Lysosomal membrane protein TMEM192 deficiency triggers crosstalk between autophagy and apoptosis in HepG2 hepatoma cells

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Abstract. As constituents of lysosomes, lysosomal membrane proteins play important roles in lysosome-related autophagy and apoptosis. In a recent proteomic study of lysosomal proteins, we identified transmembrane protein 192 (TMEM192) as a novel lysosomal membrane protein candidate. Using specific anti-TMEM192 antibody and lysosomal markers, the lysosomal localization of TMEM192 was determined by immunofluorescence. TMEM192 shows a wide expression pattern in mouse tissues. Interestingly, TMEM192 was found to be highly expressed in tumor cell lines, while it was not expressed or was detected at low levels in normal cell lines. By knockdown of TMEM192 expression using specific siRNAs, we found that TMEM192-deficient HepG2 hepatoma cells show growth inhibition and increased apoptosis. Autophagy was shown to be activated through detection of LC3II expression. Increased apoptosis was inhibited by blocking the expression of the key autophagy gene Atg7 in TMEM192-deficient HepG2 cells. The results suggest that TMEM192 is important for tumor cell growth and proliferation. TMEM192 deficiency can induce autophagy in tumor cells, and can further activate apoptosis by the mitochondrial pathway through autophagy. TMEM192 promotion of autophagy may be a new route for tumor therapy.

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Abbreviations: ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; LAMP, lysosome-associated membrane protein; siRNA, small interfering RNA; TMEM192, transmembrane protein 192

Key words: TMEM192, autophagy, apoptosis, tumor

Introduction

Autophagy refers to the highly regulated and evolutionarily conserved process of turnover and maintenance of cellular components that is required for cellular homeostasis (1). It is a multistep process where portions of the cytoplasm and organelles are engulfed in double-membrane vesicles called autophagosomes. These structures then fuse with lysosomes, resulting in the destruction of their contents by the acid hydrolases provided by the lysosome. There are three main autophagic pathways: macroautophagy, microautophagy, and chaperone-mediated autophagy (2). Autophagy is controlled by autophagy-related genes, many of which are involved in autophagosome formation. This process features conjugation systems that are well-conserved among eukaryotes: Atg12-Atg5 and Atg8 (microtubule-associated protein 1 light chain 3, LC3)-phosphatidylethanolamine systems (3).

Numerous studies have demonstrated a variety of physiological and pathophysiological roles in autophagy, such as adaptation to nutrient deprivation, intracellular clearance of protein and organelles, development, antiaging, elimination of microorganisms, cell death, tumor suppression, and antigen presentation (4-6). In tumor cells, autophagy has been recognized as an important regulator of the promotion of tumor cell survival and development and restriction of necrosis (7-9). However, recent studies suggest that the role of autophagy is more complicated, and may have diametrically opposite consequences for the tumor, depending on the circumstances (10).

Here, we demonstrate a contradictory role for autophagy in HepG2 hepatoma cells, which was induced by knockdown of the expression of transmembrane protein 192 (TMEM192), a lysosome membrane protein.

Materials and methods

Cell lines and cell culture. If not stated otherwise, LO2 and HepG2 cells were grown in DMEM (Dulbecco's modified Eagle's medium; Gibco Life Technologies) supplemented with 10% fetal calf serum (Gibco Life Technologies) and 1%

penicillin/streptomycin (Sigma, St. Louis, MO, USA) under 5% CO_2 atmosphere at 37˚C.

Western blotting. Cells were harvested in lysis buffer and subjected to western blot analysis. Signals were detected using HRP-conjugated secondary antibodies and an ECL detection reagent. Details are provided in a previous study (11). The primary antibodies included rabbit anti-human TMEM192 (Sigma-Aldrich), anti-LAMP1 (Santa Cruz Biotecnhology, Santa Cruz, CA, USA), rabbit anti-human caspase-12 (Biotime, Haimen, Zhejiang, China), rabbit anti-caspase-3, anti-P-p38 MAPK and β-actin, rat anti-human Bax and caspase-8 (Biotime). The LC3 antibody was a gift of Dr Yi Wang (Shanghai Jiao Tong University).

