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Interferon-gamma induces autophagy with growth inhibition and cell death in human hepatocellular carcinoma (HCC) cells through interferon-regulatory factor-1 (IRF-1)

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Abstract

Interferon-gamma (IFN- γ) is a pleiotropic cytokine with immunomodulatory, anti-viral, and anti-proliferative effects. In this study, we examined the effects of IFN- γ on autophagy and cell growth in human hepatocellular carcinoma (HCC) cells. IFN- γ inhibited cell growth of Huh7 cells with non-apoptotic cell death. IFN- γ induced autophagosome formation and conversion/turnover of microtubule associated protein 1 light chain 3 (LC3) protein. Furthermore, overexpression of IRF-1 also induced autophagy in Huh7 cells. Silencing IRF-1 expression with target small hairpin RNA blocked autophagy induced by IFN- γ . Silencing of the autophagy signals Beclin-1 or Atg5 attenuated the inhibitory effect of IFN- γ on Huh7 cells with decreased cell death. Additionally, IFN- γ activated autophagy in freshly cultured human HCC cells. Together, these findings show that IFN- γ induces autophagy through IRF-1 signaling pathway and the induction of autophagy contributes to the growth-inhibitory effect of IFN- γ with cell death in human liver cancer cells.

Keywords

IFN- γ ; autophagy; cell growth; cell death; HCC

1. Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer related death in the world. Its incidence is rising faster than most other cancers due to the increasing prevalence of hepatitis B and C virus infection [1–2]. Interferon is a pleiotropic cytokine with immunomodulatory, anti-viral, and anti-proliferative effects. Although type I interferon (IFN- α and IFN- β) has been used clinically in the adjuvant setting after resection of HCC [3–4], type II interferon (IFN- γ) has also been shown to have a role during HCC

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Conflicts of interest

None of the authors has any financial or other interest with regard to the submitted manuscript that might be constructed as a conflict of interest.

immunotherapy [5–6], and has a direct inhibitory effect on HCC by inducing apoptosis [7]. IFN- γ has the potential to enhance the effectiveness of HCC treatment [8]. Both type I and type II IFNs have been shown to induce nitric oxide (NO) synthesis, which can have pro- or anti- cancer effects, depending on cell type and environment [9–10].

Several key steps of the transduction pathway of IFN- γ have been investigated [11]. Initially, IFN- γ combines with its interferon- γ receptor 2 (IFNGR2) and the Janus tyrosine kinase protein family (Jak1 and Jak2) is activated. Jak1 and Jak2 phosphorylate signal transducer and activator of transcription 1 (Stat1), and phosphorylated Stat1 forms a homodimer. Stat1 dimers then translocate to the nucleus, where they bind to members of the IFN- γ -activated sequence (GAS) family enhancers. GAS enhancers induce the expression of several genes including interferon regulatory factor-1 (IRF-1), guanylate binding protein 1 (GBP1), and IFN- γ -inducible protein 10 (IP-10) [11]. Among these targets, IRF-1 is expressed in breast cancer cells [12–13] and has been identified as a tumor suppressor in addition to its function in immunomodulation and antiviral response [14]. IRF-1 is also an essential mediator in IFN- γ -induced cell cycle arrest and apoptosis in ovarian cancer cells [15–16].

However, despite these well-conserved signaling events, direct anti-proliferative effects of IFN- γ on tumor cells appear to be tissue- and cell-type specific, ranging from potent apoptosis induction to complete apoptosis resistance [7,16]. Recently, IFN- γ has been found to induce autophagy in T lymphocytes [17], macrophages [18], mammary epithelial cells [19] and HeLa cells [20]. Autophagy is an evolutionarily conserved process that is activated during cell starvation and other stresses. In this process, a double membrane is formed within the cell, and a portion of the cytoplasm and intracellular organelles are incorporated into a bound structure known as the autophagosome. The autophagosome fuses with lysosomes to form an autolysosome, in which the cell contents are degraded. Cells can survive this self-digestion to get an alternative energy source under starvation, or cells can clear unfolded proteins or microorganisms away under certain conditions [21–22]. For tumors, autophagy is a doubled-edged sword [23]. Its protective function helps tumor cells survive under stress including certain chemotherapies. However, autophagy can also be negative to cell growth [24], and autophagy-associated cell death has been demonstrated in response to various anticancer therapies [25–27]. It is unknown whether autophagy plays a role in the response of HCC cells to IFN- γ .

