]. We next investigated whether mTOR signaling is involved in VEGF induction of Livin expression. The results demonstrate that in HeLa cells or SK-MEL-28 cells VEGF stimulation causes a significant increase in mTOR kinase activity (Fig. 3B), and an elevated level of phosphorylated mTOR (Ser-2448; Fig. 3C,D; Fig. S2A,B). The phosphorylation of mTOR (Ser-2448) is required for the initiation of protein synthesis [Reynolds et al., 2002]. Besides if mTOR signaling was involved in Livin expression, pharmacologic inhibitors of mTOR would decrease Livin level. As expected, VEGF induction of Livin expression was completely blocked by cellular pretreatment with either LY294002, an inhibitor of both PI3-kinase and mTOR kinase activities [Brunn et al., 1996], or rapamycin, a specific inhibitor of mTOR signaling, in HeLa cells (Fig. 3E). Collectively, these results suggest that VEGF regulates Livin levels through mTOR signaling.

4E-BP1 PHOSPHORYLATION IS REQUIRED FOR VEGF INDUCTION OF LIVIN EXPRESSION

The two best characterized targets of mTOR are p70-S6 Kinase 1 (p70S6K) and 4E-BP1, the eukaryotic initiation factor 4E (eIF4E) binding protein 1. mTOR stimulation phosphorylates p70S6K, and

