Autophagy promotes hepatocellular carcinoma cell invasion through activation of epithelial–mesenchymal transition

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Invasion of hepatocellular carcinoma (HCC) cells is a leading cause of intrahepatic dissemination and metastasis. Autophagy is considered to be an important mediator in the invasion of cancer cells. However, the precise contribution of autophagy to cancer cell invasion and underlying mechanisms remain unclear. Autophagy was induced in HepG2 and BEL7402 cells by starvation in Hank’s balanced salt solution. Induction of autophagy inhibited the expression of epithelial markers and induced expression of mesenchymal markers as well as matrix metalloproteinase-9 stimulating cell invasion. Starvation-induced autophagy promoted the expression of epithelial–mesenchymal transition (EMT) markers and invasion in HepG2 and BEL7402 cells through a transforming growth factor-beta (TGF-β)/Smad3 signaling-dependent manner. The small interfering RNAs (siRNAs) for Atg3 or Atg7 and chloroquine inhibited autophagy of HepG2 and BEL7402 cells during starvation, resulting in suppression of EMT and diminished invasiveness of HCC cells. Administration of SIS3 also attenuated EMT and invasion of HepG2 and BEL7402 cells during starvation. Recombinant TGF-β1 was capable of rescuing EMT and invasion that was inhibited by siRNA for Atg3 and 7 in HepG2 and BEL7402 cells under starvation. These findings suggest that autophagy is critical for the invasion of HCC cells through the induction of EMT and that activation of TGF-β/Smad3-dependent signaling plays a key role in regulating autophagy-induced EMT. Inhibition of autophagy may represent a novel target for therapeutic interventions.

Introduction

Hepatocellular carcinoma (HCC) is the most frequent primary cancer of the liver and the fifth most common solid tumor and the third leading cause of cancer-related deaths worldwide (1). The prognosis for patients with HCC has improved through the use of surgical resection, chemotherapy and interventional therapy. However, metastasis resulting from the invasion of HCC significantly reduces the efficacy of these therapeutic interventions for a considerable number of patients (2,3).

Autophagy is an evolutionarily conserved physiological process in cells (4) that generates intracellular nutrients, growth factors and energy to support cell survival and cellular activities during stressors, such as starvation, hypoxia and growth factor withdrawal (4–6). Invasion and metastasis are common characteristics of cancer cells. In situ, cancer cells in solid tumors are exposed to starvation and/or hypoxia, which is due to deficient angiogenesis during tumor growth (7,8). It has been observed that autophagy accelerates invasion of cancer cells during starvation or hypoxia (9,10). However, the exact contribution of autophagy into the promotion of cancer cell invasion during starvation has not been thoroughly investigated.

Epithelial–mesenchymal transition (EMT) occurs during the progression of epithelial carcinogenesis (11,12). During EMT, epithelial cells change phenotype from epithelial to mesenchymal, which is a process that includes expression of mesenchymal markers, actin cytoskeleton reorganization, loss of cell-to-cell junctions and pseudopod formation (13). EMT increases the migratory capacity of epithelial cells (13). In addition, EMT has been shown to play a key role in the induction of cancer cell invasion and metastasis (14,15).

Transforming growth factor-beta (TGF-β)/Smad3 signaling is a key regulator of EMT in many epithelial cell types. Activation of TGF-β/Smad3 signaling in epithelial cells triggers alterations in morphological and functional phenotypes from epithelial to mesenchymal (16,17). Activation of TGF-β/Smad3 signaling is involved in the metastasis of HCC (18). However, the role of TGF-β/Smad3 signaling in the regulation of EMT and invasion of HCC has not been evaluated during autophagy induced by starvation. Therefore, the regulation of EMT and cell invasion by autophagy was investigated in two HCC cell lines during starvation.

