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Original Article

Role of autophagy in the chemopreventive effect of the IFC-305 compound in the sequential model of cirrhosis-hepatocellular carcinoma in the rat and *in vitro*

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Abstract: Hepatocellular carcinoma (HCC) can be originated from various etiologies and is preceded mostly by cirrhosis. Unfortunately, there is no effective treatment due to its late prognosis. Alterations in autophagy have been reported during the development and progression of HCC. Autophagy allows for the maintenance of a positive energy balance and the proper functioning of organelles through the selective degradation of cellular components. It has been demonstrated that autophagy suppresses spontaneous tumorigenesis in the liver. Therefore, autophagy has become a therapeutic target for effective HCC therapies. We have previously demonstrated that the adenosine-derived compound, IFC-305, has a chemopreventive effect on HCC, in addition to maintaining mitochondrial function in a sequential model of cirrhosis-HCC. Thus, the aim of this work was to determine if IFC-305 has an effect on autophagy in the sequential model of cirrhosis-HCC induced by diethylnitrosamine or *in vitro* in the HCC cell line HepG2 and mouse embryonic fibroblasts. The results of this work showed that IFC-305 modifies the levels of the BECN1, p62/SQSTM1 and LC3-II proteins that play an important role in the autophagic process. In vivo, IFC-305 regulates the levels of the PINK1 and PARKIN proteins that specifically mark mitochondria for repair or degradation. In the HepG2 cell line, its effect was accompanied by a decrease in cell viability. Interestingly, in nontumoral cells the time to autophagy induction was different compared to the HepG2 cells. This study suggests that autophagy induction may be part of the mechanism by which IFC-305 maintains mitochondrial function, thereby facilitating the prevention and reversal of HCC.

Keywords: Hepatocellular carcinoma, autophagy, IFC-305, mitochondria

Introduction

Hepatocellular carcinoma is a complex pathology that develops mainly from cirrhosis of a different etiology; ethanol consumption, hepatitis B or C virus infection (HBV or HCV, respectively), metabolic syndrome and obesity are all considered factors associated with an increased incidence of HCC [1]. The main problem with HCC is that it causes a large number of deaths mainly because there is no early detection and current therapies are not very effective [1].

Autophagy is a cellular process that helps to maintain a positive energy balance through the selective degradation of intracellular components and the renewal of cellular organelles, such as damaged mitochondria [2, 3]. Different types of cellular stress activate autophagy, such as DNA damage, mitochondrial damage, hypoxia, and nutrient deprivation, among others. In addition to cytoprotection, several functions have been related to autophagy, such as immune responses [4, 5], genome stability and tumor suppression through the induction of cell death among precancerous cells with chromosomal instability, thus preventing their proliferation [6]. Accordingly, autophagy is deregulated in various diseases, including cancer, and its modulation is a therapeutic approach.

Three types of autophagy occur in the liver: 1) Endosomal microautophagy, where the late endosomal membrane forms small invagina-

tions that later detach from the membrane and remain inside the endosome for degradation, 2) Chaperone-mediated autophagy, where cytosolic proteins are incorporated into the lysosome through a receptor called the lysosome-associated membrane protein type 2A, and 3) Macroautophagy, in which an isolation membrane forms a cup shaped structure known as the phagophore that elongates around the cytoplasmic cargo and eventually seals, giving rise to a double-membrane vesicle called the autophagosome. The autophagosome is then delivered by microtubules to lysosomes, the outer membrane of the autophagosome fuses with the lysosomal membrane to form an autolysosome, and the engulfed cargo is degraded by lysosomal hydrolases [2]. Macroautophagy is the focus of this work and will be referred to herein as autophagy.

The autophagic process is controlled by proteins known as ATGs that group into functional complexes and are encoded by autophagy related genes (*Atg*). Upon autophagy induction, the ULK1 complex is recruited to trigger the nucleation of the phagophore by phosphorylating components of the class III PI3K complex 1, which contains BECN1 (BECLIN-1) and PI3KC3 (VPS34), among other proteins. In turn, PI3KC3 produces phosphatidylinositol-3-phosphate (PI3P) that recruits the WIPI2 effector protein. WIPI2 binds to ATG16L1, recruiting the ATG5-ATG12/ATG16L complex that enhances the conjugation of the LC3-I protein to membrane-resident phosphatidylethanolamine (PE), thus forming the membrane-bound, lipidated form, LC3-II, which is required for phagophore elongation and the sealing of the autophagosome. LC3-II also attracts components of the autophagic machinery that function as cargo receptors because they contain both an LC3-interacting motif and a specific binding motif for a label in the cargo, such as ubiquitin in the case of p62/SQSTM1. Since cargo receptors are degraded together with the engulfed cytoplasmic material, the abundance of p62/SQSTM1 is indicative of a functional autophagic flux [7, 8].

The accumulation of p62/SQSTM1 during carcinogenesis has been related to the chronic activation of NRF-2 through a direct interaction with Keap1, the NRF-2 inhibitor protein [9]. NRF-2 is a transcription factor that is responsible for regulating the antioxidant response through inducible antioxidant elements in response to oxidative damage. These elements are direct or indirect free radical scavengers. The activity of NRF-2 is constitutively repressed due to its interaction with the KEAP1 cytoplasmic protein because this interaction induces the degradation of NRF-2. However, NRF-2 overexpression has been associated with different chronic inflammatory diseases, as well as the development and progression of cancer [10].

Dysregulated autophagy has been described in various metabolic disorders such as fatty liver and obesity [11]. In addition, deletions have been reported in several *Atg* genes in HCC. It has been suggested that autophagy has an important role in the suppression of spontaneous tumorigenesis in the liver because multiple tumors are induced by the deletion of the *Atg5* and *Atg7* genes [12]. Interestingly, lower expression of *Atg5*, *Atg7* and *Becn1* was observed in malignant HCC cells and in particular, the expression of *Becn1* in 44 human HCC tissue samples was decreased compared to adjacent nontumor tissue. In HCC tissue from recurrent patients, lower *Becn1* expression correlated with malignancy and poor prognosis [13]. Moreover, the promotion of inflammatory and profibrotic milieu by macrophages has been observed in autophagy-deficient Kupffer cells resulting in fibrosis and hepatocarcinogenesis [14]. Hence, dysfunctional autophagy seems to contribute to HCC pathogenesis.

The degradation of mitochondria by autophagy is known as mitophagy, which is a specialized mechanism for the elimination of malfunctioning mitochondria. Mitochondrial fission and depolarization are required for mitophagy. Fragmented mitochondria are targeted for mitophagy through labeling by the ubiquitin ligase PARKIN that is recruited by PINK1 in response to depolarization [15]. PINK1 mediates PARKIN recruitment through its association with the TOM (translocase of outer mitochondrial membrane) complex. Defective mitophagy has been found to both favor and act against tumorigenesis [16]. Takamura et al. [12] found the presence of abnormally swollen mitochondria in autophagy-deficient mice that develop liver tumors.