In this study, we show that IFN- γ induced cell growth inhibition and cell death in HCC cells. IFN- γ induced autophagy instead of apoptosis in certain HCC cells through the IRF-1 signaling pathway. Blocking autophagy attenuated the growth inhibition and cell death induced by IFN- γ . To our knowledge, this is the first report that autophagy induced by IFN- γ contributes to HCC cell growth inhibition and cell death instead of cellular protection.

2. Materials and methods

2.1. Cell culture

The human HCC cell line Huh7 was kept in our laboratory. The primary cultured HCC cells were isolated from a resected HCC patient in our laboratory as part of an institutional review board (IRB)-approved tissue banking protocol. All the cells were cultured in DMEM (Lonza) containing 10% heat-inactivated fetal bovine serum (Clontech), 100 units/ml penicillin, 100 μ g/ml streptomycin, 15 mmol/L HEPES, 200 mmol/L L-Glutamine, and incubated at 37 °C in a humidified incubator containing 5% CO₂.

2.2. Antibodies and reagents

The antibodies used were as follows: anti-SQSTM1 (p62) (Abnova); anti-LC3, anti-Becclin-1, anti-Atg5, anti-cleaved caspase-3, anti-(p)mTOR, anti-(p)AKT, anti-PI3K III (Cell Signaling); anti-IRF-1 (human or mouse) (Santa Cruz); anti-actin (Sigma); anti-Histone H3 (Abcam). The reagents used were as follows: hIFN- γ (Roche); 3-methyladenine (3-MA), acridine orange, doxorubicin, rapamycin, bafilomycin A1 (Baf A1) (Sigma); Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Vector labs).

2.3. Adenoviral Vectors

Adenoviral IRF-1 (AdIRF-1) is an E1- and E3-deleted recombinant adenovirus encoding murine IRF-1 constructed previously [28]. The control vector of Adenoviral lacZ (AdlacZ) is the same adenoviral vector encoding lacZ. AdIRF-1-shRNA was constructed recently in our laboratory, which had a microRNA hairpin structure targeting IRF-1 mRNA 3'-UTR to block IRF-1 expression [29]. The same adenoviral vector with scrambled sequence (Adsc-shRNA) was used as a control.

2.4. Cell growth and cell death assay

For cell growth assays, cells were seeded in 100 μ l medium per well at 5×10^3 /ml in 96-well flat-bottomed plates. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate cell growth according to the manual (Cell Proliferation Kit I MTT, Roche). The absorbance was determined at 550 nm with 690 nm as a reference. For cell death assay, cells were seeded in 6-well plates. After treatment, cells were harvested including the floating cells in the medium. Then cells were stained with 0.04% trypan blue (GIBCO) and the numbers of dead and live cells were counted. Also flow cytometry was used to detect cell death. All cells including floating ones were harvested and then stained with propidium iodide (BD) for 15 min in the dark. Flow cytometry analysis was done with BD LSR II machine within 1 hr.

2.5. TUNEL assay

Cell apoptosis was detected by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) with In Situ Cell Death Detection Kit, Fluorescein (Roche) according to the manual. Slides were mounted with PI and viewed with Olympus fluorescence microscopes.

2.6. Immunoblot (Western blot) analysis

Whole cell protein was extracted with Cell Lysis Buffer (Cell Signaling). The nuclear protein was extracted according to our previous report [30]. Western blot analysis was performed following a standard protocol as described previously [31–32]. β -actin or Histone H3 protein was detected as loading controls of whole cell protein or nuclear protein respectively. Blot was scanned and densitometric analysis was done by ImageJ software (NIH).