Materials and methods

Cell culture and experimental design

The human HCC cell line, HepG2, was purchased from ATCC (Manassas, VA). The HCC cell line, BEL7402, was obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone, Boston, MA) and 100 μg/ml each of penicillin and streptomycin (Gibco, Invitrogen, Carlsbad, CA) in 5% CO2 at 37°C. Autophagic activity was evaluated after 6 h of starvation performed in Hank’s balanced salt solution (HBSS, HyClone). For the LC3 turnover assay, cell lines were cultured in complete medium and HBSS both with chloroquine (5 μM, Sigma–Aldrich, St Louis, MO) for 6 h. HCC cell lines were also pretreated with chloroquine (5 μM) for 2 h to detect the effect of autophagy on EMT and invasion of HCC cells. The TGF-β/Smad3 signaling inhibitor, SIS3 (2 μmol/L, Sigma–Aldrich), was used to pretreat each cell line in complete medium and HBSS for 30 min in order to assess the role of TGF-β/Smad3 signaling in EMT and invasion of HCC cells. In addition, HCC cells transfected with small interfering RNA (siRNA)-Atgs (3 or 7) were treated with recombinant human TGF-β1 (20 ng/ml, R&D System, Minneapolis, MN) in HBSS for 6 h to evaluate the necessity of TGF-β1 in autophagy-induced EMT and invasion. Cells that were cultured in complete medium served as control.

siRNA synthesis and transfection

The cDNA sequence of the Atg3 and Atg7 gene was obtained from Genebank (NM_022488 and NM_006395) and the respective targeting sequences of three different siRNAs were designed using RNAi algorithm available online (http://www.ambion.com/techlib/misc/siRNA_finder.html). All siRNAs were synthesized and purified by GenePharma (Shanghai, China). Synthesized siRNAs were transfected into HepG2 and BEL7402 cells by TransLipid Transfection Reagent (Beijing, China) according to the manufacturer’s instruction. The siRNA (sense: 5′-GGGAAACGGACUUGGAGUG-3′, antisense: 5′-UCUUCCUGGUCUUCCUCC-3′) with the greatest silencing effect of Atg3 and the siRNA (sense: 5′-ACUAAGGGGCGAAUCGAG-3′, antisense: 5′-GCAUUUGGCCCCUAAUGAG-3′) with the greatest silencing effect of Atg7 were identified by real-time PCR and were transfected into HepG2 and BEL7402 cells for further studies. The siRNA (sense: 5′-UCAGACAUUGGCAUCGUCGU-3′, antisense: 5′-AGCUUAGGAAAAGGAAUA-3′) was served as the siRNA-vector control. After 48 h of transfection, cells were used for experiments. Cell viability was evaluated by trypan blue.

Cell apoptosis assay

Apoptosis of HCC cell lines was quantitated by flow cytometry. Cells pre-treated with chloroquine as well as transfected and non-transfected cells in
complete medium and HBSS for 6 h were harvested, washed and incubated with binding buffer containing propidium iodide (10 μg/ml) and fluorescein isothiocyanate-labeled annexin V (Annexin V-FITC) (Bender MedSystems, eBioscience, San Diego, CA) for 15 min at room temperature. Samples were analyzed by flow cytometry (BD LSR II, BD Biosciences, San Jose, CA).

Immunofluorescence
Immunofluorescence was performed on cells pretreated with chloroquine and on transfected and non-transfected cells in complete medium and HBSS for 6 h by using an avidin–biotin peroxidase complex method. Immunofluorescence for cells that were treated with SIS3 (2 μmol/l) or recombinant human TGF-β1 (20 ng/ml) was utilized to evaluate the role of TGF-β1/smad3 signaling on EMT and matrix metalloproteinase 9 (MMP-9) expression levels during starvation. Briefly, cells were fixed in 4% paraformaldehyde and permeabilized using Triton X-100. Cells were then treated with 3% hydrogen peroxide to inactivate endogenous peroxidase. Non-specific binding was blocked in phosphate-buffered saline containing 10% species-appropriate normal serum for 1 h at room temperature. Primary LC3 antibody (1:200, Cell Signaling Technology, Beverly, MA), E-cadherin antibody (1:100, Abcam, Cambridge, MA), cytokeratin 18 (CK18) antibody (1:100, Boster, Wuhan, China), fibronectin antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and MMP-9 antibody (1:200, Abcam) were applied overnight at 4°C in a humidified chamber. Cells were incubated with the appropriate secondary antibody and visualized by peroxidase substrate in conjunction with fluorescein isothiocyanate or Cy3 (Boster). The number of LC3 puncta per high power field was counted by an independent pathologist in a blinded fashion.

