IL-37 induces autophagy in hepatocellular carcinoma cells by inhibiting the PI3K/AKT/mTOR pathway

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ABSTRACT

Autophagy is an intracellular “self-eating” process that is closely related to inflammation and cellular immunity. New studies indicate that autophagy is also involved in tumor suppression. The anti-inflammatory cytokine interleukin-37 (IL-37) has been shown to have tumor-suppressive abilities in hepatocellular carcinoma (HCC). Notably, autophagy appears to play a dual role in the development of HCC and may be involved in both tumorigenesis and tumor suppression. However, the potential role of IL-37 in autophagy is currently unknown. In this study, we investigated the effect of IL-37 on autophagy in multiple HCC cell lines. In doing so, we found that IL-37 inhibits proliferation in HCC cells and also induces autophagy and apoptosis in the SMMC-7721 and Huh-7 cell lines. Further experiments revealed that IL-37 treatment reduced the levels of phosphorylated protein kinase B (p-AKT), phosphorylated mammalian target of rapamycin (p-mTOR), phosphorylated p70 ribosomal protein S6 kinase (p-p70S6K) and phosphorylated 4E-binding protein 1 (4E-BP1). Moreover, treatment with an AKT agonist, insulin-like growth factor 1 (IGF-1), reversed these IL-37-mediated effects on autophagy, and treatment with a phosphoinositide-3-kinase (PI3K)/AKT inhibitor, LY294002, mimicked the effects of IL-37. Taken together, these results indicate that IL-37 regulates autophagy in SMMC-7721 and Huh-7 cells via inhibition of the PI3K/AKT/mTOR signaling pathway.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal types of malignant tumors, and the number of affected individuals is increasing each year worldwide (Villanueva et al., 2013). Although the mortality rate associated with HCC has decreased (Chen et al., 2016) and the understanding of the underlying hepatocarcinogenic mechanisms has improved in recent years (Farazi and DePinho, 2006), the prognosis of patients with HCC is still poor. Therefore, early diagnosis and effective treatment therapies are essential.

Autophagy is an important homeostatic process that is responsible for degrading intracellular organelles and protein aggregates via a process that involves the delivery of cytoplasmic cargo to the lysosome (Levine and Kroemer, 2008). This lysosomal degradation pathway is initiated by the formation of phagophores, which fuse to form a vesicle called an autophagosome. Autophagosomes are then able to fuse with lysosomes, which degrade/digest the vesicular contents, which can then be recycled for the cell’s metabolic needs (Janku et al., 2011). Recently, autophagy has been shown to regulate inflammation and immune responses during infection (Levine et al., 2011), while also serving as a key factor in tumor-suppressive processes. Inflammation and immunity also appear to exert important effects in this process, and recent research indicates that autophagy inhibits tumor inflammation and active anti-tumor immunity in a non-autonomous manner (Zhong et al., 2016). During the development of HCC, autophagy plays a dual role: suppressing tumor formation in the initial stages and then promoting tumor growth in the subsequent progression stages (Lee and Jang, 2015). Suppression of autophagy leads to p62 accumulation, which contributes to liver tumor formation (Takamura et al., 2011), and reduced Beclin1 levels and elevated hypoxia-inducible factor (HIF)-1α expression were associated with progression of HCC (Osman et al., 2015). Autophagy is ultimately a survival mechanism and is activated in response to stress in tumor cells. Thus, increasing autophagy could produce a more hospitable environment for tumor cells (Eskelinen, 2011).