2.7. Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature, blocked with 5% BSA in PBS for 60 min, and then incubated with rabbit LC3 antibody (1:250) overnight in the cold room. Alexa Fluor 488 anti-rabbit IgG (1:500, Invitrogen) was applied for 1 h at room temperature. Slides were mounted with DAPI and viewed with Olympus fluorescence microscopes.

2.8. Acridine orange staining

Cells were washed with PBS and then stained with medium containing 1 µg/ml acridine orange for 15 minutes at 37 °C. After washing with PBS again slides were mounted and viewed with Olympus fluorescence microscopes.

2.9. Transmission electron microscopy (TEM)

Cells were fixed in cold 2.5% glutaraldehyde in PBS. The specimens were post-fixed in 1% Osmium Tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30% – 90%) and embedded in Epon. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and Reynold's lead citrate, and imaged on a JOEL JEM-1100 TEM.

2.10. Small interfering RNA

Small interfering RNA (siRNA) targeting human Beclin-1 or Atg5 was introduced using HiPerFect reagent (Qiagen). Cells were immediately transfected with 20 nmol/L siRNA after seeding. 16 h after transfection cells were changed to fresh medium and treated with IFN-γ to desired time.

2.11. Statistical analysis

Data were presented as mean ± SE. Experiments were repeated at least two times. Data were analyzed by Student t test or ANOVA, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. IFN-γ inhibits the cell growth of Huh7 HCC cells with non-apoptotic cell death

It has been reported that IFN-γ has a growth inhibitory effect on several tumors including HCC [7,33], gastric cancer [34], ovarian carcinoma [16] and breast cancer [35] and is a potent inducer of apoptosis. Here we observed the cell growth inhibitory effect of IFN-γ on the HCC cell line Huh7. IFN-γ inhibited the cell growth of Huh7 in a time- and dose-dependent manner (Fig. 1A). Also IFN-γ induced cell death through trypan blue exclusion assay. Cell death was confirmed by flow cytometry analysis with PI staining (doxorubicin is positive control) (Fig. 1B). Though induction of apoptosis is a common way for IFN-γ to suppress tumor cells, IFN-γ did not induce obvious apoptosis in Huh7 cells. Using TUNEL staining, we observed that doxorubicin induced apoptosis, while IFN-γ did not (Fig. 1C). Likewise, cleaved caspase-3 protein as a marker of apoptosis was increased in Huh7 cells after doxorubicin treatment, but not after IFN-γ treatment (Fig. 1D). Moreover, we found that IFN-γ did not induce cell cycle arrest in Huh7 cells determined by flow cytometry analysis (data not shown). Since the cell growth inhibition and cell death induced by IFN-γ was not obviously due to apoptosis in Huh7 cells, we then hypothesized that IFN-γ-induced growth inhibition might involve effects on cellular signaling pathways associated with autophagy.

3.2. IFN-γ induces autophagosome formation in Huh7 cells

IFN-γ has been reported to induce autophagy in several cell types [17–20]. Since Huh7 cell growth could be inhibited by IFN-γ without apoptosis, we investigated whether autophagy was involved. First we detected whether autophagosome formation was induced by IFN-γ in Huh7 cells. LC3 is a protein marker that is reliably localized to autophagosomes, which could be detected by immunofluorescence staining with a punctuated distribution [36]. In IFN-γ-treated Huh7 cells, nearly 40% cells have punctuated distribution of LC3 indicating autophagosomes (Fig. 2A). Rapamycin was used as a positive control as it is a strong inducer of autophagy [36]. During autophagy, some acidic vesicular organelles (AVO) are formed through the membraned cytoplasmic proteins fusing into lytic components. Acridine

orange-stained red AVOs were accumulated in the cytoplasm of IFN- γ -treated Huh7 cells, while the cytoplasm and nucleus stained green (Fig. 2B). TEM confirmed the formation of autophagosomes after IFN- γ treatment in Huh7 cells, which were recognized as characteristic double or multiple membrane vacuolar structures containing cytoplasmic contents (Fig. 2C).