Western blotting
Western blotting was used to detect the expression of LC3, p62, Atg3, Atg7, E-cadherin, CK18, fibronectin, MMP-9, TGF-β1, Smad3 or phosphorylated Smad3 in transfected and non-transfected HCC cell lines with or without starvation. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail (Roche, Branford, CT) and phosphatase inhibitor cocktail (Cell Signaling Technology). Total protein (30 μg) from each sample was electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. After being transferred to nitrocellulose membranes (Pierce, Thermo Fisher Scientific, Waltham, MA), protein samples were incubated with the corresponding primary antibodies. Blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies and the membranes were developed with SuperSignal™ chemiluminescence reagent (Pierce, Thermo Fisher Scientific) according to the manufacturer’s protocol. Protein expression levels were normalized against β-actin. Optical density of the bands was quantified using NIH Image J (Supplementary Figures III–VI, available at Carcinogenesis Online).

Quantitative reverse transcription–polymerase chain reaction
After 2 h of pretreatment with chloroquine and 48 h of transfection with siRNA-control vector or siRNA-Atgs (3 or 7), cells were cultured in complete medium and HBSS for 6 h. RNA was then isolated by TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA was reverse transcribed into first-strand cDNA using an iScript cDNA Synthesis kit (Bio-Rad, München, Germany). RNA expression was analyzed by reverse transcription–polymerase chain reaction using qSYBR Green Supermix in an iCycler Real-Time PCR Detection System (Bio-Rad). The following primer sequences were used—Atg3 sense: 5′-GGAAGAGATGAAGAAGAAA-3′, antisense: 5′-CATATACTTGGAAGTG-3′; Atg7 sense: 5′-CAGACATGTTGAGGACTTGA-3′; E-cadherin (19 sense): 5′-ACACCCCTGTGGTCTTCTTT-3′, antisense: 5′-TGTAATGTGGC-AATGCTTCTTCTC-3′; CK18 (20 sense): 5′-CAAAGCGTGTAGCTTGCTCTT-3′, antisense: 5′-GGATCCGGCAAGGGAAG-3′; fibronectin (21 sense): 5′-GGACGACTGACAGGATGATA-3′, antisense: 5′-GATGCTTTGGAAC-3′; MMP-9 (22 sense): 5′-GCAACCTGGACTCCTTCTTT-3′, antisense: 5′-TGCCCACGGAAAGAAG-3′. Expression was normalized to β-actin.

Invasion assay
Cell invasion was analyzed using Matrigel-coated invasion chambers containing 8 μm pore filters (BD Biosciences). Briefly, after transfection with siRNA-control vector or siRNA-Atgs or pretreatment with chloroquine, each cell line was seeded at a density of 1 × 10⁴ cells/well in the upper Matrigel-coated chamber of 24-well plates. HBSS was placed in the upper chamber. SIS3 (2 μmol/l) or recombinant human TGF-β1 (20 ng/ml) added to the upper chamber was utilized to detect the influence of TGF-β1/smad3 signaling on invasion. Complete medium was placed in the lower chamber as a chemotactic agent. Cells that were seeded in the upper chamber with complete medium were served as control. Invasion assay systems were incubated at 37°C with 5% CO₂ for 6 h. Cells that invaded the Matrigel to the bottom of filter were stained with 2 μg/ml 4,6-diamidino-2-phenylindole in phosphate-buffered saline and counted under a fluorescent microscope.

Statistical analysis
All data were presented as mean ± SEM. After demonstration of homogeneity of variance with Bartlett test, one-way analysis of variance, followed by Student–Newman–Keuls test where appropriate, was used to evaluate the statistical significance. Values of P < 0.05 were considered statistically significant. Experiments were performed in triplicate.

Results

Autophagy promotes invasion of HCC cells
Starvation or nutritional deficiency is a common factor that upregulates cell autophagy (4,5). HepG2 and BEL7402 cells were starved in HBSS, which contained neither nutrients nor serum for 6 h. The LC3 puncta formation assay revealed that LC3 puncta significantly increased (93 ± 5, P < 0.05; 83 ± 4, P < 0.05) in cells under starvation. Increased conversion of LC3-I to LC3-II revealed by LC3 turnover assay and decreased expression of P62 confirmed that starvation induced autophagy in HCC cells (Supplementary Figure I, available at Carcinogenesis Online).