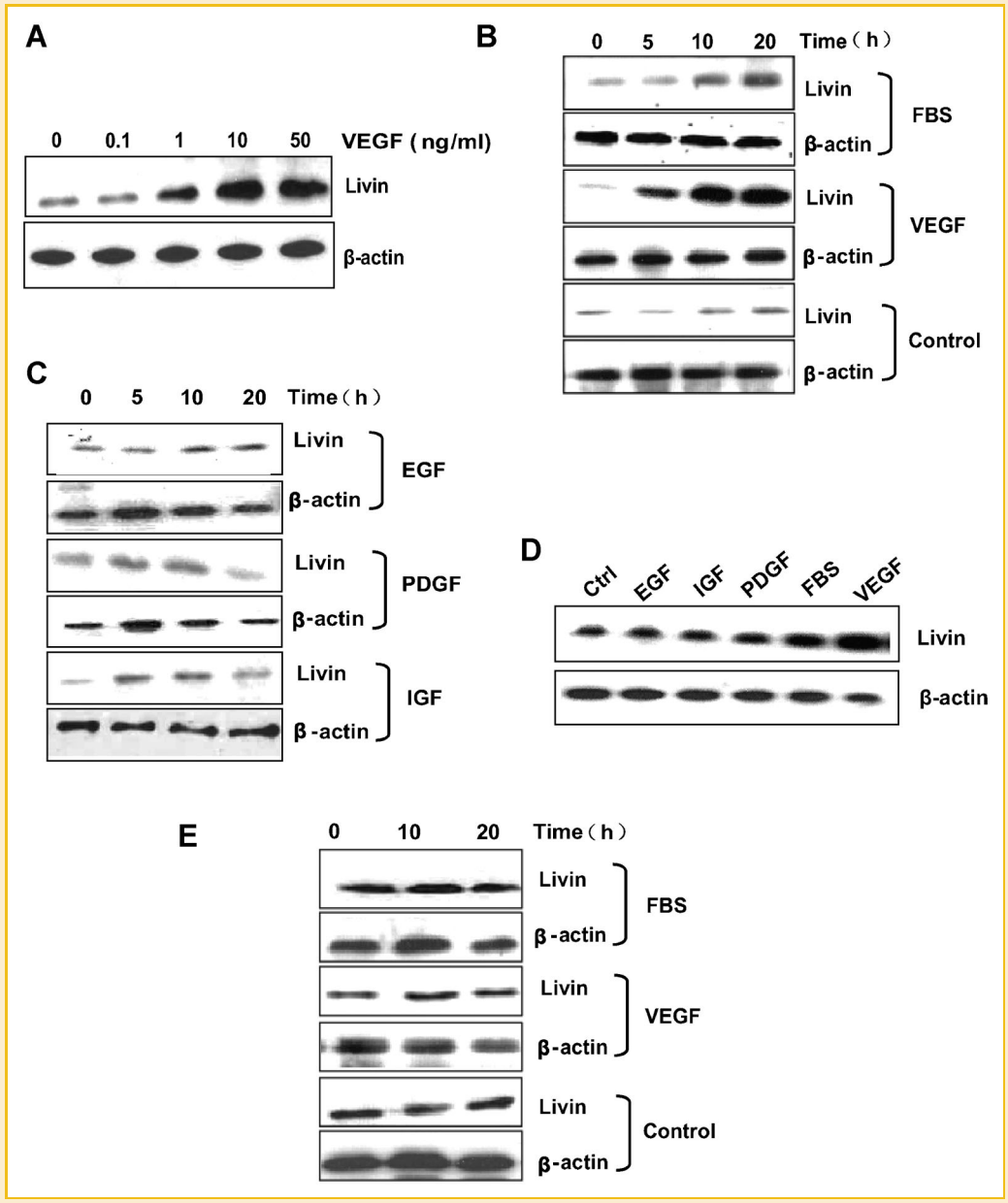

Fig. 1. Regulation of Livin expression by VEGF stimulation. A: Serum-deprived HeLa cells were treated with various concentrations of VEGF for 15 h. The expression of Livin was determined by Western blots. B,C,E: Serum-deprived HeLa cells were stimulated with VEGF (10 ng/ml), 10% FBS, PDGF (10 ng/ml), IGF-1 (10 ng/ml), or EGF (10 ng/ml) for 5, 10, or 20 h. The expression of Livin or XIAP was analyzed by Western blots. D: Serum-deprived SK-MEL-28 cells were stimulated with 10% FBS, VEGF (10 ng/ml), PDGF (10 ng/ml), IGF-1 (10 ng/ml), or EGF (10 ng/ml) for 15 h. Livin expression was detected by Western blots. β-actin expression was detected as loading control. Shown are representative images.

stimulate the initiation of protein synthesis through activation of S6 ribosomal protein and other components of the translational machinery. Meanwhile, hyperphosphorylation of 4E-BP1 carried out by mTOR inhibits 4E-BP1 binding to eIF4E, thereby promoting protein synthesis [Gingras et al., 1999; Murakami et al., 2004]. Thus, we examined whether the downstream targets of mTOR is involved in regulation of Livin expression.

HeLa cells were stimulated with VEGF or left untreated, and then the cell lysates were harvested and analyzed for changes in the phosphorylation state of 4E-BP1 and p70S6K1 protein. The results revealed that VEGF stimulation enhanced 4E-BP1 phosphorylation at all sites tested (Fig. 4A,B). In contrast, VEGF treatment has no effect on the phosphorylation state of p70 S6K1 (Fig. 4C,D) or eIF2-α (as a control; Fig. 4E,F). Taken together, these results suggest that VEGF induction of Livin levels is associated with the phosphorylation state of 4E-BP1.

4E-BP1 IS REQUIRED FOR REGULATION OF LIVIN EXPRESSION

4E-BP1 is known to be a negative regulator of protein translation, and its phosphorylation renders the inactive state [Gingras et al.,