3.3. IFN- γ promotes autophagic signals changes and autophagic flux in Huh7 cells

During the formation of autophagosomes, LC3 protein is synthesized and transformed from LC3-I to LC3-II protein [36]. Also during autophagy, p62 is incorporated into the completed autophagosome and is degraded in autolysosomes [36]. When Huh7 cells were treated with IFN- γ , LC3-II protein was induced and p62 decreased in a time- and dose-dependent manner by western blot (Fig. 3A, left panel). The concentration curve showed increased LC3-II and decreased p62 protein with increased doses of IFN- γ (100 – 1000 u/ml) compared to resting cells (Fig. 3A, right panel). To confirm the autophagy flux, we performed an LC3 turnover assay [36]. When Huh7 cells were treated with Bafilomycin A1 (Baf A1), a vacuolar H⁺-ATPase inhibitor preventing the fusion between autophagosomes and lysosomes, endogenous LC3-II was slightly increased (Fig. 3B). Addition of IFN- γ to Baf A1 further enhanced LC3-II protein levels compared to Baf A1 alone (Fig. 3B), which shows that IFN- γ enhances autophagy flux. Also, 3-MA, a well-known autophagy inhibitor, blocked autophagy in Huh7 cells induced by IFN- γ with decreased LC3-II formation in a dose-dependent manner (Fig. 3C). Finally, when Huh7 cells were treated with IFN- γ , Akt/mTOR signaling pathway was inhibited with decreased phosphorylated Akt and mTOR at 24–48 hrs, while PI3K III was activated with increased protein level detected by western blot in a time-dependent manner (Fig. 3D). The down-regulation of Akt/mTOR and up-regulation of PI3K III supports autophagy induced by IFN- γ in Huh7 cells.

3.4. IFN- γ induces autophagy in Huh7 cells through IRF-1

IRF-1 is a well-known mediator of the IFN signaling pathway, and has also been indicated as a tumor suppressor through apoptosis induction [14]. Nuclear IRF-1 was basally expressed in Huh7 cells and was strongly induced by IFN- γ 24 h – 96 h after stimulation (Fig. 4A). Nuclear IRF-1 was also induced in a dose-dependent manner by IFN- γ (Fig. 4A). Additionally, AdIRF-1 transfection successfully induced IRF-1 over production in the nucleus of Huh7 cells in a dose-dependent manner lasting up to 48 h. Basal IRF-1 expression was difficult to detect in Fig. 4B compared Fig. 4A because a rabbit anti-mouse IRF-1 antibody was used here which only weakly detects basal endogenous human IRF-1 in the Huh7 cells (in contrast to the rabbit anti-human IRF-1 antibody used in Fig. 4A). Overexpression of IRF-1 by AdIRF-1 transfection activated autophagy, as evidenced by markedly increased LC3-II and decreased p62 in Huh7 cells (Fig. 4C). Utilizing IRF-1 shRNA, we then inhibited the production of IRF-1 following IFN- γ stimulation in Huh7 cells. Knockdown of IRF-1 in Huh7 cells inhibited the ability of IFN- γ to induce autophagy, shown by decreased LC3-II and increased p62 proteins compared to scrambled (scr) shRNA. (Fig. 4D). Taken together, these results indicate that autophagy induced by IFN- γ in Huh7 cells is mediated in part by IRF-1.