The impact of starvation on HCC cell invasion was evaluated. As depicted in Figure 1A, HepG2 and BEL7402 cells, which were cultured with HBSS in the upper chambers, exhibited greater invasion rates than those cultured with complete medium (60 875 ± 1051 versus 15 813 ± 515 for HepG2 cells, P < 0.05; 60 500 ± 979 versus 15 500 ± 645 for BEL7402 cells, P < 0.05). Since starvation-induced autophagy in HCC cells, the effect of autophagy on cell invasion was further evaluated. The autophagic activity in HepG2 and BEL7402 cells was inhibited by transfection of siRNA-Atg3 and siRNA-Atg7. Silencing of Atg3 or Atg7 has been reported to effectively inhibit autophagy in other cell types (23,24). Silencing of Atg3 and/or Atg7 by siRNA was confirmed at both the RNA and protein level (Figure 1B). Lack of LC3-I to LC3-II conversion and decreased P62 expression levels confirmed inhibition of autophagy in HCC cells transfected with siRNA-Atg3 or siRNA-Atg7 during starvation (Figure 1C). Inhibition of autophagy by siRNA-Atg3 or siRNA-Atg7 significantly reduced the invasiveness of HCC during 6 h of incubation in HBSS compared with control complete medium. In addition, the pharmacological inhibitor of autophagy, chloroquine, also attenuated the invasion activity of HepG2 and BEL7402 cells under starvation (Figure 2A). However, neither inhibition of autophagy by siRNAs nor by chloroquine exhibited obvious alterations in the apoptosis rate compared with control (Figure 2B). These findings suggest that HCC cell invasion was dependent on autophagy under invasive starvation.

Autophagy promotes invasion of HCC cells by inducing EMT
EMT is reported to contribute to metastatic and invasive mechanisms of cancer cell metastasis (14,15). To explore the mechanism through which autophagy conferred invasion ability on HCC cells, the expression of epithelial and mesenchymal markers as well as MMP-9 was evaluated in HepG2 and BEL7402 cells with or without autophagy inhibition during starvation by western blotting and immunofluorescence. The epithelial markers, E-cadherin and CK18, were significantly expressed, whereas the mesenchymal marker, fibronectin, and invasion associated protein, MMP-9, were not highly expressed by HepG2 and BEL7402 cells in complete medium. During starvation, the expression of E-cadherin and CK18 was downregulated, whereas that of fibronectin and MMP-9 was upregulated in non-infected cells and cells with control siRNA-vector (Figures 3 and 4). In addition, cell morphology changed from circular-like to fibroblast (Figure 4). However, these morphological alterations and changes in the expression of epithelial and mesenchymal markers as well as MMP-9 did not occur in autophagy-deficient cells with siRNA-Atgs (3 or 7) or chloroquine during starvation compared with control (Figures 3 and 4).

TGF-β1/Smad3 signaling regulates autophagy-induced EMT
TGF-β1/Smad3 is one of the known signaling pathways that mediate EMT (16,17). We found that TGF-β1 and phosphorylated Smad3
Autophagy-dependent EMT

were not highly expressed by HepG2 and BEL7402 cells in control medium compared with starvation. However, TGF-β1 and phosphorylated Smad3 were expressed in non-transfected cells and cells transfected with control siRNA-vector during starvation. Inhibition of autophagy by siRNA-Atgs (3 or 7) or by chloroquine in HCC cells suppressed both phosphorylation of Smad3 and expression of TGF-β1 in HBSS during starvation indicating that expression of TGF-β1 and activation of TGF-β/Smad3 signaling occurred in an autophagy-dependent manner during starvation (Figure 5A).

Since inhibition of autophagy suppressed EMT and TGF-β/Smad3, we further investigated whether blockade of this signaling was capable of suppressing EMT of HCC cells with autophagy during starvation. Administration of the inhibitor of TGF-β/Smad3 signaling, SIS3 (2 μmol/l), inhibited the autophagy-induced phosphorylation of Smad3 in HepG2 and BEL7402 cells during starvation compared with control medium (Figure 5B). Furthermore, in contrast to control medium, inhibition of TGF-β/Smad3 signaling by SIS3 suppressed autophagy-dependent morphological and marker expression changes in HCC cells under starvation (Figure 5 and C). As expected, SIS3 also decreased invasive number of HepG2 and BEL7402 cells during starvation when EMT was inhibited by inactivity of TGF-β/Smad3 signaling (Figure 5D).