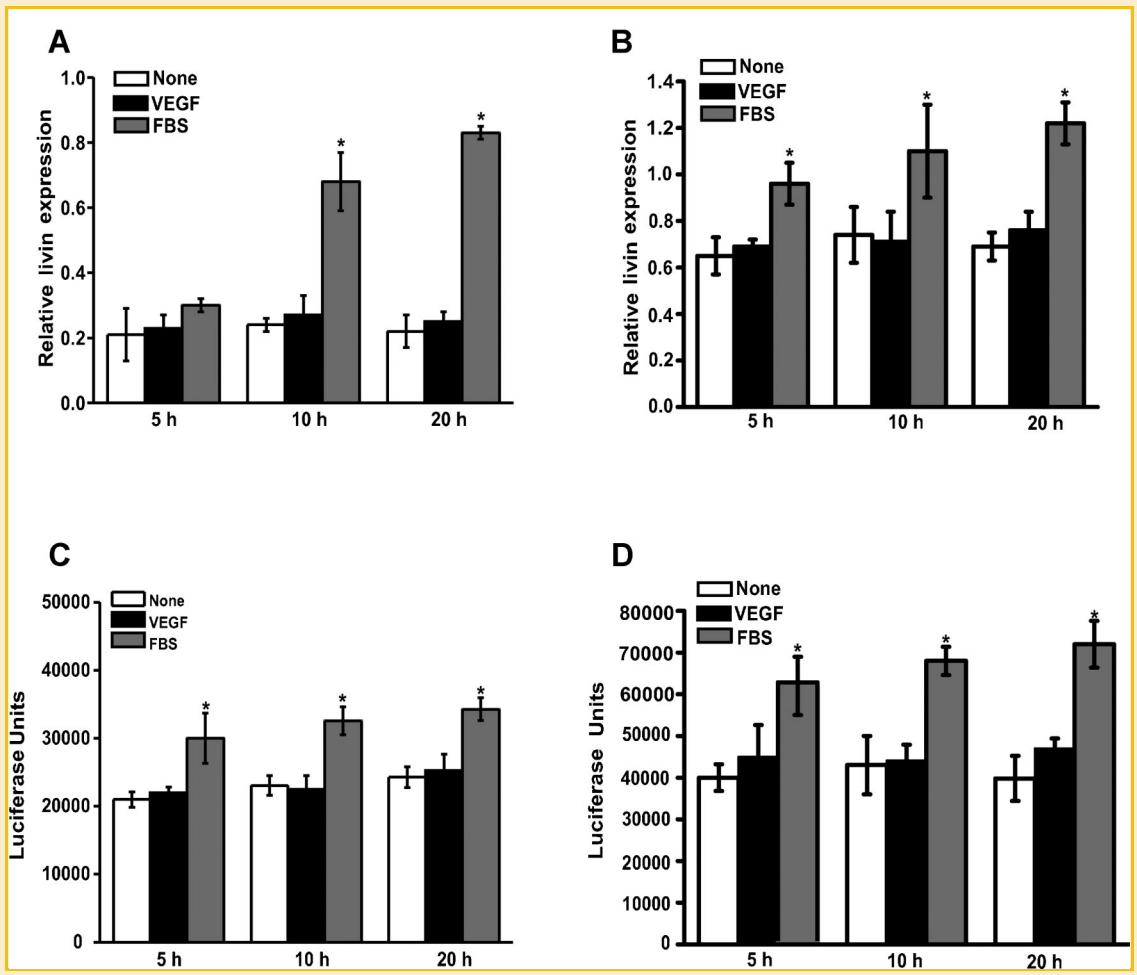

Fig. 2. Livin mRNA expression or promoter activity is not modulated by VEGF stimulation. A,B: Serum-deprived HeLa or SK-MEL-28 cells were treated with 10% FBS, VEGF (10 ng/ml) or left untreated for 5, 10, or 20 h. Livin mRNA expression was estimated by real-time PCR. β-actin was detected as the control. C,D: HeLa or SK-MEL-28 cells were cotransfected pRL-CMV plasmid (as internal control) with Livin p-LUC or pGL-basic plasmid (negative control). After 36 h transfection, the cells were stimulated with VEGF (10 ng/ml) or 10% FBS or left untreated for 5, 10, or 20 h and analyzed for Renilla luciferase-normalized luciferase activity. Data are the mean ± SEM of three independent experiments. The statistical significance was marked with * ($P < 0.05$).

1998]. Thus, 4E-BP1 silencing should mimic the phosphorylated inactive state of 4E-BP1. To address the direct contribution of 4E-BP1 to controlling Livin levels, we designed two 4E-BP1 siRNA oligoes to silence 4E-BP1 expression. HeLa cells were transfected with either 4E-BP1 siRNAs or nonsilencing siRNA (NS siRNA). Cell lysates were produced 48 h after transfection, and the levels of 4E-BP1 protein expression were determined by immunoblot analysis. As shown in Figure 5A, 4E-BP1 expression was substantially reduced in the cells transfected with 4E-BP1 siRNAs oligo 1 or oligo 2, but not with the scramble siRNA. Depletion of 4E-BP1 increased the expression of Livin levels, whereas control cells treated with NS siRNA did not affect the expression of Livin levels (Fig. 5A). In SK-MEL-28 cells, silencing 4E-BP1 also led to an elevated Livin levels (Fig. 5B). Thus, these data suggests that 4E-BP1 is an important mediator of Livin protein synthesis.

REGULATION OF CELL VIABILITY BY mTOR/LIVIN SIGNALING

To examine the effect of VEGF induction of Livin, we first interfered Livin protein expression through rapamycin treatment and Livin silencing technology, and then investigated the change of SK-MEL-28 cell viability in response to cytotoxic agent, doxorubicin. Western blots showed that VEGF stimulation caused a significant increase in Livin expression, but the increase was abrogated by rapamycin treatment (Fig. 6A). Exposure of serum-deprived SK-MEL-28 cells to doxorubicin for 15 or 30 h resulted in ~40% or 70% decrease in cell viability, in a reaction fully reversed by VEGF but not by Livin silencing or rapamycin plus VEGF treatment (Fig. 6B). Caspase-3 is partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme PARP [Nicholson et al., 1995], a key executioner of apoptosis. As expected, doxorubicin caused significant elevation in caspase-3 activity, and the increased caspase-3 activity was suppressed by VEGF stimulation. In contrast, Livin silencing or rapamycin addition suppressed caspase-3 activity enhanced by