RNA interference. Cells were transfected with a non-targeted control siRNA (small interfering RNA) or target-specific siRNAs directed to *TMEM192* sequences (Shanghai Sangon, China). Briefly, 30 nM *TMEM192* siRNA (5'-AATCTTCTG TGGTTTATTCUU-3') was transfected into cells using Oligofectamine (Invitrogen). Transfected non-targeted siRNA (20 nM, 5'-AATATCTCGTTCGTTTGTTUU-3') cells and untransfected cells were mocked as controls. After 24 h, cells were re-transfected with the indicated siRNAs for additional 24 h intervals. In the experiments of 1-5 days, cells were harvested for further analysis. The *Atg7* gene was silenced by the same procedure. The siRNA targeting *Atg7* mRNA comprised the 27-base RNA nucleotide sequence 5'-AACTTAG CCCAGTACCCTGGATGGCCT-3'. That of the control siRNA was 5'-AATAGCTCCACCTCCATGAGGGTCGCTUU-3'.

Cell proliferation assay. For growth curves, 10^6 cells were collected and transferred to a 24-well plate, and were seeded in 10% serum. Monolayers were washed and the medium was replaced by siRNA transfections as above, carried out on Days 1 and 2. Fresh medium was added on Day 4. Cell survival and growth were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) on different days, as previously described (12).

Apoptosis cell analysis by flow cytometry (FCM). To measure the quantity and the ratio of apoptotic cells, the Annexin V-FITC Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was utilized. The cells were treated as described above. Cells were harvested, fixed and stained with PI and Annexin V-FITC as described in the kit instructions. Finally, the stained cells were analyzed immediately on a FACScalibur flow cytometer (BD Biosciences).

Immunoflurorescence staining. For cells grown on coverslips in 24-well plates, the monolayer was washed twice with PBS (phosphate-buffered saline). Then the cells were fixed with 4% PFA (paraformaldehyde) for 10-30 min. After washing twice with PBS, cells were permeabilized/blocked by treatment with SS-PBS (0.2% saponin containing 10% bovine serum albumin in PBS) for 30 min. For double immunostaining, primary and secondary antibodies were overlaid on coverslips in SS-PBS for 1 h and 30 min separately, followed by 3 washes with PBS. Coverslips were mounted onto 1-mm glass slides using fluorescent mounting medium. All steps were performed at room temperature. Samples were analyzed using a Leica TCS SP2 confocal microscope. In the case of double staining with LysoTracker Red, cells were labeled with 50 nM LysoTracker Red for 2 h in serum-free DMEM and washed with PBS, then fixed with 4% PFA for 20 min at room temperature.

Quantitative real-time PCR and RT-PCR. Using 5 μg of total RNA as template, reverse transcription was performed with PrimeScript II first strand cDNA Synthesis kit (Takara). Measurement of the relative quantity of the cDNA of interest was carried out using SYBR® Premix Ex Taq™ (Takara), 300 nM of the appropriate forward and reverse primers, and 1 μl of the cDNA mixture. The sense and antisense primers used for TMEM192 were 5'-GCCATCTTGGCACTGGAAC-3' and 5'-GCTTACTCAGCAGCGCATT-3' (59˚C, 245 bp), respectively. The primers for mouse homologous TMEM192 were 5'-GTATGGATGACGACCCGCTTCT-3' and 5'-TGGG CACTTGTCTTCAGTTGGAT-3' (63˚C, 200 bp). Real-time PCR assays were performed in the Applied Biosystems 7500 Real-Time PCR System using thermal cycling parameters recommended by the manufacturer (40 cycles of 15 sec at 95˚C and 1 min at 60˚C). PCR product purity was determined by melting curve analysis. Within each plate, we included triplicates of each sample. Data were analyzed with SDS2.1 software (v.2.1.0.3, Applied Biosystems). At least three separate experiments per subject were performed. Values exceeding two standard deviations were excluded.

Statistical analysis. The data are expressed as means ± SD. Tests for significance of differences were performed by ANOVA or Student's t-test as appropriate. $P<0.05$ was considered statistically significant.