 interleukin 1 (IL-1) family member 7 (IL-1F7) was first identified in 2000 by Kumar et al. (Kumar et al., 2000) and was recently proposed to be renamed IL-37. The IL-1 family has 11 members, most of which are
pro-inflammatory cytokines, including IL-1α, IL-1β, and IL-18 (van de Veerdonk and Netea, 2013). In contrast, IL-37 has been identified as an anti-inflammatory factor that affects other pro-inflammatory signals, such as those mediated by toll-like receptor (TLR) ligands, IL-1β, tumor necrosis factor α (TNF-α), and IL-18 (Nold et al., 2010; Yang et al., 2015). To perform this function, IL-37 requires IL-18 receptor α (IL-18Rα) and single Ig IL-1-related receptor (SIGIRR/IL-1R8) (Lunding et al., 2015). A recent study indicates that IL-37 serves as a tumor suppressor in renal cell carcinoma, wherein its antitumor activity is exerted via inhibition of IL-6/STAT3 signaling (Jiang et al., 2015). Notably, IL-37 expression is reduced in patients with primary HCC and is associated with tumor progression, demonstrating that IL-37 might be a valuable prognostic biomarker (Zhao et al., 2014).

Numerous studies have demonstrated that autophagic processes can be regulated by cytokines. For example, autophagy is induced by interferon-γ (IFN-γ) and TNF-α (Gutiérrez et al., 2004; Harris and Keane, 2010; Levine et al., 2011) and inhibited by IL-4, IL-13, and IL-10 (Harris et al., 2007; Park et al., 2011). A recent study also found that IL-37 induces the expression of WD repeat and FYVE domain containing 1 (Wdfy1), which encodes an inhibitor of the mammalian target of rapamycin complex 1 (mTORC1) that is known to stimulate autophagy, and decreases the expression of mammalian target of rapamycin (mTOR) (Nold-Petry et al., 2015). Similarly, transfection of the human monocytic cell line THP-1 with IL-37 also appeared to reduce the expression of mTOR (Nold et al., 2010). Previous studies have demonstrated that mTOR (especially mTORC1) plays a major role in regulating autophagy, with phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT), AMP-activated kinase (AMPK), and extracellular signal-regulated kinase 1/2 (ERK1/2) as the key upstream effectors (He and Klionsky, 2009; Jacinto et al., 2006). Therefore, it is possible that IL-37 regulates autophagy through an mTOR-related mechanism. However, the functional role and specific mechanism of IL-37 during the autophagic process have not been studied.

In the present study, we investigated the effect of IL-37 on multiple HCC cell lines, focusing on changes in autophagic processes.

2. Materials and methods

2.1. Reagents

Recombinant human IL-37 (rhIL-37) was purchased from Protein Specialists (ProSpec, Ness-Ziona, Israel). The following purified primary antibodies were used: rabbit anti-ILC3β (cat. #L7543, Sigma-Aldrich, St.Louis, MO, USA); mouse anti-IL-37 (cat. #ab57187, Abcam, Cambridge, UK); anti-Beclin1 (cat. #3495), anti-SQSTM1 (p62) (cat. #8025), anti-Becl2 (cat. #2870), anti-Bax (cat. #14796), anti-cleaved caspase-3 (cat. #9665), anti-AKT (cat. #4691), anti-phosphorylated AKT (Ser473) (cat. #4060), anti-mTOR (cat. #2983), anti-phosphorylated mTOR (Ser2448) (cat. #5536), anti-p70 ribosomal protein s6 kinase (p70S6K) (cat. #2708), anti-phosphorylated p70S6K (Ser371) (cat. #9208), anti-4E-binding protein 1 (4E-BP1) (cat. #9644), anti-phosphorylated 4E-BP1 (Thr37/46) (cat. #2855), anti-AKT (cat. #2532), anti-phosphorylated AMPK (Thr172) (cat. #2535), and mouse anti-β-actin (cat. #3700), which were purchased from Cell Signaling Technology, Inc. (CST, Danvers, MA, USA); and rabbit anti-ERK1/2 (cat. #4370) and anti-phosphorylated ERK1/2 (Thr202/Tyr204) (cat. #4372), both purchased from Cell Signaling Technology, Inc. (CST, Danvers, MA, USA). Chloroquine (CQ) was purchased from Sigma-Aldrich (St.Louis, MO, USA). 3-methyladenine (3-MA) and LY294002 were purchased from AbMole BioScience (USA). Recombinant human insulin-like growth factor 1 (IGF-1) was purchased from R&D Systems (Minneapolis, USA).