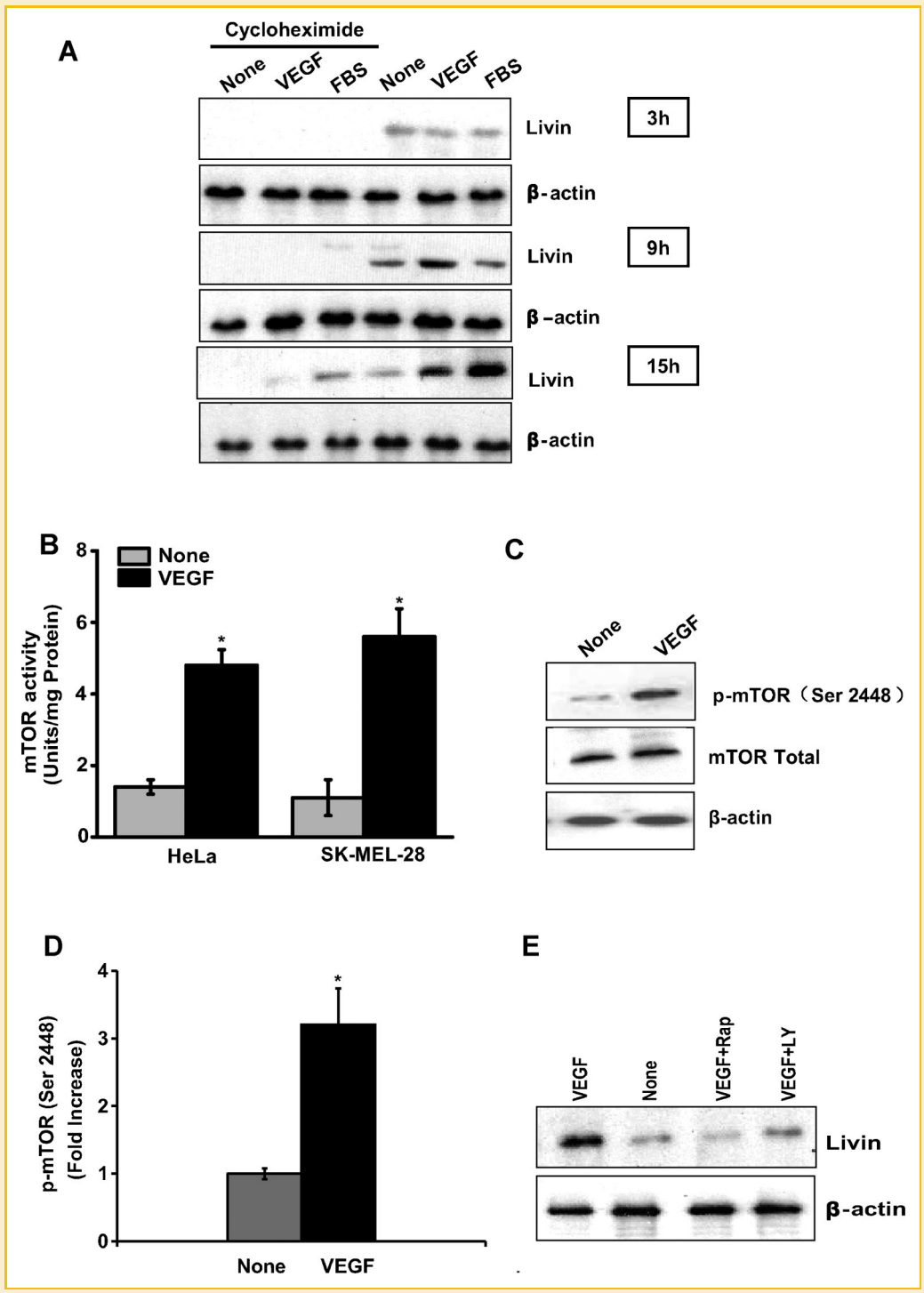

Fig. 3. VEGF modulates Livin expression at translation level via mTOR signaling. A: Effect of cycloheximide on Livin levels. Serum-deprived HeLa cells were incubated with cycloheximide (100 μg/ml), and stimulated with VEGF (10 ng/ml), 10% FBS or left untreated for 3, 9, or 15 h. Livin expression was estimated by Western blots. Shown is a representative image. B: In vitro mTOR kinase activity assay. HeLa or SK-MEL-28 cells were treated with VEGF (10 ng/ml) or left untreated for 10 h. The cell lysates were immunoprecipitated by mTOR antibody for mTOR activity assay. mTOR activity was assessed directly using an ELISA-based assay as described in the Materials and Methods Section. Data are the mean ± SEM of three independent experiments. The statistical significance was marked with * ($P < 0.05$). C,D: HeLa cells were treated as (B). phospho-mTOR (Ser-2448) was examined by Western blots (C) and densitometry analysis compared with β-actin (D). E: Serum-deprived HeLa cells were treated as indicated for 10 h. The expression of Livin was examined by Western blots. β-actin was as a loading control. Shown is a representative image.