Results

Lysosomal localization of TMEM192 by immunofluorescence. The lysosomal localization of TMEM192 was confirmed by immunofluorescence. Using specific anti-TMEM192 antibody, TMEM192 staining in HLF-I cells showed small punctuate patterns throughout the cytoplasm, predominantly in the perinuclear region. No signal was detected at the plasma membrane or in the nucleus. This suggested that TMEM192 protein was localized in an intracellular compartment (green fluorescence in Fig. 1A). Double staining with antibody against LAMP1, a marker protein for lysosomes (red fluorescence in Fig. 1A), showed colocalization with TMEM192. Double staining with the lysosome tracker red (LTR), a marker of the lysosome, also showed that it was colocalized with TMEM192 (Fig. 1B).

Tissue distribution of the TMEM192. Tissue expression analysis provides important information about the function of TMEM192. Therefore, we analyzed the expression of TMEM192 in mouse tissues and different cell lines. Using real-time PCR, we found that TMEM192 was a widely-expressed protein. Its mRNA was abundant in the kidney and liver while little or no expression was detected in other tissues in the mouse, such as the spleen and intestine (Fig. 2A and B). Interestingly and importantly, TMEM192 was highly expressed in tumor cell lines while little or low expression

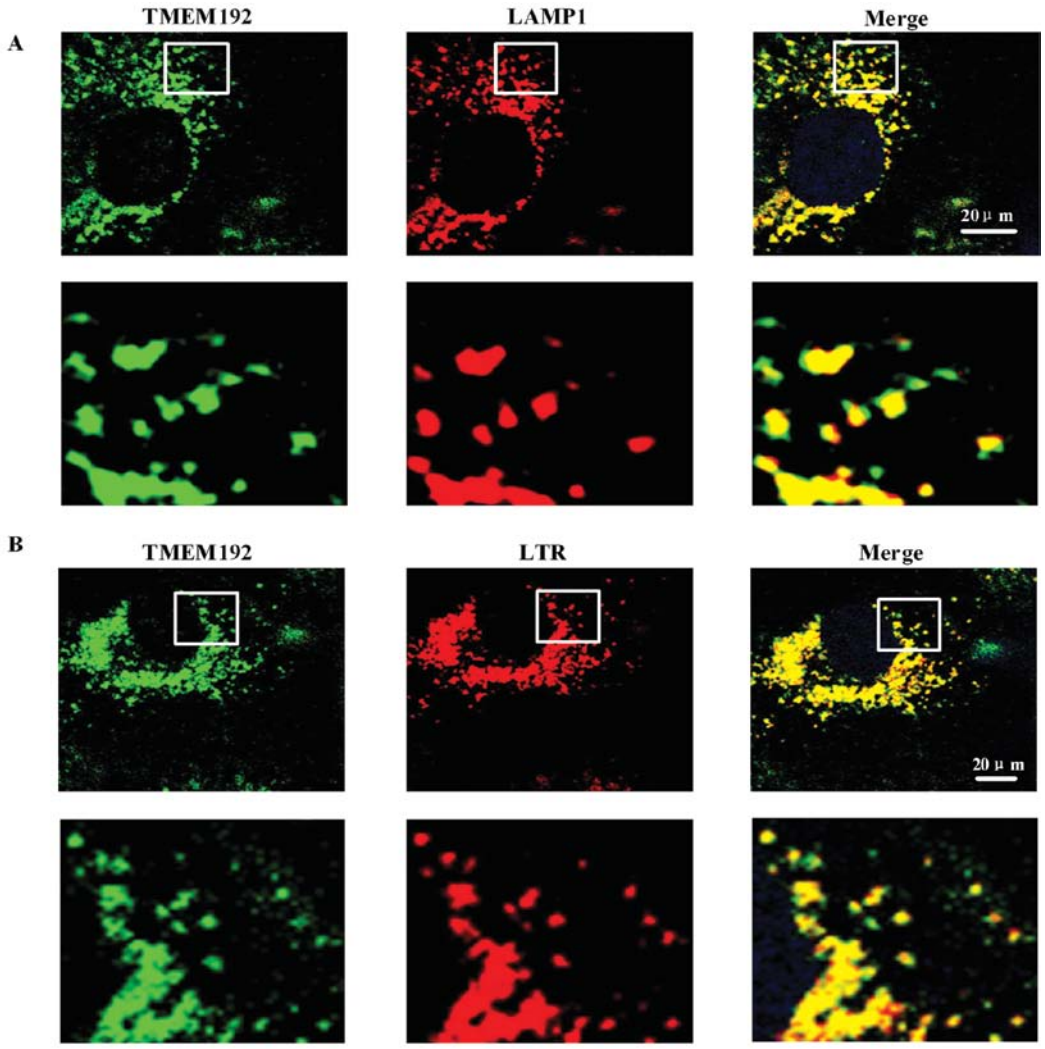

Figure 1. Double immunofluorescence staining of TMEM192 with LAMP1 and LTR. (A) TMEM192 co-localizes with the lysosomal membrane protein LAMP1. HLF-I cells were fixed, permeabilized, and incubated with antibodies to recombinant TMEM192 and LAMP1. Primary antibodies were detected with fluorescein- and Cy3-labelled secondary antibodies. (B) Colocalization of TMEM192 and the LTR. Cells were incubated for 2 h with LTR. Merge reveals colocalization of TMEM192 and LTR. Boxed areas of panel A are shown at higher magnification in the images below; bars, 10 μm.

was detected in normal cell lines (Fig. 2C). The differential expression of TMEM192 was further confirmed by RT-PCR analysis (Fig. 2D).