TGF-β1 rescues EMT and invasion in autophagy-deficient HCC cells

Since autophagy-activated TGF-β/Smad3 signaling was associated with EMT in HCC cells under starvation, we further tested whether TGF-β1 was able to rescue EMT in autophagy-deficient HCC cells under starvation. As depicted in Figure 6 and B, HepG2 and BEL7402 cells transfected with siRNA-Atgs (3 or 7) exhibited changes in the expression of EMT markers and MMP-9 under starvation compared with control medium. In contrast to control medium, administration of the exogenous TGF-β1 (20 ng/ml) downregulated expression of epithelial markers and upregulated expression of fibronectin and MMP-9 in autophagy-deficient HCC cells under starvation. Since autophagy-dependent EMT was required for invasion of HCC cells during starvation, we found that exogenous TGF-β1 was also capable of promoting invasion of HepG2 and BEL7402 cells with siRNA-Atgs.

Fig. 1. siRNA-Atgs (3 or 7) inhibit autophagy of HCC during starvation. siRNA-Atg3 and siRNA-Atg7 inhibited Atg3 and Atg7 expression and LC3 conversion as well as abrogated a decrease in P62 expression in HepG2 and BEL7402 cells under starvation. (A) Expression of mRNA for Atg3 and Atg7 was determined by quantitative reverse transcription–polymerase chain reaction. mRNA levels were normalized to β-actin. (B) Representative western blots of Atg3 and Atg7. (C) Representative western blots of P62 and LC3 conversion assay. Cells cultured in complete medium without transfection were served as control. Data are representative of three independent experiments and shown as mean ± SEM, n = 4, *P < 0.05 versus control.
J.Li et al. (3 or 7) under starvation. These results suggest that autophagy-induced TGF-β1 signaling plays a crucial role in EMT and invasion of HCC cells under starvation (Figure 6C).

Discussion

Due to metastasis that results from invasion of HCC cells, it is difficult to cure many HCC patients by current therapeutic interventions (2,3). Several publications have described a critical role for autophagy in the invasion of central neuronal system tumor cells (10). However, relatively little is known regarding the precise contribution of autophagy to the pathogenesis of HCC cell invasion. Here, we provide the first evidence that autophagy plays a central role in the invasion of HCC cells by inducing EMT under starvation. Autophagy promotes invasion of HepG2 and BEL7402 cells via inducing EMT of HCC cells under starvation. Furthermore, autophagy induces expression of TGF-β1 and activates TGF-β/Smad3 signaling during starvation. Inhibition of autophagy by siRNA-Atgs (3 or 7) or pharmacological inhibition results in inactivation of TGF-β/Smad3 signaling and the prevention of EMT and invasion of HCC cells under starvation. On the other hand, inhibition of TGF-β/Smad3 signaling pathway by SIS3 suppresses both EMT and invasion of HCC cells in the presence of autophagy during starvation. Finally, administration of exogenous recombinant TGF-β1 was sufficient to rescue the EMT and invasion of autophagy-deficient HCC cells under starvation. These findings suggest that invasion of HCC cells promoted by autophagy under starvation is dependent on TGF-β/Smad3 signaling and EMT.

Autophagy occurs in variety of malignant tumor cells, including HCC cells (25), glioma cells (10) and glioblastoma stem cells (26). Under these pathological conditions, autophagy can be triggered by various stimuli, such as starvation, hypoxia and growth factor.

Fig. 2. Inhibition of autophagy prevented invasion of HCC cells. Absence of autophagy in cells transfected with siRNA-Atg3 or siRNA-Atg7 or pretreatment of chloroquine had no notable effect on cellular activity and apoptosis rate after 6h of starvation in complete medium. However, there was a significant reduction of the invasiveness of HCC cells during starvation. (A) Invasive number of HepG2 and BEL7402 cells with or without siRNA-Atgs (3 or 7) and chloroquine. (B) Representative flow cytometry of HepG2 and BEL7402 cells with or without siRNA-Atgs (3 or 7) and chloroquine. Cells cultured in complete medium were served as control. Data are representative of three independent experiments and shown as mean ± SEM, n = 4, *P < 0.05 versus control.