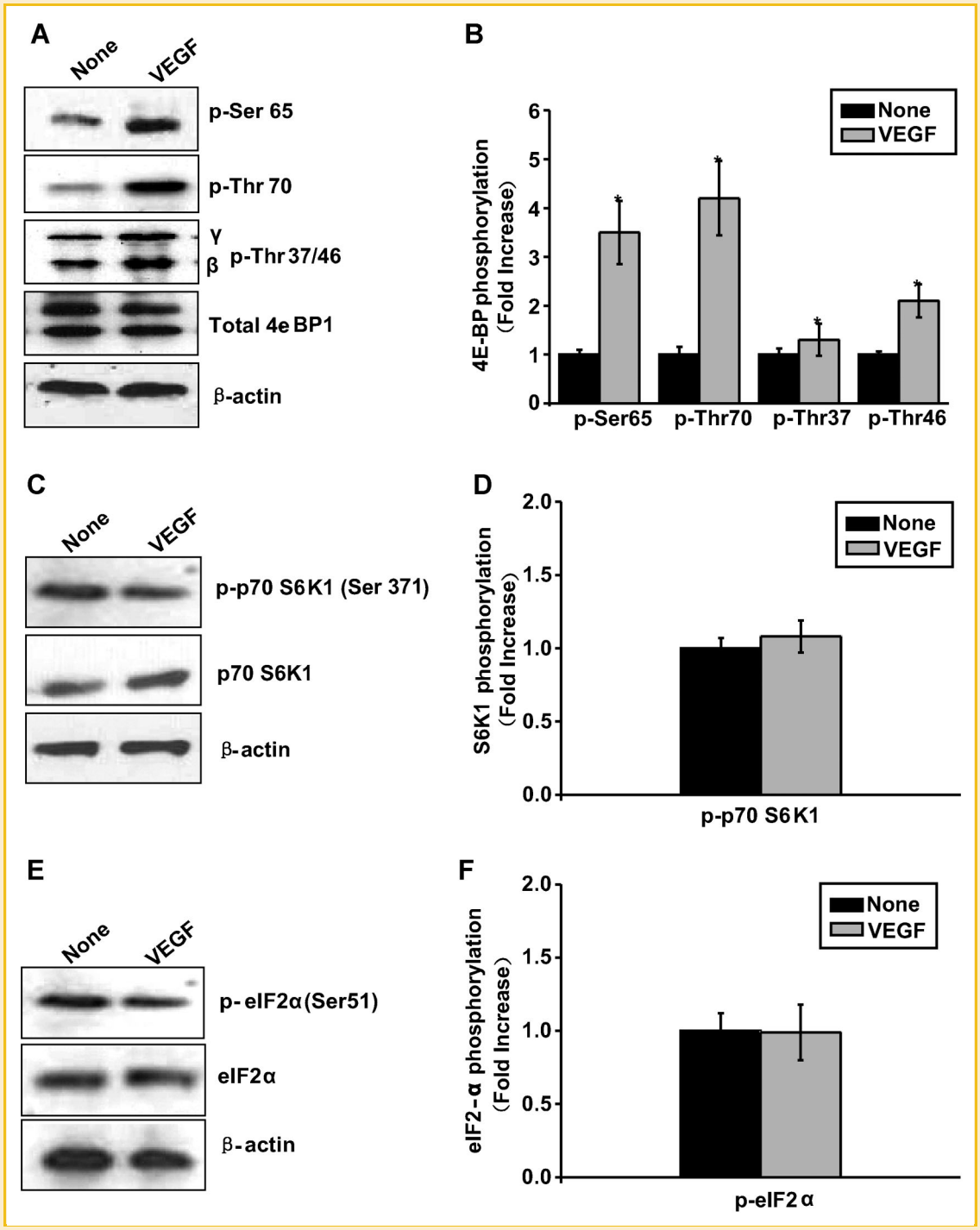

Fig. 4. VEGF stimulation in HeLa cells enhances 4E-BP1 phosphorylation. HeLa cells were stimulated with VEGF (10 ng/ml) or left untreated for 10 h. Cell lysates were examined for phospho-4E-BP1 (Thr-37/Thr-46, Thr-70, p-Ser65), phospho-p70 S6K1(Ser 389), p-eIF2α (Ser 51) by Western blots (A,C,E) and β-actin-normalized densitometry analysis (B,D,F). $^{*}P < 0.05$ when compared with untreated group by ANOVA. Densitometry analysis is representative of three independent experiments.

doxorubicin (Fig. 6C). We also found that exposure of SK-MEL-28 cells to rapamycin did not induce loss of cell viability for the first 15 h (Fig. 6B). However, a 30-h treatment with rapamycin resulted in ~50% decrease in cell viability, and doxorubicin addition could further decrease cell viability (Fig. 6B). Moreover, decreased cell viability was accompanied by decreased Livin expression levels (Fig. 6D), implicating that doxorubicin plus rapamycin combination will be more effective than either treatment alone in reducing SK-MEL-28 cell viability in the presence of VEGF. In addition, inhibition of Livin expression in HeLa cells could also accelerate the