TMEM192 siRNA induces apoptosis in HepG2 tumor cell lines by upregulating Bax, caspase-3 and p38 MAPK and activating autophagy. According to the experiment above, TMEM192 is highly expressed in tumor cells. To elucidate the potential molecular function of TMEM192, we analyzed the effect of TMEM192 deficiency in HepG2 hepatoma cells and normal LO2 cell lines, by silencing *TMEM192* expression with specific siRNA targeting the *TMEM192* gene. RT-PCR with specific primers demonstrated a marked reduction in the levels of *TMEM192* mRNA in cells transfected with the target siRNA (data not shown). This was consistent with the protein expression data (Fig. 3C). Our results using MTT and FCM have shown that TMEM192 deficiency in HepG2 cells induced growth inhibition and increased apoptosis while LO2 cells were unaffected (Fig. 3A and B). Intracellular TMEM192 has been shown to localize in the lysosome, and may have a function in the lysosomes. In the present experiments, HepG2 cells transfected with TMEM192 siRNA were shown to activate autophagy by detecting the LC3 protein (Fig. 3C). LC3 exists in two forms: the free mature form (LC3-I) and the faster lapidated LC3 (LC3-II). The presence of LC3-II band confirms that the reaction of LC3 conjugation to phospholipids was activated. Caspase-12 is an ER stress-related protein. These cells also showed the presence of an activated degraded caspase-12 fragment (48 kDa, Fig. 3C). Caspase-3, normally present as a zymogen (32 kDa), was detected in its activated 17 kDa form, while caspase-8 was detected only as the zymogen. Synthesis of Bax protein was activated 48 h after transfection. In addition, we found that p38-MAPK was phosphorylated (Fig. 3C). These results indicate that TMEM192 deficiency induces autophagy and apoptosis of HEPG2 cells. It is likely that the apoptosis occurred by upregulating the expression of Bax and caspase-3, as well as the phosphorylation of p38-MAPK.

Blocking autophagy inhibits apoptosis of HEPG2 cells induced by TMEM192 deficiency and downregulates caspase-12, capase-3 and Bax. Atg7 is a crucial autophagy gene.