(3 or 7) under starvation. These results suggest that autophagy-induced TGF-β1 signaling plays a crucial role in EMT and invasion of HCC cells under starvation (Figure 6C).
Autophagy-dependent EMT withdrawal (4–6). As the blood supply enriched solid tumor, starvation and/or hypoxia that results from deficient angiogenesis or usage of chemotherapeutics appears to be responsible for autophagy in HCC (7,8). Autophagy is a genetically regulated and finely orchestrated process of selective cell survival and cell apoptosis. Both starvation and hypoxia have been shown to be crucial initiators of this process (4). In this study, HCC cell autophagy was successfully achieved by starvation in HBSS (Supplementary Figure I, available at Carcinogenesis Online). In contrast, silencing of Atg3 or Atg7 or pretreatment with chloroquine increases resistance of HCC cells to starvation-induced autophagy.

Autophagy has been implicated in the pathogenesis of cancer cell invasion. In glioblastoma stem cells, autophagy accelerates their

Fig. 3. Autophagy-induced EMT in HCC cells during starvation. After transfection of siRNA-Atg3, siRNA-Atg7 or pretreatment of chloroquine, HepG2 and BEL7402 cells were starved in HBSS for 6h. Inhibition of autophagy by siRNA-Atgs or chloroquine prohibited EMT of hepatocarcinoma cells under starvation. (A) Representative western blots of epithelial markers, E-cadherin and CK18, as well as MMP-9 and mesenchymal marker, fibronectin, in these cells. (B) Expression of mRNA for E-cadherin and CK18 as well as MMP-9 and fibronectin was determined by quantitative reverse transcription–polymerase chain reaction. mRNA levels were normalized to β-actin. Cells cultured in complete medium were served as control. Data are representative of three independent experiments and shown as mean ± SEM, n = 4, *P < 0.05 versus control.
invasion by regulating DRAM1 and p62 (26). Inhibition of autophagy impairs invasion of glioma cells and hypoxia triggers autophagy to regulate cancer cell invasion (9,10). However, the direct role of autophagy in the invasion of HCC cells is unclear. In this study, inhibition of HCC cellular autophagy was achieved by silence of Atg3 or Atg7 and chloroquine. This experimental model makes it possible to define the direct role of autophagy in invasion of HCC cells. In this model, silencing of Atgs (3 or 7) or chloroquine was sufficient to inhibit autophagy of HCC cells, leading to reduced invasiveness during starvation. These findings suggest that autophagy plays an active role in the initiation of HCC cell invasion. Accordingly, inhibition of autophagy in HCC cells represents a potential target for therapeutic intervention to prevent their invasion.

Autophagy from hypoxia has been documented to mediate the invasion of several cell lines by hypoxia-inducible factor-1α (9,27). However, in this study, the role of hypoxia in stimulating autophagy was performed in identical volumes of complete medium and HBSS (4 ml in a T25 flask). Hypoxia-inducible factor-1α was expressed at similar low levels in HCC cells in both complete medium and starved culture system (Supplementary Figure II, available at Carcinogenesis Online), indicating that the other mediators of autophagy-induced invasion exist in HCC cells under starvation.

Furthermore, the epithelial markers, E-cadherin and CK18, were downregulated, whereas fibronectin and MMP-9 were upregulated in HepG2 and BEL7402 cells during starvation-induced invasion. Inhibition of autophagy by siRNA-Atgs (3 or 7) or by pharmacological inhibitor abrogated the alterations in EMT marker expression, suggesting that EMT occurred in HCC cells during starvation-induced autophagy.