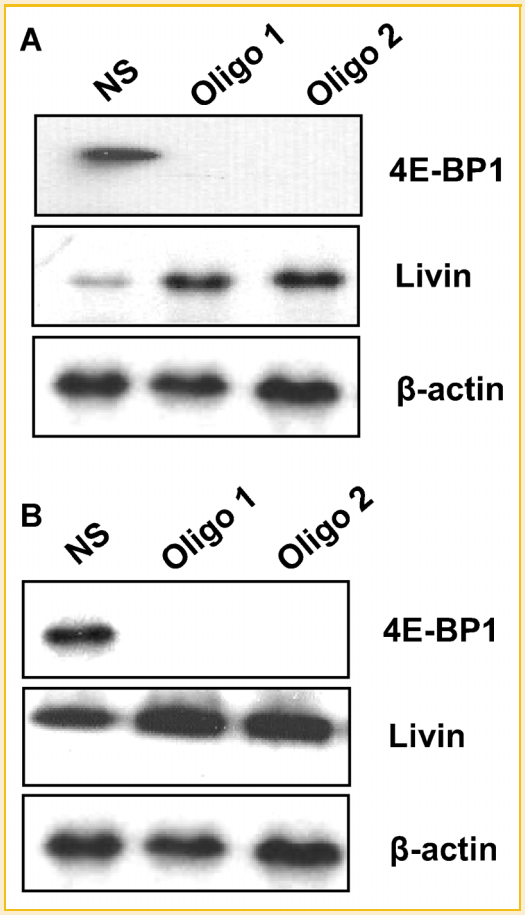

Fig. 5. 4E-BP1 silencing contributes to elevated expression of Livin levels. A,B: HeLa or SK-MEL-28 cells were transfected with 4E-BP1 siRNA oligo 1, oligo 2 or nonsilencing (NS) control siRNA. Thirty-six hours later, the expression of Livin or 4E-BP1 was estimated by Western blots. β-actin was detected as the loading control. Shown is a representative image.

apoptosis progression in response to the addition of cytotoxic agent (Fig. S3A,B). Taken together, these results suggest that cell viability can be regulated by VEGF/mTOR/Livin signaling in SK-MEL-28 and HeLa cells.

DISCUSSION

The basal level of apoptosis is tightly controlled by endogenous IAPs in mammalian cells. Dysregulation of IAPs expression can result in tumorigenesis and chemoresistance [Salvesen and Duckett, 2002]. Livin is a member of the IAP gene family, which encodes negative regulatory proteins that prevent cell apoptosis [Kasof and Gomes, 2001]. Down-regulation of Livin expression will increase apoptotic rate, reduce tumor growth potential and sensitize tumor cells to chemotherapeutic drugs [Liu et al., 2007]. Thus, understanding Livin regulation contributes to developing specific Livin inhibitors. In human NSCLC cell lines, Livin is a direct target gene of β-catenin/TCF signaling pathway [Yuan et al., 2007]. β-catenin/TCF complex binds to Livin promoter which ultimately promotes Livin mRNA expression. In neuroblastoma, MycN is found to be a novel interactor of Livin promoter [Dasgupta et al., 2009]. In addition, Livin protein can undergo site-specific cleavage by effector caspase-3 and -7 to produce a large COOH-terminal subunit containing both the BIR and RING domains to alter its function [Chang and Schimmer, 2007]. In this study, we delineate a novel regulation mechanism of Livin expression. We found that Livin expression can be up-regulated by VEGF stimulation at translational levels. But the protein stability of Livin, the transcriptional of Livin mRNA or Livin promoter activity is not affected by VEGF treatment.

VEGFs are crucial regulators of vascular development during embryogenesis (vasculogenesis) as well as blood vessel formation (angiogenesis). They play great roles in multiple cellular processes including cell migration, survival, and proliferation [Olsson et al., 2006]. Previous studies have revealed that VEGF treatment can prevent apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling [Gupta et al., 1999]. In neuroblastoma cells, VEGF treatment contributes to survivin expression which is essential for the anti-apoptosis response [Beierle et al., 2005]. VEGF can induce Mcl-1 up-regulation and protect multiple myeloma cells against apoptosis [Le Gouill et al., 2004]. In addition, VEGF-induced phosphorylation of Bcl-2 influences B lineage leukemic cell response to apoptotic stimuli [Wang et al., 2005]. Base on these evidences, tumor therapies that are based on neutralizing anti-VEGF antibodies and small-molecular weight tyrosine-kinase inhibitors that target the VEGFRs have been developed. The data presented here demonstrated that VEGF stimulation results in elevated expression of Livin levels, while inhibition of VEGF signaling prevents Livin induction. These findings provide a novel mechanism for tumor therapies achieved by inhibiting VEGF signal-transduction pathways that are exaggerated in pathological angiogenesis.

mTOR plays a central role in mediating responses to many stimuli such as nutrients, hormones, and stress that result in the regulation of multiple cellular processes involved in cell growth and metabolism. mTOR is an important regulator of protein synthesis by activation or inhibition of several downstream translation initiation and elongation factors directly or indirectly through changing their phosphorylation state [Sarbassov et al., 2005]. Present study demonstrates that VEGF treatment can result in mTOR signaling activation, which in turn mediates elevated expression of Livin levels. Livin is suggested to be a novel effector of mTOR signaling. Previous study demonstrated that survivin, another member of IAP family, is regulated by IGF/mTOR signaling [Vaira et al., 2006]. Both Livin and survivin are crucial for the anti-apoptotic effect in mammalian cells, while they are regulated by mTOR signaling pathway. The finding further emphasizes the important role of mTOR signaling in cell apoptosis.