3.5. Blocking autophagy attenuates the growth inhibition and cell death induced by IFN- γ in Huh7 cells

Since IFN- γ triggered growth inhibition and non-apoptotic cell death in Huh7 cells with autophagy, we wanted to examine whether blocking autophagy abrogated the IFN- γ - induced growth inhibition and cell death. Huh7 cells were transfected with siRNA targeted to autophagic signals Beclin-1 or Atg5 and then treated with IFN- γ . Silencing Beclin-1 (Fig. 5A, left panel) or Atg5 (Fig. 5A, right panel) attenuated autophagy induced by IFN- γ in Huh7 cells with decreased LC3-II and increased p62 proteins compared to scrambled (scr)

shRNA. Also silencing Beclin-1 or Atg5 partially reversed the cell growth inhibition by IFN- γ in Huh7 cells determined by MTT assay (Fig. 5B). Finally, siRNA silencing of Beclin-1 or Atg5 also partially reversed the cell death induced by IFN- γ in Huh7 cells determined by trypan blue exclusion (Fig. 5C). Therefore, we conclude that autophagy activation contributes to IFN- γ -induced growth inhibition and cell death in the human HCC cells.

3.6. IFN- γ induces autophagy in primary cultured human HCC cells

To investigate the effect of IFN- γ on primary HCC cells, we used a freshly cultured human HCC cell line, HCCG1, isolated from a resected HCC tumor of a patient. We stimulated the cells with IFN- γ and examined autophagic signaling by western blot and TEM. IFN- γ induced a dramatic increased LC3-II protein in a dose-dependent manner (Fig. 6A). LC3 turnover assay confirmed the autophagic flux in these cells which was increased by the combination of IFN- γ and Bafilomycin A1 (Fig. 6B). TEM showed the presence of autophagosomes following IFN- γ treatment (Fig. 6C). These data suggest that autophagy induced by IFN- γ in HCC cells can occur in primary liver cancer cells as well as established liver HCC cell lines.

4. Discussion

In this study, we investigated a novel pathway of IFN- γ in eliciting growth inhibition in human HCC cells. Previous studies have shown the ability of IFN- γ to induce apoptosis as a mechanism of tumor suppression [7,16,35]. The major and novel findings of this study are: 1) IFN- γ induced growth inhibition and non-apoptotic cell death in Huh7 HCC cells; 2) Autophagy was activated by IFN- γ in Huh7 cells and involved IRF-1 signaling pathways; 3) Autophagy induced by IFN- γ promoted cell growth inhibition and cell death instead of cytoprotection; 4) IFN- γ -induced autophagy was also observed in primary human HCC cells.

IFN- γ has been reported to induce apoptosis in many tumors including HCC [7,33], gastric cancer [34], pancreatic carcinoma [37], breast cancer [38–39], and ovarian carcinoma [16]. In this study, cell growth inhibition and non-apoptotic cell death was observed in Huh7 cells stimulated with IFN- γ . Doxorubicin-stimulated apoptosis in Huh7 cells confirmed that the apoptotic signaling pathways were intact in these cells (Fig. 1C–1D). Additionally, IFN- γ did not induce obvious cell cycle arrest in Huh7 cells determined by flow cytometry analysis with PI staining (data not shown). Since we could not demonstrate apoptosis in response to IFN- γ in Huh7 cells, we hypothesized that autophagy may be exerting a role in mediating growth inhibition and cell death selectively in Huh7 cells. Indeed, IFN- γ -induced autophagy was demonstrated in Huh7 cells shown by fluorescent staining for LC3 protein and AVO, as well as autophagosome formation visualized on TEM (Fig. 2A–2C). The autophagy induced by IFN- γ in Huh7 cells was further indicated by formation of LC3-II protein in a time- and dose-dependent manner. Moreover, stimulation of Huh7 cells with IFN- γ decreased p62 protein levels in a time- and dose-dependent manner, which is also indicative of active autophagy (Fig. 3A). LC3 turnover assay further confirmed the autophagic flux, suggesting that the accumulation of autophagosomes were not induced by the blockade of autolysosome formation or degradation (Fig. 3B). Also the IFN- γ -induced autophagy could be inhibited by 3-MA, a well-known autophagy inhibitor (Fig. 3C). The changes of Akt/mTOR and PI3K III signals displayed that autophagy could be activated through such pathways by IFN- γ in Huh7 cell (Fig. 3D). In addition to Huh7 HCC cell line, we also demonstrated that IFN- γ induced autophagy in primary human HCC cells from a freshly isolated HCC specimen (Fig. 6).