2.2. Cell culture

The human HCC cell lines SMMC-7721, Huh-7, and HepG2 and the human liver cell line LO2 were purchased from the American Type Culture Collection (ATCC, Maryland, USA). Cells were maintained in Roswell Park Memorial Institute (RPMI) medium 1640 basic (Gibco, Grand Island, USA) supplemented with 1% penicillin/streptomycin (Gibco, Grand Island, USA) and 10% fetal bovine serum (Gibco, Grand Island, USA). All cell lines were cultured at 37 °C in a humidified incubator with 5% CO₂.

2.3. Reagent treatment

RhIL-37 was reconstituted to a concentration of 100 ng/ml in phosphate-buffered saline (PBS). IGF-1 was reconstituted at 200 μg/ml in sterile PBS. SMMC-7721 and Huh-7 cells were seeded in six-well plates with the same cell number for each experiment. For IL-37 treatment, cells were incubated with various concentrations of IL-37 (0, 50, 100, and 200 ng/ml) for 12 h as previously described (Sakai et al., 2012). The cells were treated with 3-MA (5 μmol/l) (Young et al., 2015) and CQ (10 μmol/l) (Hori et al., 2015). For IGF-1 treatment, cells were incubated with various concentrations (0, 100, and 200 ng/ml) (Liu et al., 2015) for the lengths of time specified. The cells were also treated with LY294002 (20 μmol/l) (Zhang et al., 2015).

2.4. Cell viability assay

Cell viability was examined using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) according to the manufacturer’s protocol. Cells were plated in 96-well plates and incubated with different concentrations of IL-37 (0, 50, 100, and 200 ng/ml) at 37 °C in a humidified incubator with 5% CO₂ for 24, 48, or 72 h. Then, the supernatant was removed and the cells were incubated in RPMI-1640 medium containing 10 μl CCK-8 for an additional 2 h at 37 °C. The optical density (OD) was measured at 450 nm using a microplate reader (Biotek, USA).

2.5. Transmission electron microscopy (TEM)

SMMC-7721 and Huh-7 cells were seeded in 10-cm cell culture dishes and treated with 100 ng/ml IL-37 for 12 h. The cells were then washed 3 times with 0.1 M PBS. The cells were then post-fixed in 1% osmium tetroxide at 4 °C for 2 h, followed by staining with Millipore-filtered uranyl acetate and dehydration in a graded series of ethanol and acetone. We then infiltrated, embedded, and polymerized the samples in ethylene resin. Semi-thin (0.5 μm) and ultrathin (60 nm) were prepared, stained with uranyl acetate and lead citrate, and then observed using a Hitachi Model H-7500 transmission electron microscope (Hitachi, Tokyo, Japan). Photographs were taken using a Gatan 780 system (Gatan, Pleasanton, CA, USA).

2.6. Western blot analyses

SMMC-7721 and Huh-7 cells were washed with cold PBS and harvested. Total protein was extracted from the cells, using RIPA buffer (Beyotime, Shanghai, China) with 1% phenylmethylsulfonylfluoride (PMSF, Beyotime, Shanghai, China), split on ice for 30 min, and centrifuged for 15 min at 12,000 rpm in 4 °C to remove cell debris. Protein concentrations were measured by the BCA method (Beyotime, Shanghai, China) in 96-well plates. Then, the proteins were boiled after being mixed with protein loading buffer (protein:buffer = 4:1) (Beyotime, Shanghai, China). Proteins were separated on 8%-12% SDS-PAGE gels (Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). Membranes were blocked for 2 h at room temperature (RT) with 5% skim milk in
TBST (20 mmol/l Tris-HCl, 137 mmol/l NaCl, and 0.1% Tween-20) or with 5% bovine serum albumin (BSA) (Biosharp, Hefei, China) in TBST for phosphorylated proteins. The membranes were then incubated overnight with diluted antibodies at 4 °C. All antibodies were diluted 1:1,000 to 1:800 with primary antibody diluent (Beyotime Biotechnology, shanghai, China) before use. Subsequently, the membranes were washed with TBST three times (10 min each) and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit or anti-mouse IgG, 1:5,000) for 2 h at RT. Then, the membranes were washed with TBST three times (10 min each). The proteins were detected with an enhanced chemiluminescence (ECL) kit (Advansta, Menlo Park, CA, USA) and vilber Fusion FX7 Spectra (Fusion FX7, Franck). Protein expression was quantified using Fusion software.