Our group has previously demonstrated that the compound IFC-305 has a chemopreventive effect in the sequential model of cirrhosis-HCC induced by diethylnitrosamine (DEN) in the Wistar rat; this effect is related to diminished cellular proliferation [17]. In the HCC model induced by DEN there are functional and meta-

Table 1. Design of the experimental groups

Group	Treatment	
	16 weeks	6 weeks
Control	Saline solution	
HCC	DEN+ saline solution	
HCC+IFC-305	DEN+IFC-305	
CP	DEN	Saline solution
CP+IFC-305	DEN	IFC-305

Five experimental groups (n=6) were included as indicated. HCC (hepatocellular carcinoma), CP (cancer progression). The control group received saline solution only. DEN was administered by i.p. injection at 50 mg/kg, once a week for 16 weeks and the IFC-305 compound was administered at 50 mg/kg, 3 times weekly during the DEN administration intoxication (HCC+IFC-305), or for 6 weeks after DEN administration was stopped (CP+IFC-305).

bolic alterations. Moreover, fission process is favored leading to damaged mitochondrial. Treatment with IFC-305 had a protective effect favoring the fusion process and the recovery of morphology, and thus improving mitochondrial function [18].

Considering the implications of dysfunctional autophagy in HCC, the accumulation of damaged mitochondria in HCC and the recovery of mitochondrial function by IFC-305 administration in the sequential model of cirrhosis-HCC, in this work we hypothesized that the IFC-305 compound restores autophagic function. We found that the IFC-305 compound induces autophagy both in the experimental model of cirrhosis-HCC and *in vitro*, explaining part of its chemopreventive mechanism.

Materials and methods

Chemicals

IFC-305 is the aspartate salt of adenosine prepared with adenosine free base (MP Biomedicals, LLC, Illkirch, France) and L-aspartic acid (MP Biomedicals, Inc, Eschwege, Germany) [19, 20]. Diethylnitrosamine, sucrose, EDTA, Trizma base, KCl, $MgCl_2$, luminol, and cumaric acid were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Sodium pentobarbital was purchased form PiSA (PiSA Agropecuaria, Hidalgo, México).

Animal treatment and experimental groups

Male Wistar rats (weighing 200 g) were obtained from and housed at the Animal Facility of the National Autonomous University of Mexico (UNAM), and all procedures were conducted according to our institutional guidelines for the care and use of laboratory animals. Rats (*n*=6 per group) were divided in two different groups: hepatocellular carcinoma (HCC) and cancer progression (CP). They were treated as follows: A) the HCC groups were i.p. injected with DEN (Sigma-Aldrich) at 50 mg/kg of body weight, once a week, and saline solution or IFC-305 at 50 mg/kg of body weight, 3 times weekly (HCC+IFC-305) for 16 weeks, plus 2 a week wash out period; B) the CP groups were i.p. administered DEN at 50 mg/kg of body weight, once a week for 16 weeks and then received saline solution or IFC-305 (CP+IFC-305) at 50 mg/kg of body weight, 3 times weekly for 6 weeks (**Table 1**). For euthanasia, the animals were anesthetized with sodium pentobarbital and subsequently underwent cardiac puncture, followed by removal of the liver.

Mitochondria isolation

Liver samples were homogenized (1:10 w/v) in a medium containing 250 mM sucrose, 1 mM EDTA, 10 mM Trizma base, and 0.1% BSA, at pH 7.3. The tissue homogenate was centrifuged at 755 *g* for 5 min to remove the nuclei and plasma membrane fragments. The supernatant was filtered through organza fabric and centrifuged at 8400 *g* for 10 min to obtain the mitochondrial pellet. Mitochondria were resuspended in 250 mM sucrose, 1 mM EDTA, and 10 mM Trizma base, pH 7.3.

Electron microscopy

Liver samples for electron microscopy were fixed with glutaraldehyde (6%) and stained with osmium tetroxide (1% phosphate buffered saline solution) according to Mascorro et al. [21].

Isolation and culture of mouse embryonic fibroblasts (MEFs)

According to a standard protocol [22], mouse embryonic fibroblasts from wild type CD1 or GFP-LC3 transgenic mice (C57BL/6J) [23] were isolated at E13.5. MEFs were seeded in Dulbecco's Modified Eagle Medium + GlutaMAXTM, 10% FBS and 100 U/mL Penicillin/Streptomycin. Media and supplements were purchased from GIBCO® Life TechnologiesTM, Grand Island, NY, USA.

HepG2 treatment with IFC-305 and determination of the membrane potential (ΔΨm) with JC-1 staining

HepG2 cells (American Type Culture Collection, USA) were cultured in EMEM (ATCC, USA), 10% FBS and 100 U/mL Penicillin/Streptomycin. Cells (10×10^3 cells/well) were cultured in black 96-well plates for 24 h and then IFC-305 was added at 5 mM for 1, 2, 4, 6 or 24 h. After each timepoint, cells were stained with JC-1 (Cayman Chemical #15003, USA) at 3 μM in culture medium for 20 min at 37°C and 5% CO_2. Then, cells were washed with PBS, and maintained in PBS. JC-1 is able to form aggregates within the mitochondria when their membrane potential is high or remains as monomers in cells containing mitochondria with low membrane potential. Fluorescence of J-aggregates and J-monomers was measured with excitation/emission wavelengths of 535/590 and 485/535, respectively, with a Cytation plate reader (Bio-Tek, USA). For confocal microscopy, cells were seeded in 35 mm Fluorodish plates (World Precision Instruments, Inc) (20×10^4 cells/well) and treated with IFC-305 as above and analyzed by confocal microscopy using a Leica TCS SP5 microscope. Scale bars represent 30 μm. The chloroquine treatment 25 μM for 30 min was used as an autophagy inhibitor to demonstrate the inhibition of the effect of IFC-305 on the recovery of ΔΨm.

Western blot assays

Mitochondrial and total protein extracts were used. Volumes equivalent to 50 μg of protein were electrophoresed on polyacrylamide gels (10% for BECN1 and p62/SQSTM1; 15% for LC3: 12% for PINK1 and PARKIN) and separated proteins were transferred onto PVDF (Immobilon P). Next, blots were blocked with 5% skim milk and 0.05% Tween-20 for 30 min at room temperature and independently incubated overnight at 4°C with selective antibodies (1:1000 dilution) against BECN1 and p62/SQSTM1 (Cell Signaling, cat. D40C5 and 5114S, respectively), LC3 (MBL, cat. PD014), PINK1, PARKIN, HSP60, and ACTIN (Santa Cruz Biotechnology, cat. sc-33796, cc-133167, sc-136291, and sc-47778, respectively). On the following day, the membranes were washed and then exposed to a secondary peroxidase-labeled antibody at a dilution of 1:10000 (Jackson ImmunoResearch) in the blocking solution for 1 h at room temperature. Blots were washed and the protein signal was developed using the ECL detection system. Densitometric analyses of bands were performed with Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Sulforhodamine B assay for HepG2 cells

For cytotoxicity assays, HepG2 cells were seeded into 96-well plates (10×10^4/well) 24 h before treatment. Then, HepG2 cells were cultured with 5 mM IFC-305 for 24 hours and the cell density was evaluated by the Sulforhodamine B colorimetric assay for cytotoxicity screening (SRB) [24]. Briefly, cells were fixed with 10% (wt/vol) trichloroacetic acid and stained with a 0.04% (wt/vol) SRB solution in acetic acid for 60 min. Then, the plates were rinsed with 1% (vol/vol) acetic acid to remove unbound dye. The protein-bound dye was dissolved in 10 mM Tris pH 10.5 base solution and the absorbance was measured at 510 nm using a microplate reader.