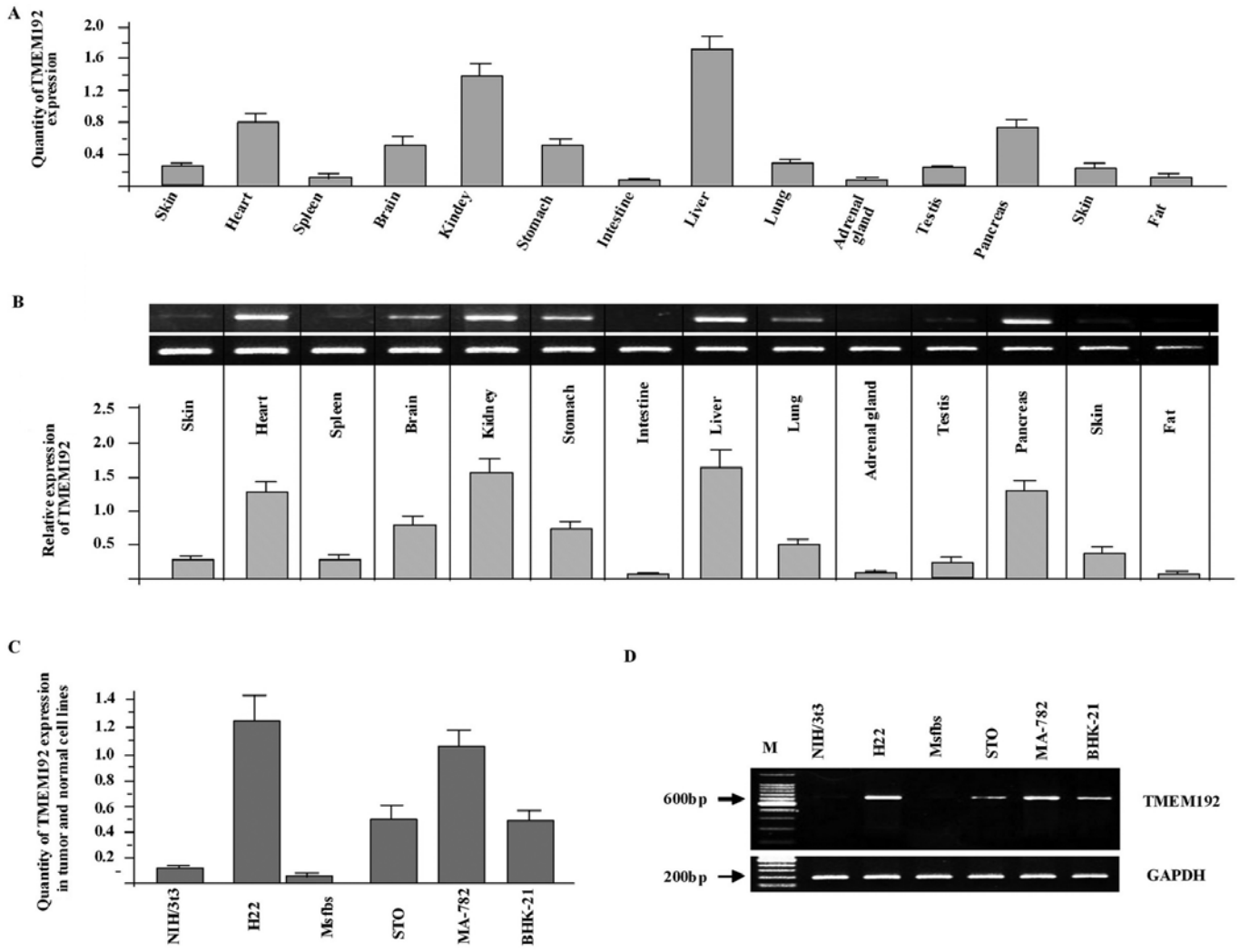

Figure 2. Tissue distribution of TMEM192. (A) Real-time PCR analysis of TMEM192 mRNAs in mouse tissues. (B) RT-PCR analysis of the TMEM192 mRNAs in the tissues indicated (A). Normalized cDNAs derived from different mouse tissues were subjected to PCR to amplify a fragment (200 bp). Normalization was confirmed using primers specific for GAPDH. (C) The detection of TMEM192 expression by real-time PCR in different cell lines. (D) RT-PCR analysis of TMEM192 mRNAs in the above cell lines. GAPDH was used as an internal control.

We used Atg7 siRNA to investigate whether blocking the autophagy would affect the apoptosis of HepG2 cells induced by TMEM192 deficiency. In this experiment, the Atg7 mRNA was silenced successfully using Atg7 siRNA (Fig. 4A). In TMEM192-deficient HepG2 cells, autophagy and apoptosis were inhibited after transfection with Atg7 siRNA. Blocking of the activation of caspase-12, Bax and caspase-3 was also observed (Fig. 4B). These results indicate that TMEM192 siRNA induced apoptosis in HepG2 cells through autophagy and the ER pathway, and this effect was closely related to the mitochondrial pathway, all of which can be suppressed by downregulation of autophagy by silencing the Atg7 gene.