IRF-1 is a master transcriptional factor that regulates gene expression during innate and adaptive immunity [40–41], and IRF-1 expression itself is known to be regulated by type I (IFN- α/β) and type II (IFN- γ) interferons [40]. IRF-1 plays an important role in IFN- γ -induced apoptosis in tumor cells via downstream signals including iNOS [7], p21 [33], p53 upregulated modulator of apoptosis (PUMA) [34] and Fas-associated Death Domain (FADD) [38]. Since IFN- γ was able to elicit autophagy in Huh7 cells, and since IFN- γ is a major activator of IRF-1 gene expression, we explored a possible mechanistic role for IRF-1 in mediating IFN- γ -induced autophagy. IRF-1 was basally expressed in Huh7 cells, and was strongly induced by IFN- γ in a dose-dependent manner. Both endogenous expression of IRF-1 induced by IFN- γ , and exogenous expression of IRF-1 with AdIRF-1 transfection, activated autophagy in Huh7 cells (Fig. 4C). The importance of IRF-1 in mediating IFN- γ -induced autophagy was shown when shRNA silencing IRF-1 resulted in decreased LC3-II protein and increased p62 protein, indicating diminished autophagy (Fig. 4D). Specificity was shown with scrambled shRNA that had no effect.

As a tumor suppressor gene, IRF-1 is expressed at a low level in cancers such as esophageal cancer [42], gastric cancer [43] and HCC [44–45]. IRF-1 induces apoptosis in some tumor cells [14]. In our study, autophagy induced by IFN- γ was also mediated by IRF-1. To the best of our knowledge, this is the first report that IRF-1 regulates autophagy in addition to apoptosis, and expands the downstream effector pathways of the IFN- γ /IRF-1 signaling axis, which maybe a candidate for cancer therapy. Recent studies have also shown that IRF-1 contributes to liver injury during warm ischemia/reperfusion (I/R), and liver transplant preservation (cold I/R) injury, although the role of autophagy was not addressed in these studies [46–47].

The role of autophagy in tumor development and therapy is an area of active investigation. Under certain conditions, such as hypoxia, starvation, and stress from anti-tumor therapies, tumor cells employ autophagy to sequester and degrade damaged organelles and cell contents to provide energy and promote cell survival [21–22]. It is generally accepted that autophagy is a survival response for tumor cells undergoing anti-tumor therapies. In fact, inhibition of autophagy promoted the cell growth inhibition and apoptotic cell death in certain cancer cells [48–49]. However, some studies have suggested that tumor cell growth inhibition and tumor cell death is actually promoted by autophagy induced by transforming growth factor-beta (TGF- β) [25], Concanavalin A [26], or OSU-03012 [27]. Our findings that IFN- γ inhibited HCC cell growth and promoted cell death along with induction of autophagy in Huh7 cells is consistent with this notion. This point is bolstered by the findings that siRNA targeting the crucial autophagy effectors Beclin-1 or Atg5 attenuated autophagy with less growth inhibition and cell death induced by IFN- γ stimulation (Fig. 5).

In conclusion, our findings show that IFN- γ induces autophagy in human HCC cells, mediated in part by the IRF-1 signaling pathways. Further, autophagy contributes to the cell growth inhibition and cell death induced by IFN- γ . This work underscores the complex pleiotropic effects of IFN- γ in regulating the autophagy pathway in liver cancer cells.

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Abbreviations

IFN-γ	interferon-gamma
HCC	hepatocellular carcinoma
IRF-1	interferon regulatory factor-1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide
PI	propidium iodide
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling
TEM	transmission electron microscopy
AVOs	acidic vesicular organelles
LC3	microtubule-associated protein 1 light chain 3
3-MA	3-methyladenine

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Fig. 1. IFN- γ inhibits the cell growth of Huh7 HCC cells with non-apoptotic cell death

(A) Cell growth. Huh7 cells were treated with 500 IU/ml IFN- γ and assayed at different time points, or treated with different concentration of IFN- γ and assayed at 96 h. Cell growth was assessed by MTT assay. The data are presented as the means $\pm$ SE of four samples and are representative of three independent experiments with similar results. * P <0.05 and ** P <0.01 vs. control.