4E-BP1 is an inhibitor of 5′ cap-dependent protein translation, which is known to play an important role in cellular response to changes in environmental conditions such as altered nutrient levels and various physiological stresses [Clemens, 2001]. 4E-BP activity is regulated post-translationally by mTOR signaling. Activated mTOR hyperphosphorylates 4E-BP, and promotes the 5′ cap-dependent

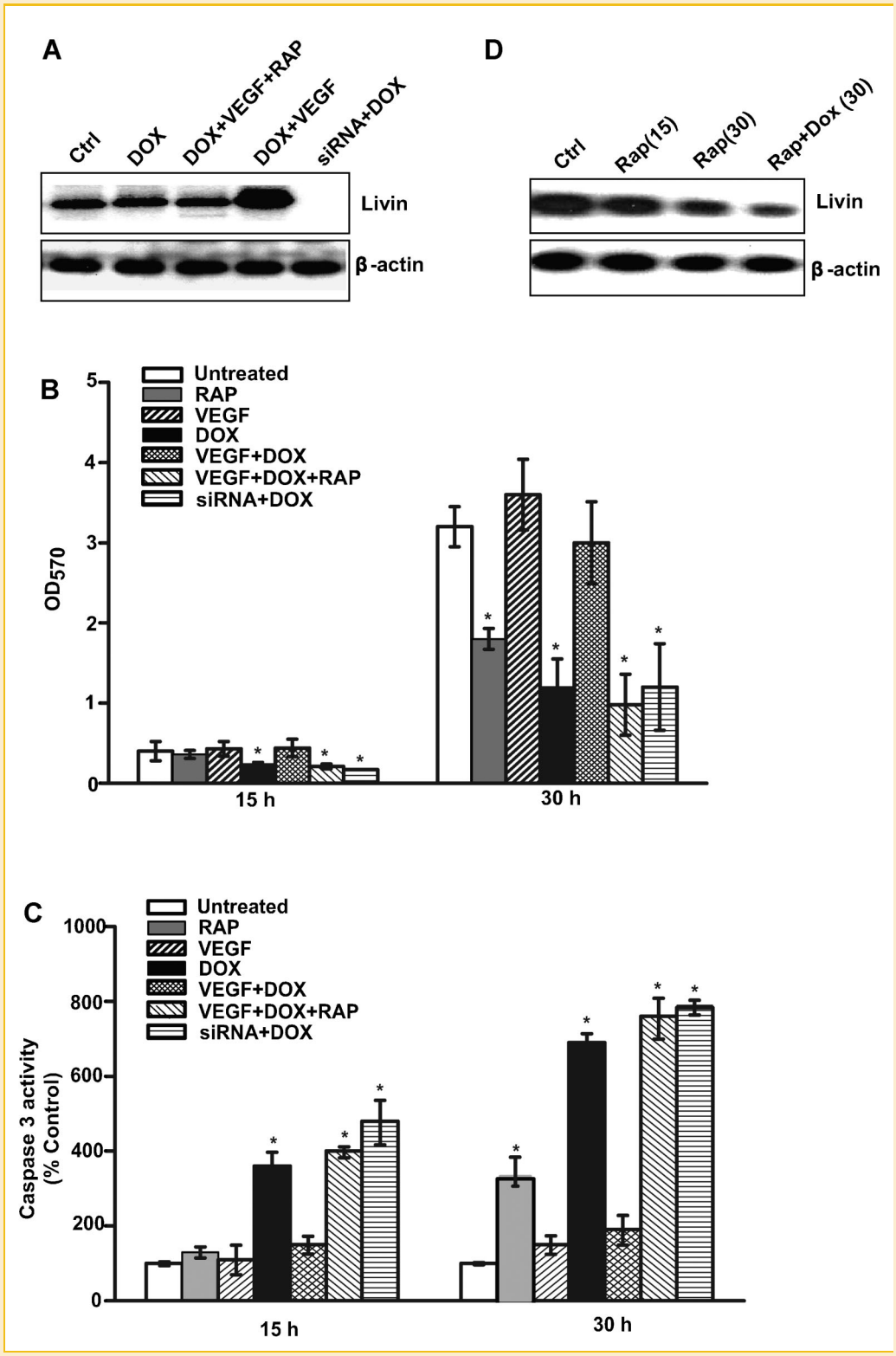

Fig. 6. Effect of VEGF/mTOR/Livin signaling on SK-MEL-28 cell viability. A: Serum-deprived SK-MEL-28 cells were left untreated or treated with rapamycin (RAP, 20 nM), stimulated with VEGF (10 ng/ml) in the presence or absence of doxorubicin (Dox, 5 μM). β-actin-normalized Livin levels were determined by Western blots. Shown is a representative image. B,C: Serum-deprived SK-MEL-28 cells were treated as shown. The cell viability was analyzed by MTT after 15 or 30 h treatment. The activity of caspase 3 was determined using Caspase-Glo® 3 assay kit (Promega) according to the manufacturer's protocol. All data are expressed as means ± SEM of three independent experiments. The statistical significance was marked with * ($P < 0.05$). D: Serum-deprived SK-MEL-28 cells were treated with RAP (20 nM) for 15 or 30 h in the presence or absence of Dox (5 μM). The expression of Livin levels was estimated by Western blots. β-actin was detected as loading control. Shown is a representative image.

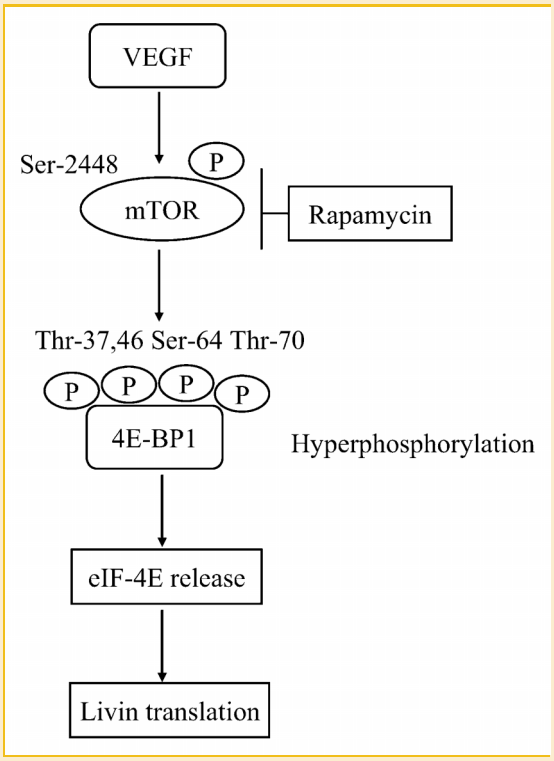

Fig. 7. Model for VEGF-mediated Livin expression modulation. The study supports the following hypothesis. VEGF stimulation enhances mTOR activity, and inhibits 4E-BP1 activity by enhancing phosphorylation at Thr-37, 46, Ser-64, and Thr 70. The phosphrylation of 4E-BP1 in turn leads to its dissociation from the translational initiation eIF4E. Finally, eIF4E activation is responsible for increased Livin expression.