Cyto-ID staining

IFC-305 was added to MEFs at 5 mM for 1, 2, 4 or 6 h. After each timepoint, cells were stained with CYTO-ID® (Autophagy Detection Kit, Enzo Life Sciences, cat. 51031-K200, Farmingdale, NY, USA) using 1 μL CYTO-ID/mL culture medium for 20 min at 37°C and 5% CO_2. Then, the cells were washed with PBS, maintained in PBS supplemented with 10% FBS and analyzed by confocal microscopy using a Leica TCS SP5 microscope (with UV-405 and Arg-488 nm lasers). Scale bars represent 30 μm.

Statistical analysis

Data are expressed as the mean values +/- standard error of the mean (S.E.M.). Comparisons were carried out by analysis of variance, followed by Tukey's test, as appropriate, using Graph Pad Prism 5.0 (Graph Pad Software Inc, La Jolla, CA) for Windows. Differences were considered statistically significant when $P\leq 0.05$.

Results

IFC-305 induces autophagy in the HCC experimental model in vivo

To assess whether IFC-305 has any effect on autophagy in vivo, we followed the scheme shown in **Table 1**. The presence of citrate synthase and VDAC1 was determined as positive controls and β-actin as mitochondrial purity. **Figure 1G** shows the presence of citrate syn-

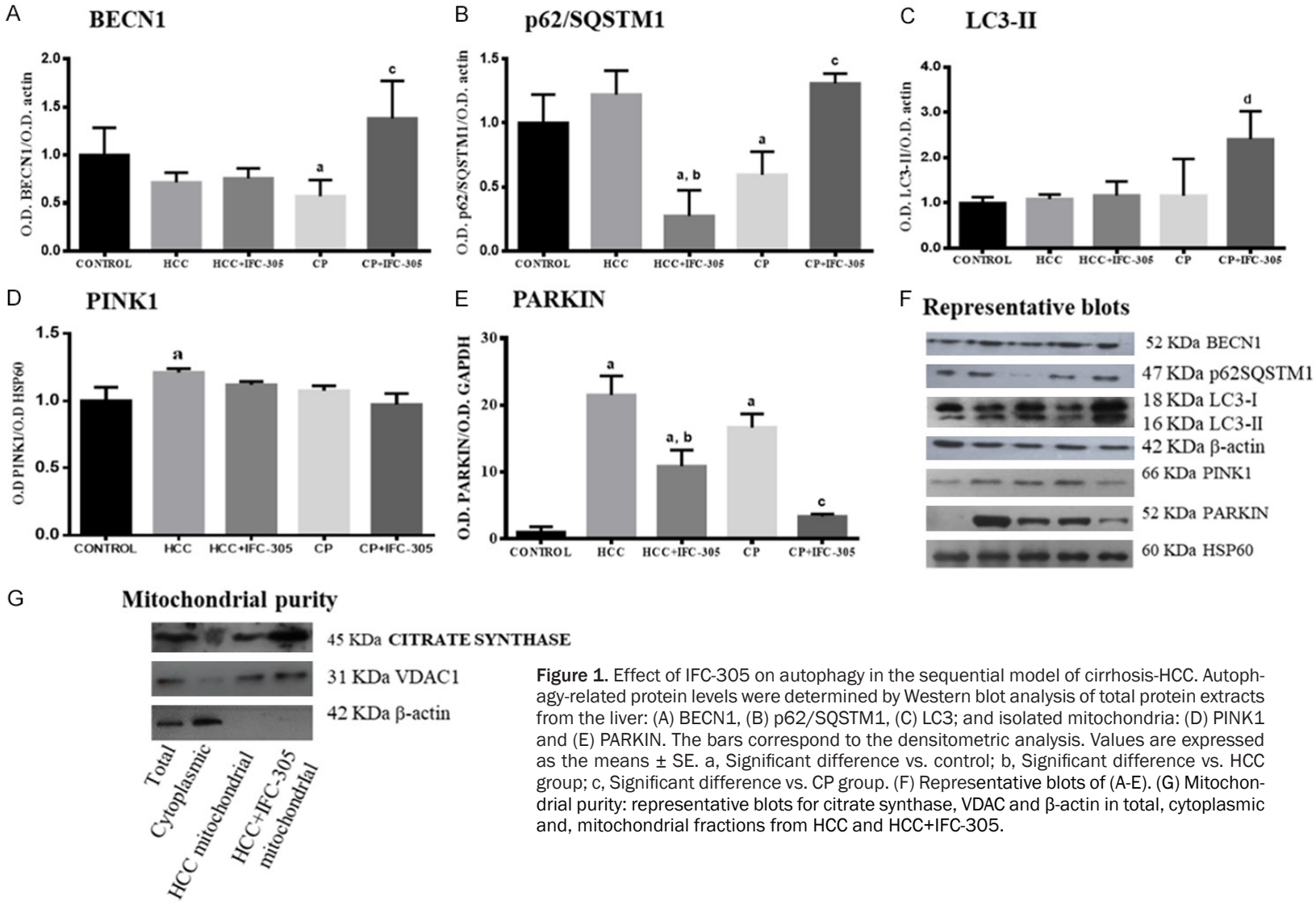

Figure 1. Effect of IFC-305 on autophagy in the sequential model of cirrhosis-HCC. Autophagy-related protein levels were determined by Western blot analysis of total protein extracts from the liver: (A) BECN1, (B) p62/SQSTM1, (C) LC3; and isolated mitochondria: (D) PINK1 and (E) PARKIN. The bars correspond to the densitometric analysis. Values are expressed as the means ± SE. a, Significant difference vs. control; b, Significant difference vs. HCC group; c, Significant difference vs. CP group. (F) Representative blots of (A-E). (G) Mitochondrial purity: representative blots for citrate synthase, VDAC and β-actin in total, cytoplasmic and, mitochondrial fractions from HCC and HCC+IFC-305.