(B) Cell death by trypan blue exclusion and PI staining. Huh7 cells were treated with or without 500 IU/ml IFN- γ for 48 h and then collected for trypan blue exclusion assay. All cells were stained with PI and cell death was analyzed by flow cytometry. The ratio between number of dead cells and total cells was calculated. Cells treated with 1 μ mol/L Doxorubicin

(Dox) was used as positive control. $**P<0.01$ vs. untreated cells. Data were means $\pm$ SE of triplicates.

(C) TUNEL staining. Huh7 cells were treated as in Fig. 1B and then assessed with TUNEL staining. Apoptotic cells were labeled with green fluorescence, and the nuclei were stained red with PI (*Bars*, 20 μ m). At least three random fields were counted and averaged, $**P<0.01$ vs. untreated cells. (D) Western blot for cleaved caspase-3. Huh7 cells were treated with or without 500 IU/ml IFN- γ from 6 h to 72 h, or treated with different concentration of IFN- γ for 48 h. Whole cell protein was extracted and cleaved caspase-3 was assayed by western blot. Huh7 cells were treated with 1 μ mol/L Doxorubicin (Dox) for 48 h as positive control.

Fig. 2. IFN- γ induces autophagosome formation in Huh7 cells

(A) LC3 immunofluorescence staining. Huh7 cells were cultured on coverslips and treated with 500 IU/ml IFN- γ for 48 h, or with 40 nmol/L rapamycin (Rap) as positive control. After LC3 immunofluorescent staining, nuclei were stained blue with DAPI. The punctuated distribution of green fluorescence indicated the formation of autophagosomes (Bars, 10 μ m). At least three random fields were counted and averaged, * P <0.01 vs. untreated cells. (B) Acridine orange staining. Cells were treated as in Fig. 2A and then stained with 1 μ g/ml acridine orange. The cytoplasm and nucleus stained bright green, whereas acidic compartments such as AVOs stained orange (Bars, 10 μ m). At least three random fields were counted and averaged, * P <0.01 vs. untreated cells.

(C) TEM. Huh7 cells were treated as in Fig. 2A and then assayed by TEM. Autophagosome are seen (marked by arrows) as the rounded vacuolar structures with double or multiple membranes containing cytoplasmic contents (*Bars*, 500 nm).

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Fig. 3. IFN- γ induces autophagic signals changes and autophagic flux in Huh7 cells

(A) LC3 conversion and p62 degradation. Huh7 cells were treated with 500 IU/ml IFN- γ and harvested at different time points, or treated with different concentration of IFN- γ for 48 h. LC3 and p62 protein were detected by western blot from whole cell protein.

(B) LC3 turnover assay. Huh7 cells were treated with 1,000 IU/ml IFN- γ for 24 h and then 40 nmol/L Bafilomycin A1 (Baf A1) was added for further 4 h. LC3 protein was detected by western blot. The value under the blot is the ratio of LC3-II to β -actin and normalized to control.

(C) Huh7 cells were pretreated with 3-MA and 1 h later IFN- γ was added for 48 h. LC3 protein was detected by western blot. The value under the blot is the ratio of LC3-II to β -actin and normalized to control.

(D) Huh7 cells were treated with 500 IU/ml IFN- γ for 3 – 48 hrs. Autophagy regulators mTOR, AKT, and PI3K III were detected by western blot.

Fig. 4. IFN- γ induces autophagy in Huh7 cells through IRF-1

(A) IFN- γ induced nuclear IRF-1 production in Huh7 cells. Cells were treated with 500 IU/ml IFN- γ for 24 h to 96 h, or treated with different concentration of IFN- γ for 48 h. Nuclear protein was extracted and IRF-1 was detected by western blot.