2.7. Flow cytometry analysis

Flow cytometric analysis of propidium iodide (PI) staining was performed to measure the cell cycle. SMMC-7721 and Huh-7 cells were treated with or without IL-37 and then harvested and fixed with ice-cold 75% ethanol at 4 °C overnight. The fixed cells were stained with PI and the mixture was incubated in the dark at RT for 30 min. Then, the stained cells were analyzed by fluorescence-activated cell sorting (FACS) flow cytometry.

Flow cytometric analysis of Annexin V-fluorescein isothiocyanate (FITC) and PI double staining was performed to measure apoptosis. SMMC-7721 and Huh-7 cells were harvested after treatment with IL-37 and resuspended in 500 μl of PBS. The resuspended cells were stained with Annexin V-FITC and PI, and the mixture was incubated in the dark at RT for 10 min. Annexin V-FITC binding was detected in all stained cells at excitation and emission wavelengths of 488 and 530 nm, respectively.

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Student’s t test and one-way analysis of variance (ANOVA) were performed to analyze differences between experimental groups. Statistical analysis was performed with SPSS 18.0 software (SPSS, Chicago, IL, USA), with a p < 0.05 considered statistically significant.

3. Results

3.1. IL-37 inhibits proliferation of HCC cells

To investigate the effect of IL-37 on cell growth, we first measured the expression of IL-37 in normal human liver cells and HCC cells. As shown in Fig. 1A, the expression of IL-37 in LO2 cells was higher than that in HepG2 cells and was significantly higher than that found in SMMC-7721 and Huh-7 cells. To further examine whether IL-37 has an effect on the growth of HCC tumors, we evaluated cell viability using a CCK-8 assay (Fig. 1B). The viability of HCC cells decreased in a concentration-dependent manner after treatment with IL-37, and this effect was particularly notable for the SMMC-7721 and Huh-7 cells. We then chose 100 ng/ml IL-37 as an optimal concentration for cell proliferation. Cell proliferation is reflected by the cell cycle. To examine whether IL-37 affects the cell cycle in SMMC-7721 and Huh-7 cells, we evaluated the cell cycle by flow cytometry. The proliferation index (PI) and S-phase cell fraction (SPF), which respectively are defined as the percentage of G2/M and S phase cells or S phase cells among all cells (G0/G1 + S + G2/M), reflect the cell cycle. The proportion of cells in the G0/G1 phases was increased and that of cells in the G2/M and S phases was decreased in IL-37-treated cells (Fig. 1C). The PI and SPF in the IL-37-treated cells were lower than those in the control cells. Taken together, these data demonstrate that IL-37 inhibits proliferation in HCC cells.