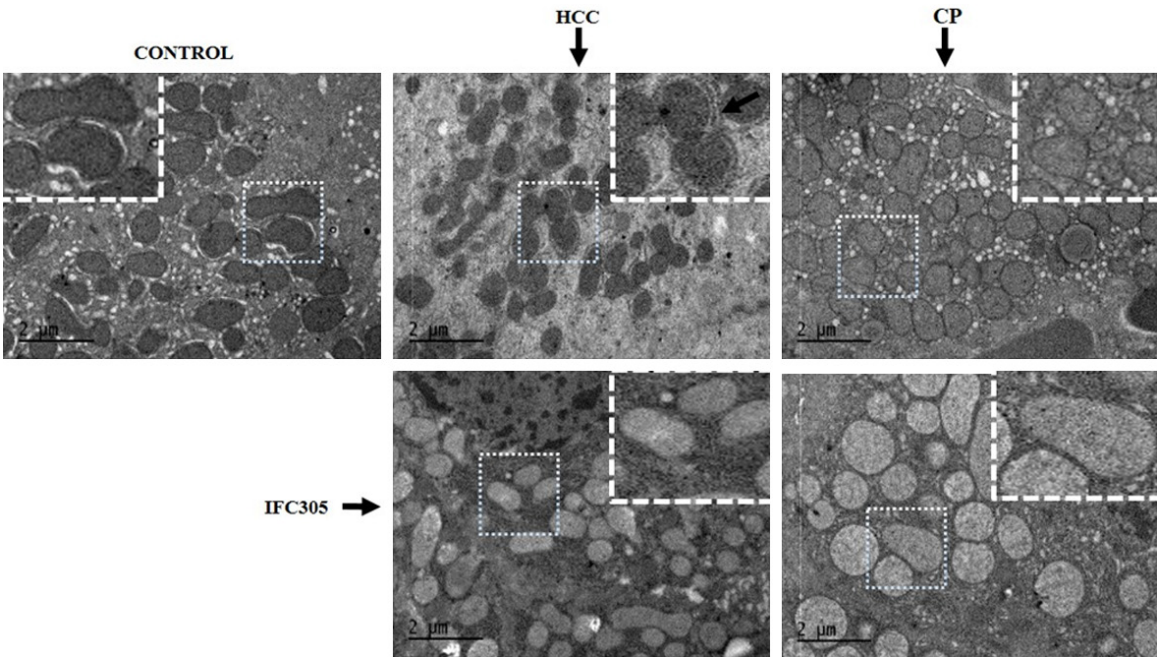

Figure 2. Morphological changes evaluated by electron microscopy. Each panel is representative of the indicated group. Liver slices were fixed with glutaraldehyde and stained with osmium tetroxide. The dotted-outline squares represent a magnification of a mitochondrion.

thase and VDAC in the total and mitochondrial fractions. On the other hand, in the mitochondrial extracts the presence of β-actin was not detected. This result indicates the mitochondrial fraction purity. To determine if autophagy is induced by the IFC-305 compound, which has a chemopreventive function in the sequential model of cirrhosis-HCC, we compared the levels of BECN1, p62/SQSTM1 and LC3 with or without IFC-305. We found that the level of BECN1 was reduced in the CP group and the IFC-305 treatment increased the levels of this protein (CP+IFC-305); there were no significant changes in the level of this protein in the HCC or HCC+IFC-305 groups (**Figure 1A**). The abundance of p62/SQSTM1, on the other hand, was not altered with 16 weeks of DEN administration (HCC group) but was significantly decreased with IFC-305 treatment (HCC+IFC-305); however, at week 22, the level of p62/SQSTM1 was reduced in the CP group and increased with IFC-305 treatment (CP+IFC-305) (**Figure 1B**). The levels of the lipidated form of LC3 (LC3-II) were only modified in the group where the rats were treated with IFC-305 when the DEN administration was stopped (CP+IFC-305) (**Figure 1C**). These results suggest that IFC-305 indeed induces autophagy. To evaluate whether mitophagy in particular is being induced, we decided to determine whether the PINK1 and PARKIN proteins, which mark depolarized and damaged mitochondria for degradation by mitophagy, are recruited to the mitochondria in response to IFC-305 treatment. There was an increase in the PINK1 mitochondrial content in the HCC group, while no changes were detected in the other experimental groups (**Figure 1D**). Interestingly, an increased PARKIN content was detected in the HCC and CP groups, and the treatment with IFC-305 inhibited and diminished this effect (HCC+IFC-305 and CP+IFC-305, respectively) (**Figure 1E**). These results indicate that even though damaged mitochondria are properly recognized by PINK1, mitophagy is interrupted in HCC giving rise to dysfunctional mitochondria accumulation, which suggest that IFC-305 treatment restores the mitophagic flux. We confirmed by electron microscopy that engulfment of damaged mitochondria is impaired in HCC, as mitochondria with surrounding membranes were observed (pointed with an arrow, **Figure 2**), suggesting incomplete autophagosome formation. By contrast, circular mitochondria were observed in the CP group. The treatment with IFC-305 led to the presence of normally shaped mitochondria and the absence of surrounding membranes, as expected for

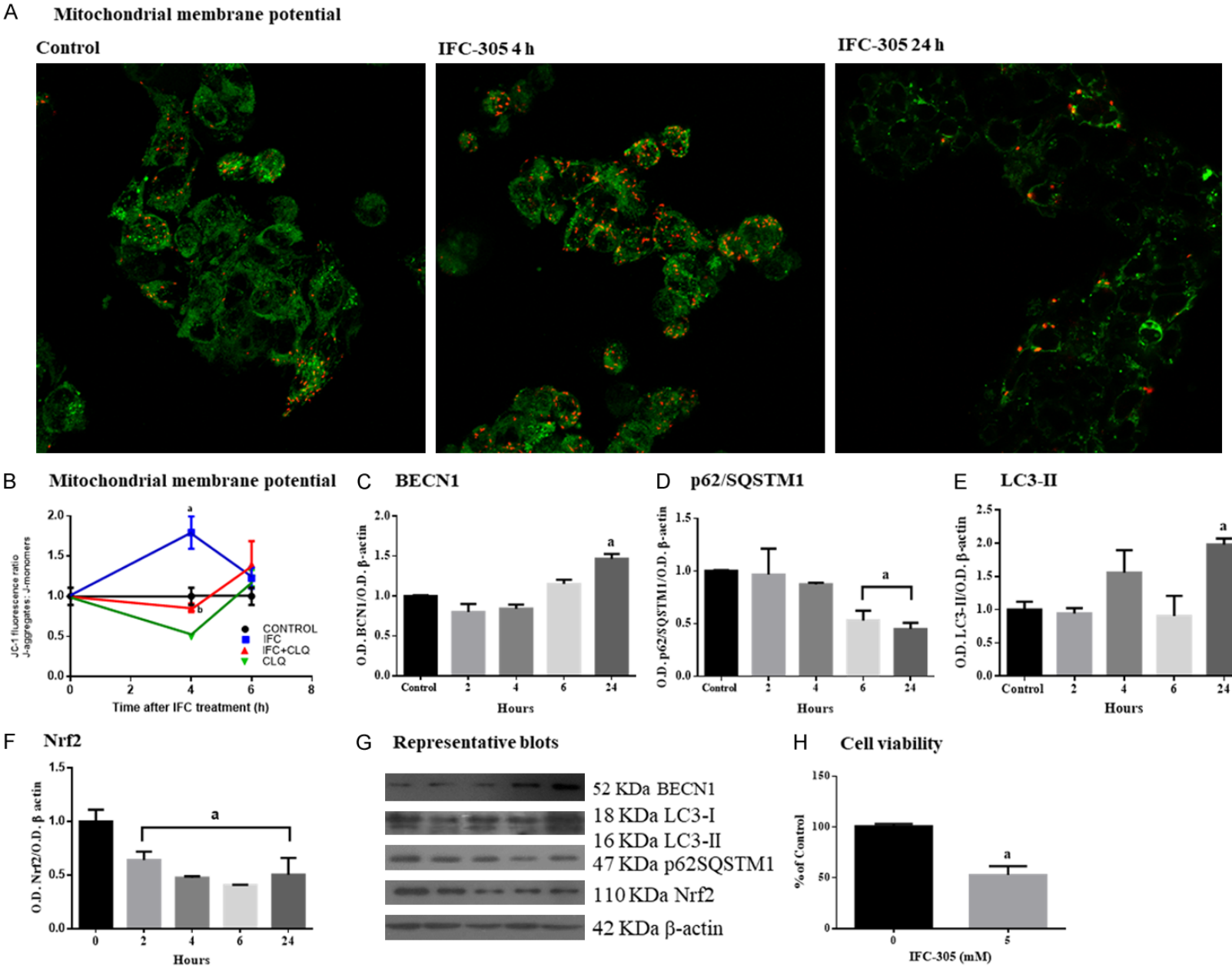
A
Mitochondrial membrane potential
Control
IFC-305 4 h
IFC-305 24 h
B
Mitochondrial membrane potential
JC-1 fluorescence ratio
J-aggregates: J-monomers
Time after IFC treatment (h)
CONTROL
IFC
IFC+CLQ
CLQ
C
BECN1
O.D. BCN1/O.D. β-actin
Hours
D
p62/SQSTM1
O.D. p62/SQSTM1/O.D. β-actin
Hours
E
LC3-II
O.D. LC3-II/O.D. β-actin
Hours
F
Nrf2
O.D. Nrf2/O.D. β actin
Hours
G
Representative blots
52 KDa BECN1
18 KDa LC3-I
16 KDa LC3-II
47 KDa p62SQSTM1
110 KDa Nrf2
42 KDa β-actin
H
Cell viability
%of Control
IFC-305 (mM)