Discussion

In previous proteomic analyses, TMEM192 was identified in lysosomal membranes (13). The exogenous and endogenous localization of TMEM192 was further verified (14), which suggest that TMEM192 is a lysosomal membrane protein. In the present study, using immunofluorescence staining approaches, we also demonstrated that TMEM192 colocalized with the lysosomal marker protein, LAMP1, and the lysosomal tracker, LTR. TMEM192 has tissue-specific expression. In mouse, its ortholog is highly expressed in the liver and brain but shows little or low expression in spleen and other tissues. Importantly, TMEM192 was highly expressed in tumor cell lines, whereas lower expression was detected in normal cell lines. This distribution may suggest the important function of TMEM192. In order to investigate the potential role of TMEM192 in tumor cell line proliferation and growth, we obtained TMEM192-deficient HepG2 cells using RNAi. HepG2 cells deficient in TMEM192 showed growth inhibition.

Autophagy refers to the highly regulated and evolutionarily conserved process of turnover and maintenance of cellular components that is required for cellular homeostasis (1). Autophagy and apoptosis are partners in cell life and death. They crosstalk, interact and share common regulatory pathways (15). In this study, our results show that TMEM192 siRNA activated autophagy accompanied by an increased

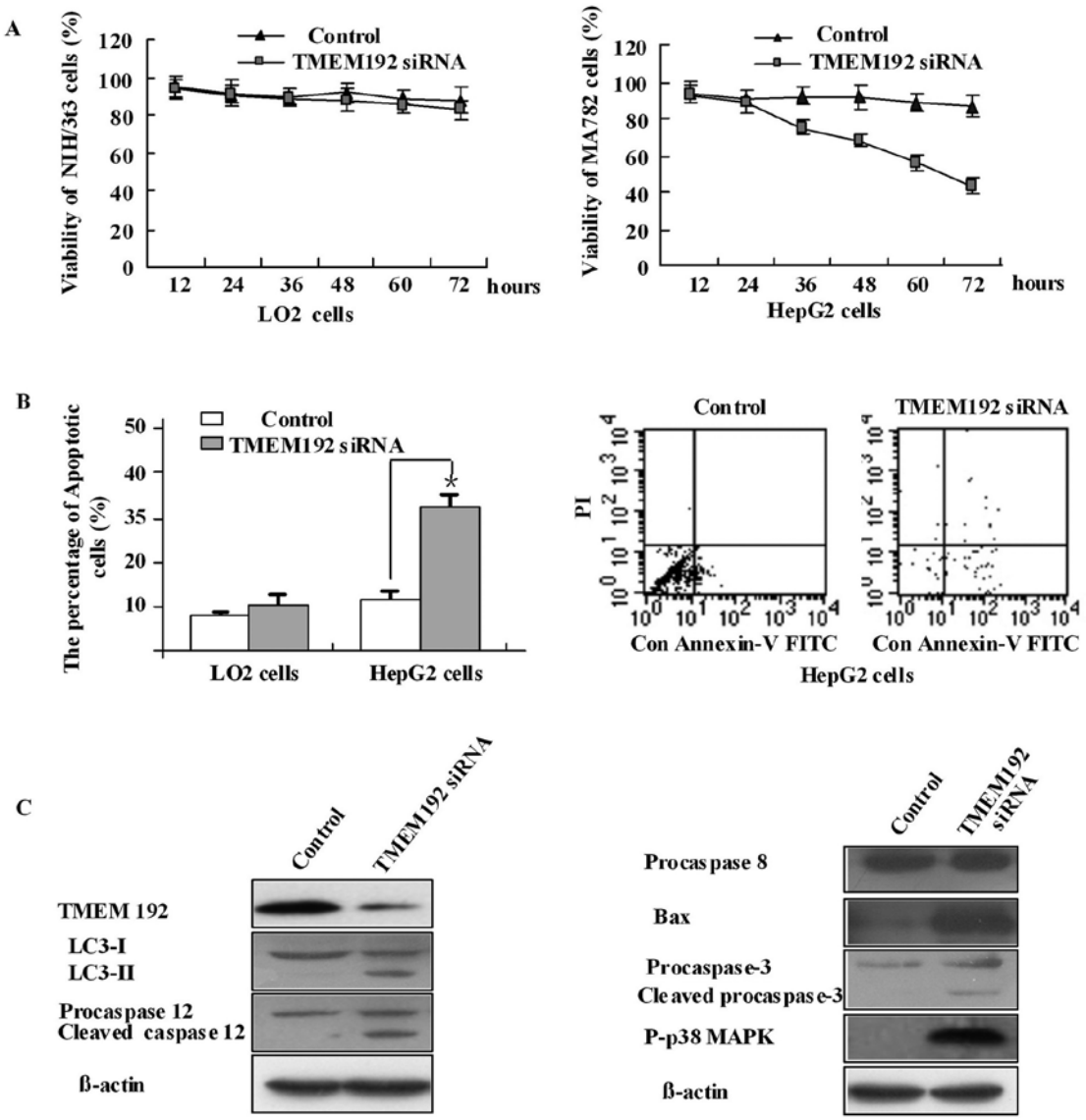

Figure 3. Knockdown of TMEM192 expression induces growth inhibition, autophagy and apoptosis in HepG2 cells. (A) LO2 and HepG2 cells growth curve. The cells were cultured and MTT detecting in 1-6 days respectively. For detecting, two cell lines were cultured and transfected with TMEM192 siRNA after 24 h. In order to maintain the RNAi effect, cells were transfected additionally with target siRNA after each 24-h interval, which treatment was up to the 6th day. (B) Percent of apoptotic cells determined by FCM in 3 days after transfection with target siRNA. Data are expressed as the average of triplicate samples ± SD (n=9, *P<0.05). (C) HepG2 cells were treated for 72 h with TMEM192 siRNA. Apoptosis related proteins and LC3 were detected by western blotting. β-actin was used as the internal control. All experiments were repeated in triplicate.