3.2. IL-37 induces autophagy in SMMC-7721 and Huh-7 cells

Autophagy is an important degradation mechanism that is critical during the regulation of cell growth. Previous studies have suggested that IL-37 may have some effects on cell autophagy. In this experiment, we first investigated the relationship between IL-37 and autophagy in SMMC-7721 and Huh-7 cells. TEM is the gold standard for identifying autophagosome formation. In our TEM analysis, we identified some vesicles inside the cells and observed an increase in the production of autophagosomes after treatment with IL-37 (Fig. 2A and B). During autophagy, LC3-I is converted to LC3-II, and a high LC3-II/LC3-I ratio is a hallmark of this process. p62 and Beclin1 are also used as markers of autophagy. Thus, to further examine whether IL-37 treatment induced autophagy, we measured the LC3-II/LC3-I ratio as well as p62 and Beclin1 expression following IL-37 treatment. Notably, the LC3-II/LC3-I ratio and the protein levels of Beclin1 appeared to increase after treatment with IL-37 in a concentration-dependent manner in both SMMC-7721 and Huh-7 cell lines (Fig. 2C and D). Consistent with this, the expression of p62 decreased following addition of IL-37, indicating enhanced autophagy flux (Fig. 2C and D). Moreover, to further determine whether IL-37 induces autophagy, we used 3-MA and CQ, which are inhibitors of autophagy. The LC3-II/LC3-I ratio was significantly decreased in IL-37/3-MA-co-treated SMMC-7721 and Huh-7 cells and increased in IL-37/CQ-co-treated SMMC-7721 and Huh-7 cells (Fig. 2E and F). Together, these data further indicate that IL-37 induces autophagy in SMMC-7721 and Huh-7 cells.

3.3. IL-37 induces apoptosis in SMMC-7721 and Huh-7 cells

There are multiple connections between the autophagic and apoptotic processes. In fact, Beclin1 has been shown to interact with the anti-apoptotic multidomain protein Bcl-2 via its BH3 domain, an amphipathic α-helix that binds to the hydrophobic cleft of Bcl-2 (Maiuri et al., 2007). This association of Beclin1 with Bcl-2 blocks the induction of autophagy. In our experiments, we found that the expression of Beclin1 increased in response to IL-37 treatment (Fig. 3A). To further evaluate whether treatment with IL-37 induces autophagy, we stained IL-37-treated SMMC-7721 and Huh-7 cells with Annexin V/PI and quantified the number of cells undergoing apoptosis by flow cytometry. In doing so, we found that IL-37 induced apoptosis in early (Annexin V+/PI-) and late (Annexin V+/PI+) stages, with higher levels being observed in the latter (Fig. 3A and B). To further confirm these results, we measured the expression of the apoptosis related proteins Bax and cleaved caspase-3 as well as the level of anti-apoptotic protein Bcl-2 in the cells. The expression of both Bax and cleaved caspase-3 was increased, whereas the level of Bcl-2 was decreased following treatment with IL-37 in SMMC-7721 and Huh-7 cells (Fig. 3C and D). Overall, these results demonstrate that IL-37 does in fact induce apoptosis in SMMC-7721 and Huh-7 cells.

3.4. IL-37 triggers autophagy by inhibiting PI3K/AKT/mTOR signaling

Previous studies have shown that inhibition of AKT or ERK and activation of AMPK can positively regulate autophagy (Oh et al., 2016; Perluigi et al., 2015; Zhou et al., 2016). To determine the specific signaling pathway involved in IL-37-mediated autophagy in SMMC-7721 and Huh-7 cells, we measured the activation of key signal proteins, including ERK, AKT, and AMPK. We found that treatment with IL-37 had little effect on p-ERK and p-AMPK expression, but did cause an observable decrease in p-AKT expression (Fig. 4A and B), indicating that AKT may be an important factor in mediating the effects of IL-37. Furthermore, studies have shown that mTOR is a key regulator of autophagy, especially the mTORC1 complex. We found that expression of p-mTOR and two downstream substrates, which were named p70S6K and 4E-BP1, decreased following IL-37 treatment in both the SMMC-7721 and Huh-7 cell lines (Fig. 4C). Consistent with this, the
LC3-II/LC3-I ratio and Beclin1 levels were also increased, whereas p62 expression was decreased (Fig. 4C and D). These data suggest that inhibition of mTOR and its downstream substrates also contributes to the induction of autophagy by IL-37 and that the PI3K/AKT/mTOR pathway could be involved in IL-37-induced autophagy in SMMC-7721 and Huh-7 cells.