Figure 3. Autophagy modulation by the IFC-305 compound in HepG2 cells. IFC-305 was added to cultured HepG2 cells for 0, 2, 4, 6, and 24 h. (A) Mitochondrial membrane potential was determined by staining with JC-1 dye; (B) Timecourse of JC-1 fluorescence as determined by the J-aggregates:J-monomers ratio and the effect of chloroquine. Autophagy-related protein levels were determined by Western blot analysis of total protein extracts from HepG2 cells (C) BECN1, (D) p62/SQSTM1, (E) LC3 and (F) NRF2. (G) Representative blots. (H) Cell density determination by the sulforhodamine B (SRB) assay based on the measurement of cellular protein content after 24 hours of treatment with 5 mM IFC-305.

healthy mitochondria, suggesting that any damaged mitochondria had already been degraded. In **Figure 2**, magnified images of some mitochondria are shown in the dotted squares for a better appreciation.

IFC-305 induces autophagy in HepG2 cells

Because the abnormal mitochondria found in abundance in the *in vivo* model of HCC disappear with IFC-305 administration, we decided to evaluate whether the IFC-305 compound restores the mitochondrial membrane potential (ΔΨm) in an HCC cell line. To do this, HepG2 cultured cells were treated with 5 mM IFC-305 for 2, 4, 6, and 24 h and the ΔΨm was evaluated. **Figure 3A** shows the JC-1 signal in the untreated and 4 and 24 h IFC-305-treated HepG2 cells. In the IFC-305-treated cells there was a greater red fluorescence signal indicating a recuperation of the ΔΨm. This result was corroborated with the J-aggregates versus J-monomers ratio, which indicated the recovery of the ΔΨm. As shown in **Figure 3B**, the maximum peak is observed at 4 hours after treatment with IFC-305, while this peak diminished after 24 hours. In addition to the effect on fluorescence at 24 hours, a different HepG2 phenotype can also be observed; the green and red JC-1 signals are reduced after 24 hours of treatment with IFC-305. This could indicate that there is an alteration in the cellular integrity due to a modification in the membranes or cell death. Hence, IFC-305 partially restores the ΔΨm in HepG2 cells. This effect was prevented by the autophagy inhibitor chloroquine (CQL) as it compound avoid the increment in the ΔΨm induced by the IFC-305 treatment suggesting that the effect observed in mitochondria is due to an induction of autophagy.

To determine whether the positive effect of the IFC-305 compound on mitochondrial function is related to autophagy induction in the HepG2 cells, we measured the amount of some ATG proteins by Western blot. The levels of the BECN1 and LC3-II proteins were significantly increased after 24 h of IFC-305 treatment (**Figure 3C**), while p62/SQSTM1 progressively diminished, with significant decreases after 6 and 24 h of IFC-305 treatment (**Figure 3D**). Because p62/SQSTM1 affects the abundance of NRF-2 by interacting with PEAK1, and HCC protects against oxidative stress, we decided to evaluate if there were changes in the amount of NRF2 induced by IFC-305 treatment. Indeed, the level of NRF2 diminished after 2 h of IFC-305 treatment and was maintained at the same level out to 24 h (**Figure 3F**). Representative blots are presented in **Figure 3G**. In summary, the IFC-305 compound induced autophagy in transformed HepG2 cells, however, it also decreased cell viability (**Figure 3H**).

IFC-305 induces autophagy in primary mouse embryonic fibroblasts

To evaluate the effect of the IFC-305 compound on autophagy in healthy, nontumoral cells, we measured BECN1, p62/SQSTM1 and LC3 protein content in primary mouse embryonic fibroblasts (MEFs). We incubated the cells with 5 mM IFC-305 for 0, 1, 2, 4 and 6 h. We observed a progressive increase in BECN1 up to 4 h of IFC-305 treatment; after 6 h, the BECN1 content recovered to the normal level (**Figure 4A**). An augmented p62/SQSTM1 level was observed beginning at 1 h of IFC-305 treatment, which was sustained until 4 h, but returned to normal levels by 6 h (**Figure 4B**). LC3-I and LC3-II significantly increased at 1 h and then slightly decreased at 2 and 4 hours, with levels lower than the control group at 6 h. Representative blots are shown in **Figure 4D**. Together, these observations suggested that IFC-305 induced a functional autophagic flux in MEFs.