(B) AdIRF-1 transfection induced over-expression of nuclear IRF-1 in Huh7 cells. Cells were transfected with 50 MOI AdIRF-1 from 24 h to 96 h, or transfected with different MOI of AdIRF-1 for 48 h. Cells transfected with AdLacZ (50 MOI) for 48 h were used as negative control (NC).

(C) IRF-1 overexpression increased LC3-II protein and decreased p62 protein in Huh7 cells. Cells were transfected with different MOI of AdIRF-1 for 48 h. Cells were transfected with AdLacZ as negative control or treated with 500 IU/ml IFN- γ as positive control. LC3 and p62 proteins from whole cell lysate were detected by western blot.

(D) IRF-1 silencing attenuated the increase of IRF-1 and LC3-II proteins, and decrease of p62 protein induced by IFN- γ in Huh7 cells. Cells were transfected with AdIRF-1-shRNA or Adscr-shRNA (negative control) of 50 MOI. 16 h later cells were changed to fresh medium with or without 200 IU/ml IFN- γ . 48 h after transfection, cells were harvested and IRF-1, LC3, and p62 proteins were detected by western blot. The value under the blot is the ratio of IRF-1, LC3-II or p62 to β -actin and normalized to control respectively.

Fig. 5. Autophagy blocking attenuates the growth inhibition and cell death induced by IFN- γ in Huh7 cells

(A) Beclin-1 or Atg5 siRNA transfection suppressed autophagy induced by IFN- γ in Huh7 cells. Cells were transfected with 20 nmol/L Beclin-1 siRNA, Atg5 siRNA or negative control scrambled (scr) siRNA immediately after seeding. 16 h later cells were changed to fresh medium with or without 200 IU/ml IFN- γ . 48 h after transfection, cells were harvested and Beclin-1, Atg5, LC3 and p62 proteins were detected by western blot. The value under the blot is the ratio of Beclin-1, Atg5, LC3-II or p62 to β -actin and normalized to control respectively.

(B) Autophagy blocking attenuated the growth inhibition by IFN- γ in Huh7 cells. Cells were seeded on 96-well plates and immediately transfected with siRNA as in Fig. 6A. 16 h later cells were changed to fresh medium and treated with or without 500 IU/ml IFN- γ . 48 h later, cells were transfected and treated with IFN- γ again. Cell growth was assessed by MTT assay after treatment with IFN- γ for 96 h. Data were means $\pm$ SE of 6 samples.

(C) Autophagy blocking attenuated the cell death induced by IFN- γ in Huh7 cells. Cells were seeded on 6-well plates and immediately transfected with siRNA as in Fig. 6A. 16 h later cells were changed to fresh medium and treated with or without 500 IU/ml IFN- γ . 48 h later, cells were harvested including the floating cells in the medium and then stained with 0.04% trypan blue. The numbers of dead and live cells were counted respectively. Also cells were stained with PI and cell death was analyzed by flow cytometry. Data were means $\pm$ SE of triplicates.

Fig. 6. IFN- γ induces autophagy in primary cultured human HCC cells

(A) LC3 conversion. HCCG1 cells were treated with different doses of IFN- γ for 48 h respectively. LC3 protein was detected from whole cell lysate by western blot.

(B) LC3 turnover assay. HCCG1 cells were treated with 1 000 IU/ml IFN- γ for 24 h and then added 40 nmol/L Bafilomycin A1 (Baf A1) or not for further 24 h. LC3 protein was detected by western blot. The value under the blot is the ratio of LC3-II to β -actin and normalized to control respectively.

(C) TEM. HCCG1 cells were treated with or without 500 IU/ml IFN- γ for 48 h and then assayed by TEM. Autophagosomes were seen (marked by arrows) as the rounded vacuolar

structures with double or multiple membranes containing cytoplasmic contents (*Bars*, 500 nm).

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