3.5. Activation of AKT signaling reversed IL-37-induced autophagy and inhibition of AKT signaling mediated effects similar to IL-37-induced autophagy

IGF-1 is known to bind to the IGF-1 receptor (IGF-1R), which is one of the tyrosine kinase receptors that activate the PI3K/AKT signaling pathway upon stimulation. Thus, to further confirm the role of PI3K/AKT/mTOR signaling during IL-37-induced autophagy, we first tested
the expression of p-AKT after treatment with IGF-1. IGF-1 treatment increased the expression of p-AKT in a concentration-dependent manner in SMMC-7721 and Hep-7 cells, with optimal changes observed at a concentration of 100 ng/ml and a treatment time of 1 h (Fig. 5A). mTOR expression was also increased, whereas the LC3-II/LC3-I ratio was decreased following IGF-1 treatment. Notably, these effects are opposite to those induced by IL-37 treatment. We further investigated the expression of p-AKT and p-mTOR following treatment with both IL-37 and IGF-1 in SMMC-7721 (Fig. 5B) and Hep-7 cells (Fig. 5C). Compared to cells treated with only IL-37, cells treated with both IL-37 and IGF-1 had a significantly decreased LC3-II/LC3-I ratio. These data indicate that activation of AKT signaling reversed the effects of IL-37-induced autophagy in SMMC-7721 and Hep-7 cells. Moreover, we measured the expression of p-AKT, p-mTOR, and LC3 following
**Quantitation of cellular apoptosis.** Data are expressed as the mean ± SD (n = 3); *p < 0.01 compared with the SMMC-7721 cell control group, **p < 0.05 compared with the Huh-7 cell control group. (C) Western blot analysis showing the expression of Bcl-2, Bax, cleaved caspase-3, and β-actin in SMMC-7721 and Huh-7 cells after treatment with 100 ng/ml IL-37 for 12 h. (D) The relative expression of Bcl-2, Bax, and cleaved caspase-3 is consistent with the results in Fig. 3C. All data are expressed as the mean ± SD (n = 3).

**4. Discussion**

HCC is one of the most prevalent malignant tumors and is associated with a high lethality rate. Although recent research has made some progress in uncovering the molecular mechanisms of HCC, the prognosis for patients with HCC remains very poor. In recent years, there have been a number of studies published concerning the effects of autophagy on HCC. It appears that autophagy plays a dual role in the development of HCC. As a physiological survival mechanism under certain cellular conditions, autophagy is related to the survival of tumor cells. It has been linked to p62 accumulation, which has a positive effect on tumor formation (Takamura et al., 2011). However, excess autophagy may result in cell death and suppression of cell proliferation (Su et al., 2015). Thus, regulation of autophagy in HCC is an interesting avenue for developing potential treatment options. Indeed, ginsenoside Rh2 (GRh2), a component of red ginseng that has been shown to have anti-inflammatory properties, was recently shown to substantially inhibit the growth of HCC cells through autophagy-related processes (Yang et al., 2016).

Previous studies demonstrated that IL-37 mediates various anti-cancer effects in multiple types of cancer, including HCC (Jiang et al., 2015; Zhao et al., 2014). Other cytokines are also associated with autophagy. For example, IL-6 has been shown to inhibit starvation-induced autophagy by activating the STAT3/Bcl2 pathway, and this process involves Beclin1 and VPS34 (Qin et al., 2015). IL-17A also appears to inhibit autophagy through phosphorylation of p38 mitogen-activated protein kinase (MAPK) and increased expression of Bcl2 (Liu et al., 2013; Said et al., 2014). Moreover, IL-33 has been shown to regulate autophagy in degenerative tissues in addition to controlling macrophage migration into these tissues (Wu et al., 2015). Recent reports have also provided evidence that IL-37 can reduce the level of mTOR, which is similar to the effect of starvation on autophagy (Nold-Petry et al., 2015). Some scholars have speculated that IL-37 may affect autophagy via the mTOR/ULK1 pathway (Wu et al., 2016). However, the specific association between IL-37 and autophagy and the mechanism underlying this effect are poorly understood.