To further confirm IFC-305-induced autophagy, we directly detected the presence of autophagosomes stained with Cyto ID®; **Figure 4E** shows representative images. An induction of autophagosome formation was observed with 5 mM IFC-305 treatment for 2 h, which

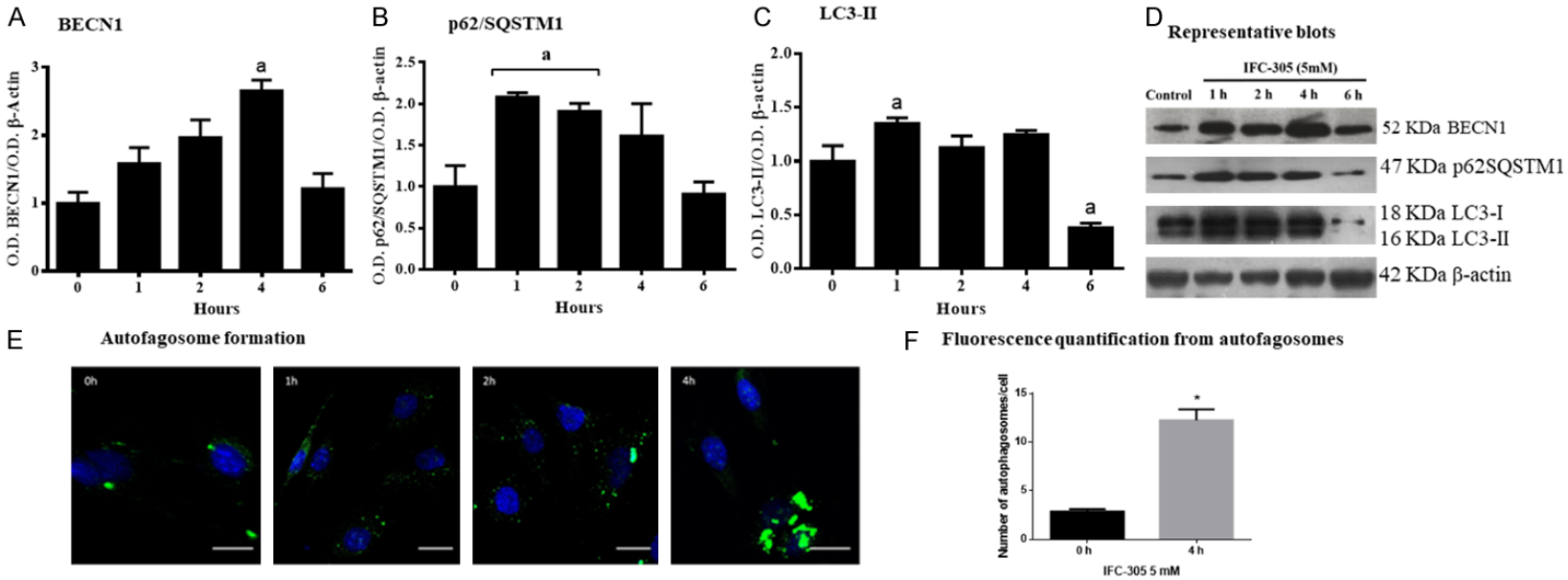

Figure 4. IFC-305 induces autophagy in MEFs. IFC-305 was added to MEFs at 5 mM for 1, 2, 4 or 6 h. Autophagy-related protein levels were determined by Western blot analysis of total protein extracts from MEFs: (A) BECN1, (B) p62/SQSTM1, (C) LC3. (D) Representative blots. (E) Representative images of autophagosome formation in MEFs stained with Cyto ID® and nuclear Hoechst staining after 12, 2 or 4 h of treatment with 5 mM IFC-305. (F) Fluorescence quantification from the autophagosomes in (E).

increased after 4 h. This effect was diminished at 6 h, as expected with an active autophagic flux because CytoID fluorescence diminishes in acidic pH such as that in autolysosomes. Fluorescence quantifications from autophagosomes are shown in **Figure 4F**.

Discussion

Autophagy is a recycling process that promotes cell survival during stress and serves as organelle quality control, such as the process of mitophagy that helps to maintain functional mitochondrial. In addition, autophagy seems to be protective against cancer [27]. However, the exact mechanism is not yet known, however, it has been proposed to be dependent on the context of the cell involved [28].

BECN1 has been implied as a tumor suppressor [29, 30]. Here, we showed an important increase in the level of BECN1 in the group treated with IFC-305 after the discontinuation of DEN administration, suggesting the induction of autophagy in our *in vivo* model. Previously, the expression of BECN1 was been found to be downregulated in HCC patients, and this finding was correlated with the severity of HCC, cirrhosis and vascular invasion [31]. Zou et al. demonstrated that induction of BECN1 could mediate autophagy as a mechanism to promote apoptosis in HCC cultured cells [32]. This result suggests that HCC severity is lower when treated with IFC-305 compared to the group that only received vehicle due to autophagy induction.

As seen in the HCC groups, IFC-305 treatment affected protein levels, as demonstrated by the decreased amount of p62/SQSTM1 in the HCC+IFC-305 group compared to the HCC group. It has been recently shown that p62/SQSTM1 is required to induce HCC in mice [33] and that it is needed for NRF2 activation to protect HCC from oxidative stress in a preneoplastic stage [34]. This effect could explain the differences founded in the HCC and CP groups, remembering that the latter corresponds to a cancer progression state rather than an initial stage. The ability of IFC-305 to diminish the amount of p62/SQSTM1 protein supports its previously reported chemopreventive effect [17].

Recently, the absence of LC3 was associated with immediate mortality after surgical HCC resection. Therefore, in addition to the role that these proteins have in autophagy, they may also serve as prognosis indicators in HCC patients undergoing liver resection [35]. The results from our *in vivo* model may suggest the importance of mitophagy induction to degrade dysfunctional mitochondria that accumulate in the HCC and CP groups. On the other hand, we observed a significant increase only in the levels of PINK1, which suggests that mitochondria with decreased membrane potential are labeled, an effect that we have demonstrated previously [18]. We know that one of the most important steps, although not unique, in continuing the process of repairing or degrading damaged mitochondria through mitophagy is the subsequent translocation of PARKIN and the ubiquitination of various proteins, which precedes LC3-mediated degradation via the autophagy machinery [36]. One of the most notorious changes in the *in vivo* model was the increase in PARKIN protein levels in the HCC and CP groups, which were decreased with IFC-305 treatment (HCC+IFC-305 and CP+IFC-305). This effect could suggest that mitochondria marked by PINK1 have indeed been repaired or degraded by mitophagy. In addition to the important role that PARKIN plays in mitophagy, this protein has also been associated with 8-hydroxy-2'-deoxyguanosine, a marker of mutagenicity and damage to mitochondrial and nuclear DNA (mtDNA and nDNA, respectively). Its colocalization has been found in models of chronic and acute alcoholism in rats [37, 38]. Mitochondria can promote tumorigenesis because they play an important role in the overproduction of reactive oxygen species when they are nonfunctional and favoring fission. As a result, mitophagy may function as a tumor suppressor because it is the main way to correct or degrade dysfunctional mitochondria [39].