EMT has been documented to be a mechanism that induces metastasis of cancer cells (14,15). Dedifferentiation of HCC by EMT may represent reactivation of the embryonic development program (28). EMT triggers epithelial cells to express mesenchymal markers and downregulate epithelial marker expression (17). E-cadherin is the epithelial molecular marker, as it is responsible for establishment of the adherens junction, which forms a continuous adhesive belt below the apical surface (29). Its extracellular domain mediates interactions with E-cadherin molecules on adjacent cells to form intercellular junctions (29,30). Loss of E-cadherin by EMT results in detachment of intercellular junctions and decreases adhesive force (29). At the same time, downregulation of other junctional adhesion molecules by EMT leads to dissociation of cellular interconnected strands and matrix barriers as well as loss of apical-basolateral polarity (31,32). On the other hand, expression of MMP-9 and the intermediate filament...
Fig. 5. Autophagy-activated TGF-β1/Smad3 signaling promotes EMT and invasion of HCC cells during starvation. Starvation induces expression of TGF-β1 and phosphorylation of Smad3. Inhibition of autophagy by siRNA-Atg3, siRNA-Atg7 or pretreatment of chloroquine downregulated TGF-β1 expression and inactivated TGF-β1/Smad3 signaling in HCC cells during starvation. (A) Representative western blots of TGF-β1, phosphorylated Smad3 and Smad3 in HepG2 and BEL7402 cells with or without siRNA-Atgs (3 or 7) and chloroquine. Inactivation of TGF-β1/Smad3 signaling by SIS3 (2 μmol/l) notably inhibited EMT and invasion of hepatocarcinoma cells under starvation. (B) Representative western blots of E-cadherin, CK18, fibronectin and MMP-9 in HepG2 and BEL7402 cells with or without SIS3. (C) Representative immunofluorescent staining for E-cadherin, CK18, fibronectin and MMP-9 in HepG2 and BEL7402 cells with or without SIS3. (D) Invasive number of HepG2 and BEL7402 cells with or without SIS3. Cells cultured in complete medium served as a control. All data are representative of three independent experiments and shown as mean ± SEM, n = 4, *P < 0.05 versus control.
protein, fibronectin, contributes to the degradation of basement membrane, pseudopod formation and cytoskeleton remodeling (33,34). All of these phenotypic changes arising from EMT facilitate cancer cell invasion. Therefore, loss of E-cadherin, expression of fibronectin and MMP-9 as well as pseudopod formation stimulated starvation-induced EMT may be prerequisites for invasion of HCC cells. Prevention of EMT by inhibition of autophagy through siRNA-Atg3 or chloroquine was able to abolish starvation-promoted invasion of HCC cells. Although a recently published model shows that metastatic tumors are composed of non-EMT cancer cells (35), according to the cell cooperativity model of Takanori et al., EMT cancer cells with enhanced invasive phenotypes digest surrounding matrix and penetrating adjacent connective tissues. Non-EMT cells then migrate together with EMT cells during metastasis (35). Hence, these data suggest that autophagy-activated EMT mediates the starvation-promoted invasion of HCC cells.

In addition to induction of EMT of HCC cells during starvation, expression of TGF-β1 was elevated and TGF-β1/Smad signaling was activated by autophagy. TGF-β1/Smad signaling is activated through phosphorylation of TGF-β receptor complex (19). Another important finding of this study is that TGF-β1/Smad signaling is pivotal for EMT and invasion of HCC cells. This is based on the observation that inhibition of TGF-β1/Smad signaling pathway by SIS3 suppresses both EMT and invasion of HCC cells during starvation-induced autophagy. However, administration of exogenous recombinant TGF-β1 rescues the EMT and invasion of autophagy-deficient HCC cells under starvation. Consequently, our results suggest that autophagy modulates the EMT-induced invasiveness of HCC via the TGF-β1/Smad signaling axis during starvation.

In support of our findings, indicating that autophagy plays a key role in the progression of HCC, other studies have reported that autophagy protects HCC through a beclin-1-dependent mechanism (36). In addition, HCC and colorectal carcinoma patients also showed a positive correlation to the expression of the autophagic marker, beclin-1 (37).

In summary, the findings reported here indicate that starvation-induced autophagy plays a crucial role in the invasion of HCC cells through activation of EMT, which involves the activation of TGF-β signaling. The targeted manipulation of starvation-induced autophagy and cell signaling for EMT might prevent HCC cell invasion.

Supplementary material

Supplementary Figures I–VI can be found at http://carcin.oxfordjournals.org/.

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