In the present study, we first confirmed that IL-37 is indeed able to inhibit HCC cell proliferation. As autophagy is involved in cell growth processes, we hypothesized that the effects of IL-37 on proliferation may be due to changes in autophagy. To test this hypothesis, we first performed TEM to show that IL-37 positively regulates autophagy, as revealed by the autophagosomes formed inside cells, increased LC3-II and Beclin1 protein levels, and decreased expression of p62 protein in HCC cells. We chose 100 ng/ml as the optimal concentration of IL-37. The autophagy induced by 100 ng/ml IL-37 was more prominent than that induced by 200 ng/ml IL-37, possibly because of the short half-life of autophagosomes, with a parallel acceleration of degradation. High concentrations of IL-37 may accelerate the formation of autophagosomes, with a parallel acceleration of degradation, and the autophagy induced by 200 ng/ml IL-37 may have represented a partial degradation. We then confirmed the effect of IL-37 on autophagy through treatment with the autophagy inhibitors 3-MA and CQ.

The connection between autophagy and apoptosis is complex, but the interaction between the BH3 domain of Beclin1 and Bcl-2 has been shown to play an important role (Maiuri et al., 2007). As the expression of Beclin1 was increased following treatment with IL-37, we considered whether IL-37 is involved in apoptosis. Indeed, our data indicate that IL-37 does induce early (Annexin V+/PI-) and late (Annexin V+/PI+) apoptosis.
apoptosis in HCC cells. Consistent with this finding, the expression of Bax and cleaved caspase-3 was also increased, whereas that of Bcl-2 was decreased. Thus, we suspect that IL-37 induces autophagy and apoptosis simultaneously in HCC cells.

Autophagy is triggered by numerous complicated signaling cascades, including the PI3K/AKT/mTOR and the AMPK pathways (Huo et al., 2016; Nunes et al., 2016). In the present study, we showed that levels of p-AKT, p-mTOR, p70S6K, 4E-BP1, and p62 were decreased, whereas the LC3-II/LC3-I ratio was significantly increased following IL-37 treatment, indicating that the autophagy triggered by IL-37 is mTOR-dependent. Our data also demonstrate that treatment with IGF-1, a stimulator of the PI3K/AKT/mTOR signaling pathway, reverses the IL-37-mediated changes in p-AKT expression, whereas the expression of the respective proteins in the LY294002-treated cells was similar to that in IL-37-treated cells. This indicates that the effects of IL-37 are indeed mediated via this signaling cascade.

Our findings suggest that IL-37 likely induces cell autophagy and apoptosis, resulting in the death of HCC cells, while also inhibiting cell proliferation. This is the first report that highlights this correlation between IL-37 and autophagy, and further study of IL-37 as a treatment for HCC is warranted. Moreover, although the proapoptotic effects of IL-37 may be largely dependent on autophagy, these interactions are...
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Relative expression of p-AKT

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p-AKT

AKT

p-mTOR

mTOR

LC3B-I

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SMMC-7721

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Relative expression of proteins

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p-AKT

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p-AKT

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LC3B-I

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β-actin

SMMC-7721

Huh-7

Relative expression of proteins

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Fig. 5. Activation of AKT signaling reversed IL-37-induced autophagy and inhibition of AKT signaling mimicked the effect of IL-37-induced autophagy. (A) Western blot analysis of AKT expression in SMMC-7721 and Huh-7 cells treated with or without IGF-1 at the specified concentrations for 1 or 30 min. The p-AKT/AKT, p-PI3K/PI3K, and p-PI3K-Akt/PI3K-Akt ratios were calculated. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group. **p < 0.05, ***p < 0.001 compared with the IL-37-treated group. (B) Western blot analysis of AKT, mTOR, LC3, and p62 in SMMC-7721 cells treated with or without IGF (100 ng/ml) or IL-37 (100 ng/ml). The p-AKT/AKT, p-PI3K/PI3K, LC3B-II/LC3B-I, and p62–actin ratios were calculated. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the control group. All data are expressed as the mean ± SD (n = 3).

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