We compared the *in vivo* effect of IFC-305 with its effect on an HCC cell line to verify if this compound also induces autophagy *in vitro*. Recently, it has been demonstrated that autophagy inhibits the proliferation of HepG2 cells and that some of the autophagy markers could be useful as prognostic factors [32, 35, 40]. Here, we showed an increase in the levels of BECN1 and LC3-II and a decrease in the level of p62/SQSTM1. Moreover, we evaluated the $\Delta\Psi m$ by JC-1 staining, which selectively enters mitochondria with a polarized mem-

brane potential and forms complexes known as J-aggregates with red fluorescence that decrease the red/green fluorescence intensity ratio. We observed a ΔΨm recuperation after 4 h of IFC-305 treatment and a depolarization at 24 h. Interestingly, we observed a significant decrease in the levels of the NRF2 transcription factor beginning within the first hour of IFC-305 treatment. This decrease probably occur because decreased p62/SQSTM1 levels inhibit the NRF-2-KEAP1 interaction, which results in its polyubiquitination and degradation by the proteasome [41]. This result suggests that a lower antioxidant defense compromised the cellular viability, which would explain the observed changes in the JC-1 signal and thus, the changes in cell morphology. Some studies have found that increased NRF2 expression is related to proliferation, invasion, chemoresistance, tumor size and differentiation metastasis in HCC [42-44].

Finally, this study evaluated the ability of the IFC-305 compound to induce autophagy in nontumoral cells. Here, we demonstrate, through the evaluation of autophagy markers, that IFC-305 is able to induce autophagy in MEFs, showing a peak in autophagosome formation after 4 hours of treatment with the compound. This effect was corroborated by the autophagosome formation observed in GFP-LC3 MEFs (data not shown). The autophagy induction by IFC-305 could be part of the protective mechanism that has been demonstrated for this compound, including preventing the accumulation of nonfunctional mitochondria or allowing for their repair through mitophagy. We have previously reported the effect of IFC-305 on the function, metabolism and dynamics of mitochondria in the sequential model of cirrhosis-HCC [18]. Further studies are required to elucidate the autophagy-related mechanism by which the inhibition of tumor cell proliferation by IFC-305 is carried out. An important characteristic that we would like to highlight is that the times of autophagy induction in the HepG2 cell line and in the MEFs are different; in the latter we find that autophagy is induced more rapidly, possibly because the metabolic characteristics of these cells are different.

In conclusion, IFC-305 is a compound that has the ability to induce autophagy. In the *in vivo* HCC model, this effect may help to recover mitochondrial function by preventing progression and favoring the reversal of HCC [17, 18]. In the in vitro model, the induction of autophagy may partially favor the recovery of the ΔΨm in HepG2 cells, this result was supported by the fact that chloroquine, an autophagy inhibitor, avoided this effect; however, the main effect of the IFC-305 compound was a decrease in the number of viable cells. In this study, we found that the induction of autophagy by the IFC-305 compound may be an important component in the recovery of mitochondrial function, as well as in the prevention and reversal of HCC.

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Disclosure of conflict of interest

None.

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References

[1] Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM. Estimates of worldwide burden of cancer in 2008: globocan 2008. Int J Cancer 2010; 127: 2893-2917.

[2] Madrigal-Matute J and Cuervo AM. Regulation of liver metabolism by autophagy. Gastroenterology 2016; 150: 328-339.

[3] Dikic I and Elazar Z. Mechanism and medical implications of mammalian autophagy. Nat Rev Mol Cell Biol 2018; 19: 349-364.

[4] Zhang H and Baehrecke EH. Eaten alive: novel insights into autophagy from multicellular model systems. Trends Cell Biol 2015; 25: 376-387.

[5] Cecconi F and Levine B. The role of autophagy in mammalian development: cell makeover rather than cell death. Dev Cell 2008; 15: 344-357.
[6] Nassour J, Radford R, Correia A, Fuste JM, Schoell B, Jauch A, Shaw RJ and Karlseder J. Autophagic cell death restricts chromosomal instability during replicative crisis. Nature 2019; 565: 659-663.
[7] Zaffagnini G and Martens S. Mechanisms of selective autophagy. J Mol Biol 2016; 428: 1714-1724.
[8] Yu L, Chen Y and Tooze SA. Autophagy pathway: cellular and molecular mechanisms. Autophagy 2018; 14: 207-215.
[9] Bartolini D, Dallaglio K, Torquato P, Piroddi M and Galli F. Nrf2-p62 autophagy pathway and its response to oxidative stress in hepatocellular carcinoma. Transl Res 2018; 193: 54-71.
[10] Xu D, Xu M, Jeong S, Qian Y, Wu H, Xia Q and Kong X. The role of Nrf2 in liver disease: novel molecular mechanisms and therapeutic approaches. Front Pharmacol 2018; 9: 1428.
[11] Schneider JL and Cuervo AM. Liver autophagy: much more than just taking out the trash. Nat Rev Gastroenterol Hepatol 2014; 11: 187-200.
[12] Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K and Mizushima N. Autophagy-deficient mice develop multiple liver tumors. Genes Dev 2011; 25: 795-800.
[13] Ding ZB, Shi YH, Zhou J, Qiu SJ, Xu Y, Dai Z, Shi GM, Wang XY, Ke AW, Wu B and Fan J. Association of autophagy defect with a malignant phenotype and poor prognosis of hepatocellular carcinoma. Cancer Res 2008; 68: 9167-9175.
[14] Sun K, Xu L, Jing Y, Han Z, Chen X, Cai C, Zhao P, Zhao X, Yang L and Wei L. Autophagy-deficient Kupffer cells promote tumorigenesis by enhancing mtROS-NF-kappaB-IL1alpha/beta-dependent inflammation and fibrosis during the preneoplastic stage of hepatocarcinogenesis. Cancer Lett 2017; 388: 198-207.
[15] Wallace DC and Fan W. Energetics, epigenetics, mitochondrial genetics. Mitochondrion 2010; 10: 12-31.
[16] Yi CH, Pan H, Seebacher J, Jang IH, Hyberts SG, Heffron GJ, Vander Heiden MG, Yang R, Li F, Locasale JW, Sharfi H, Zhai B, Rodriguez-Mias R, Luithardt H, Cantley LC, Daley GQ, Asara JM, Gygi SP, Wagner G, Liu CF and Yuan J. Metabolic regulation of protein N-alpha-acetylation by Bcl-xL promotes cell survival. Cell 2011; 146: 607-620.
[17] Velasco-Loyden G, Perez-Martinez L, Vidrio-Gomez S, Perez-Carreon JI and Chagoya de Sanchez V. Cancer chemoprevention by an adenosine derivative in a model of cirrhosis-hepatocellular carcinoma induced by diethylnitrosamine in rats. Tumour Biol 2017; 39: 1010428317691190.
[18] Chavez E, Lozano-Rosas MG, Dominguez-Lopez M, Velasco-Loyden G, Rodriguez-Aguilera JR, Jose-Nunez C, Tuena de Gomez-Puyou M and Chagoya de Sanchez V. Functional, metabolic, and dynamic mitochondrial changes in the rat cirrhosis-hepatocellular carcinoma model and the protective effect of IFC-305. J Pharmacol Exp Ther 2017; 361: 292-302.
[19] Chagoya de Sanchez V and Hernández-Muñoz Ri. Método para preparar aspartato de adenosina a partir de la adenosina, composición farmacéutica que las contiene y uso de las mismas en el tratamiento de enfermedades hepáticas fibrosantes. Patent N° 207422. Mexico City: Instituto Mexicano de la Propiedad Intelectual; 2002.
[20] Chagoya-Hazas V, Hernandez Munoz R and Villa-Trevino S, inventors. Use of Adenosine Aspartate for the preparation of pharmaceutical products for the treatment of liver cancer. EE.UU. Patent Nº. 8,507,459 B2. Washington (DC): U.S. Patent and Trademark Office; 2013.
[21] Mascorro JA LM and Yates RD. Rapid infiltration of biological tissues nucleoside. The latter could be particularly true regarding utilizing h-hexenyl succinic anhydride (HXSA)/vinyl ciclohexane dioxthe formation of the extracellular matrix, 7 which is similar ide (VCD), and ultra-low viscosity embedding medium. Proc Ann Meeting Soc Am 1976; 34: 346-347.
[22] Xu J. Preparation, culture, and immortalization of mouse embryonic fibroblasts. Curr Protoc Mol Biol 2005; Chapter 28: Unit 28.1.
[23] Mizushima N, Yamamoto A, Matsui M, Yoshimori T and Ohsumi Y. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol Biol Cell 2004; 15: 1101-1111.
[24] Vichai V and Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 2006; 1: 1112-1116.
[25] Schiffer E, Housset C, Cacheux W, Wendum D, Desbois-Mouthon C, Rey C, Clergue F, Poupon R, Barbu V and Rosmorduc O. Gefitinib, an EGFR inhibitor, prevents hepatocellular carcinoma development in the rat liver with cirrhosis. Hepatology 2005; 41: 307-314.
[26] Lozano-Rosas MG, Chávez E, Velasco-Loyden G, Domínguez-López M, Martínez-Pérez L and Chagoya de Sánchez V. Liver induction of S-adenosylmethionine levels and its metabolism as a mechanism of chemoprevention of an adenosine derivative in a cirrhosis-hepatocellular carcinoma induced by diethylnitrosamine in rats. Cancer Biol Ther 2019.