expression of LC3-II. In addition, apoptosis was induced as evidenced by caspase-12 activation. Autophagy and apoptosis were inhibited after treatment with Atg7 siRNA, which indicates that the autophagy pathway plays a critical role in apotosis of HepG2 cells.

Studies have shown that caspase activation through the death receptor pathway, the mitochondrial pathway, and the ER pathway causes cell apoptosis. Caspase-8 is the primary initiator of the death receptor pathway, while caspase-3 is the main caspase effector in the mitochondrial pathway (16,17). Bax plays an important role in the mitochondrial apoptosis pathway (18,19). In HepG2 cells, we found that RNAi of TMEM192 increased the expression of Bax and caspase-3, as well as the phosphorylation of p38 MAPK, without affecting the levels of caspase-8. When Atg7 siRNA was used to block the autophagy, however, expression of Bax and caspase-3 was significantly downregulated. Since caspase-12 is a key factor in ER stress, we therefore, hypothesized that TMEM192 siRNA induces ER stress in HepG2 cells by activating caspase-12, and further activates Bax and the phosphorylation of p38 MAPK. In turn this induces a translocation of Bax from the cytosol into the mitochondria promoting the release of cytochrome c, which finally activates caspase-3 resulting in tumor cell apoptosis.

Autophagy is controlled by autophagy-related genes, many of which are involved in autophagosome formation. This process features conjugation systems that are well-conserved among eukaryotes: Atg5-12 and Atg8 (LC3)-phosphatidylethanolamine systems. In this study, we have shown that TMEM192 deficiency can induce autophagy concurrently with apoptosis in HepG2 hepatoma cells. Previous studies have shown that there is crosstalk between