translation [Gingras et al., 2001]. VEGF treatment results in enhancement of mTOR activity, as indicated by an increase in phosphorylation at Ser-2448 and mTOR activity assay. 4E-BP1 is phosphorylated at Thr-37, Thr-46, Ser-65, and Thr-70 residues, and the hyper-phosphorylation of 4E-BP1 renders it inactive leading to its dissociation from the translation initiation factor eIF4E. Finally, eIF4E is released and activated, and the initiation of Livin translation is enhanced (Fig. 7).

mTOR pathway constitutes a "sensor" network for environmental nutrients or stress conditions, thus affecting cell viability [Wullschleger et al., 2006]. Present study indicates that targeted inhibition of VEGF/mTOR contributes to decreasing Livin levels and promoting cell apoptosis, suggesting that Livin/mTOR signaling plays an important role in regulation of cell viability. Preclinical studies have revealed that inhibition of VEGF-dependent signal transduction cascades with pharmacological compounds is a potent antiangiogenic strategy and in particular when combined with a cytotoxic treatment modality [Riesterer et al., 2004]. We found that cytotoxic reagent combined with mTOR inhibitor is more efficient than mTOR inhibitor alone in reduction in cell viability. Thus, modulation of VEGF/mTOR signaling combined with cytotoxic treatment will be a more promising way for cancer treatment.

In summary, VEGF treatment results in increased Livin expression through mTOR signaling. Inhibition of mTOR with doxorubicin alone or in combination with rapamycin, or knockdown the downstream target, 4E-BP1, can abolish VEGF induction of Livin expression, and decrease cell viability and promote apoptosis progression. Thus, pharmacologic inhibition of VEGF/mTOR/Livin signaling may provide an additional strategy to suppress the cell viability of cancer cell. Ablation of Livin translation contributes to remove an anti-apoptotic mechanism potentially associated with the aggressive tumor behavior in vivo.

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