[27] Finley LW and Haigis MC. The coordination of nuclear and mitochondrial communication during aging and calorie restriction. Ageing Res Rev 2009; 8: 173-188.
[28] Liu L, Liao JZ, He XX and Li PY. The role of autophagy in hepatocellular carcinoma: friend or foe. Oncotarget 2017; 8: 57707-57722.
[29] Boland ML, Chourasia AH and Macleod KF. Mitochondrial dysfunction in cancer. Front Oncol 2013; 3: 292.
[30] Avalos Y, Canales J, Bravo-Sagua R, Criollo A, Lavandero S and Quest AF. Tumor suppression and promotion by autophagy. Biomed Res Int 2014; 2014: 603980.
[31] Qiu DM, Wang GL, Chen L, Xu YY, He S, Cao XL, Qin J, Zhou JM, Zhang YX and Qun E. The expression of beclin-1, an autophagic gene, in hepatocellular carcinoma associated with clinical pathological and prognostic significance. BMC Cancer 2014; 14: 327.
[32] Zou M, Lu N, Hu C, Liu W, Sun Y, Wang X, You Q, Gu C, Xi T and Guo Q. Beclin 1-mediated autophagy in hepatocellular carcinoma cells: implication in anticancer efficiency of oroxylin A via inhibition of mTOR signaling. Cell Signal 2012; 24: 1722-1732.
[33] Chen J ZB, Wong N, Lo AW, To KF, Chan AW, Ng MH, Ho CY, Cheng SH, Lai PB, Yu J, Ng HK, Ling MT, Huang AL, Cai XF and Ko BC. Sirtuin 1 is upregulated in a subset of hepatocellular carcinomas where it is essential for telomere maintenance and tumor cell growth. Cancer Res 2012; 72: 4138-4149.
[34] Umemura A, He F, Taniguchi K, Nakagawa H, Yamachika S, Font-Burgada J, Zhong Z, Subramaniam S, Raghunandan S, Duran A, Linares JF, Reina-Campos M, Umemura S, Valasek MA, Seki E, Yamaguchi K, Koike K, Itoh Y, Diaz-Meco MT, Moscat J and Karin M. p62, upregulated during preneoplasia, induces hepatocellular carcinogenesis by maintaining survival of stressed HCC-initiating cells. Cancer Cell 2016; 29: 935-948.
[35] Lin CW, Lin CC, Lee PH, Lo GH, Hsieh PM, Koh KW, Lee CY, Chen YL, Dai CY, Huang JF, Chuang WL, Chen YS and Yu ML. The autophagy marker LC3 strongly predicts immediate mortality after surgical resection for hepatocellular carcinoma. Oncotarget 2017; 8: 91902-91913.
[36] Hamacher-Brady A and Brady NR. Mitophagy programs: mechanisms and physiological implications of mitochondrial targeting by autophagy. Cell Mol Life Sci 2016; 73: 775-795.
[37] Eid N, Ito Y, Horibe A and Otsuki Y. Ethanol-induced mitophagy in liver is associated with activation of the PINK1-Parkin pathway triggered by oxidative DNA damage. Histol Histopathol 2016; 31: 1143-1159.
[38] Eid N, Ito Y and Otsuki Y. Triggering of parkin mitochondrial translocation in mitophagy: implications for liver diseases. Front Pharmacol 2016; 7: 100.
[39] Wilde L, Tanson K, Curry J and Martinez-Outschoorn U. Autophagy in cancer: a complex relationship. Biochem J 2018; 475: 1939-1954.
[40] Hu P, Cheng B, He Y, Wei Z, Wu D and Meng Z. Autophagy suppresses proliferation of HepG2 cells via inhibiting glypican-3/wnt/beta-catenin signaling. Onco Targets Ther 2018; 11: 193-200.
[41] Kansanen E, Kuosmanen SM, Leinonen H and Levonen AL. The Keap1-Nrf2 pathway: Mechanisms of activation and dysregulation in cancer. Redox Biol 2013; 1: 45-49.
[42] Gao AM, Ke ZP, Shi F, Sun GC and Chen H. Chrysin enhances sensitivity of BEL-7402/ADM cells to doxorubicin by suppressing PI3K/Akt/Nrf2 and ERK/Nrf2 pathway. Chem Biol Interact 2013; 206: 100-108.
[43] Lee SE, Yang H, Jeong SI, Jin YH, Park CS and Park YS. Induction of heme oxygenase-1 inhibits cell death in crotonaldehyde-stimulated HepG2 cells via the PKC-delta-p38-Nrf2 pathway. PLoS One 2012; 7: e41676.
[44] Zhang M, Zhang C, Zhang L, Yang Q, Zhou S, Wen Q and Wang J. Nrf2 is a potential prognostic marker and promotes proliferation and invasion in human hepatocellular carcinoma. BMC Cancer 2015; 15: 531.