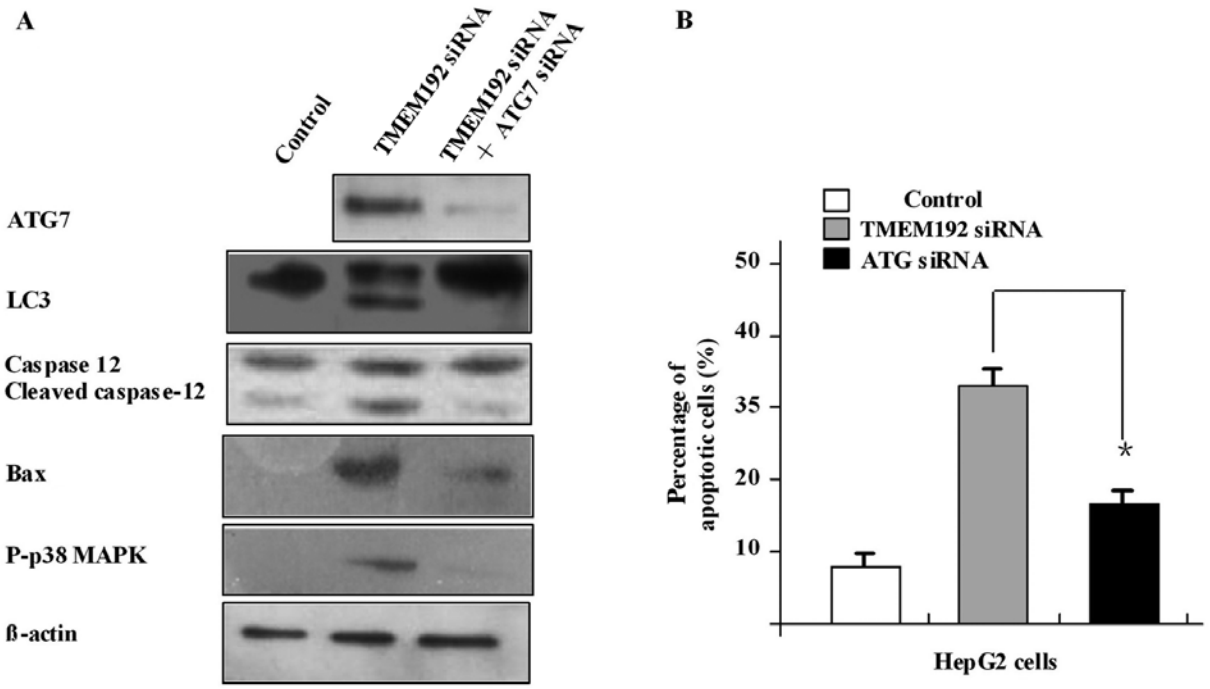

Figure 4. Blocking autophagy inhibits apoptosis in HepG2 cells transfected with TMEM192 siRNA. (A) HepG2 cells were treated with control siRNA, TMEM192 siRNA, and TMEM192 siRNA + Atg7 siRNA respectively. Cells were harvested and western blotting assay was performed 72-h post-transfection. Results were repeated in triplicate, and β-actin was used as the internal standard. (B) Percentages of apoptotic cells detected by FCM after 72-h treatment with siRNA. Results are expressed as the average of triplicate samples ± SD (N=9, $^{*}P<0.05$).

apoptosis and autophagy (15). Although the two processes are markedly different, several pathways regulate both the autophagic and the apoptotic machinery and autophagy can cooperate with apoptosis. For example, alterations in the mitochondrial membrane potential-induced Bax/Bak ratio can signal to both apoptosis and mitochondrial autophagy (20). Indeed, earlier studies have shown that mitochondria that have sustained stress-induced damage can be removed by selective mitochondrial autophagy (21-24). There is a consensus that autophagy itself is usually a survival mechanism (25). However, it is possible that autophagy can contribute to the induction of some death responses, such as apoptosis, in light of the fact that the coordinate activation of both autophagic and apoptotic signals were induced by TMEM192 siRNA. By knockdown of the Atg7 expression, we found that autophagy was inhibited by the reduction of LC3-II expression. Interestingly, caspase-12 and Bax-independent apoptosis was reversed. The impact of autophagy on apoptosis is highly context-dependent, with compelling evidence that autophagy can either inhibit or promote apoptosis, depending on the system. In tumor cells, the relations between autophagy and apoptosis are more complicated (10,26).

Lysosomal membrane proteins have been associated with autophagy. For example, deficiency of mice or cells in lysosomal protein, such as LAMP1 and LAMP2, resulted in increased autophagy, while other proteins, such as LAPTM4B, exhibited promotion of autophagy. In the tumor cells, TMEM192 deficiency can also activate the autophagy signaling pathway which can promote apoptosis independently of the caspase-12 and Bax pathway. None of these results were found in normal cells (data not shown). In summary, this study offers proof of crosstalk between autophagy and apoptosis. TMEM192 promotion of autophagy may be a new route for tumor therapy.

Acknowledgements

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