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Rottlerin induces autophagy which leads to apoptotic cell death through inhibition of PI3K/Akt/mTOR pathway in human pancreatic cancer stem cells

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ABSTRACT

Multiple lines of evidence support the idea that autophagy plays an essential role in the development of drug resistance, self-renewal, differentiation, and tumorigenic potentials of cancer stem cells (CSCs). Rottlerin (ROT) is widely used as a protein kinase C-delta (PKC-δ) inhibitor. Recent studies revealed that ROT induces apoptosis through engagement of mitochondria. However, it is not known whether ROT-induced apoptosis is associated with other mechanisms such as autophagy. Here we found that ROT induced autophagy followed by induction of apoptosis via inhibition of PI3K/Akt/mTOR pathway and activation of caspase cascade in human pancreatic CSCs. ROT induced a dose- and time-dependent inhibition of cell survival and induction of cytoplasmic vacuolations. The conversion of microtubule-associated protein LC3-I to LC3-II, and increased accumulations of *Atg7* and Beclin-1 were also observed in CSCs treated with ROT. Prolonged exposure of CSCs to ROT eventually caused apoptosis which was associated with the suppression of phosphorylated Akt (Ser473) and mTOR (Ser2448), downregulation of XIAP, cIAP-1, Bcl-2 and Bcl-X_L, induction of Bax, activation of caspase-3 and -9, and concomitant degradation of PARP. ROT-induced apoptosis was enhanced by dominant negative AKT, Akt1/2 inhibitor, and rapamycin. Our study also demonstrates that gene silencing of Atg7 and Beclin1, or cotreatment of the autophagosome inhibitor, 3-methyladenine, inhibited ROT-induced autophagy and accelerated ROT-induced apoptosis. The knockdown of PKC-δ did not block ROT-induced autophagy and cell death, suggesting these effects of ROT were exerted through PKC-δ-independent pathway. In summary, our data demonstrate that ROT can induce autophagy which leads to cell death in pancreatic CSCs.

1. Introduction

Autophagy is considered as a cell survival pathway that plays roles in development [1], immunity [2], and cell death [3] and has been implicated in neurodegeneration, autoimmunity, and cancer [4]. Recent studies have reported on the induction of autophagy at early stage after treatments with chemotherapeutic agents [5,6]. Accumulating evidence suggests that cancer cells tend to have reduced autophagy relative to their normal counterparts and premalignant lesions [7]. However, several recent studies revealed that Ras-driven cancer have elevated basal autophagy, required for growth of cells [8,9]. Espina et al. also found elevated basal autophagy in ductal carcinoma in situ [10]. Autophagy takes place at basal levels but is also regulated developmentally and/or by environmental stimuli, such as nutrient/energy availability, hypoxia and reactive oxygen species. Several *Atg* proteins have been implicated in autophagosome formation [11]. Of these, *Atg7* is required to recruit other proteins to the autophagosomal membrane and to form the autophagic vacuole in a pathway [12,13]. Moreover, an important autophagy-regulatory gene such as Beclin-1 functions as a haploinsufficient tumor suppressor gene [14], further emphasizing the clinical importance of autophagic cell death. In some circumstances, both autophagy and apoptosis have been observed in human cancers [15,16], and both may be interconnected by some signaling pathways [17]. Despite these advances, the relationship between autophagy and apoptosis is still not well understood.

Cancer stem cells (CSCs) comprise a subset of hierarchically organized, rare cancer cells with the ability to initiate cancer of genetically modified murine models [18]. CSCs may be responsible

Abbreviations: CSCs, cancer stem cells; PARR, (ADP-ribose) polymerase (PARP); PKC-δ, protein kinase C delta; ROTT, rottlerin.

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for tumor onset, self-renewal/maintenance, mutation accumulation, and metastasis [19]. We have recently reported the existence of pancreatic CSCs in humans and $Kras^{G12D}$ mice [20]. The existence of CSCs could explain the high frequency of cancer relapse and resistance to chemotherapy. The PI3K/Akt/mTOR signaling pathway is a key regulator of physiological cell processes which include proliferation, differentiation, apoptosis, motility, metabolism, and autophagy. In CSCs, autophagy plays an essential role in the regulation of drug resistance, self-renewal, differentiation, and tumorigenic potential [21], suggesting autophagy could be a promising therapeutic target in a subset of cancers. Thus activating autophagy may abrogate the resistance of CSCs to chemotherapy and could lead to the development of novel therapeutic approaches for the treatment of various cancers.

Rottlerin (ROT) has been used as a protein kinase C-delta (PKC-δ) signaling pathway inhibitor to verify the biological function of PKC-δ [22]. It inhibits cell proliferation and induces apoptosis through mitochondrial membrane depolarization [23]. However, it also acts as an uncoupler of mitochondrial oxidative phosphorylation in a PKC-δ-independent manner [24]. Recently, in several human cancer cells, ROT has been shown to induce a starvation response, which is a key regulator of autophagy causing its induction [25]. Since pancreatic cancer contains pancreatic CSCs, we sought to examine the molecular mechanism by which ROT induces autophagy in pancreatic CSCs.

The main objective of the paper is to examine the molecular mechanisms by which ROT induces autophagy in pancreatic CSCs. Here we reported that ROT-induced early autophagy is mainly dependent on induction of autophagosomes, conversion of LC3-I to III, induction of *Atg7* and Beclin-1 and inhibition of Bcl-2 and Bcl-X_L. Eventually, ROT induced apoptosis through inhibition of PI3K/Akt/mTOR pathway and activation of caspases. ROT-induced apoptosis was enhanced by dominant negative AKT, Akt1/2 inhibitor, and rapamycin (mTOR inhibitor). Moreover, inhibition of *Atg7* and Beclin-1 enhanced apoptosis-inducing potential of ROT. These findings strongly suggest that ROT-induced autophagy may play some role as a survival mechanism against apoptosis.

2. Methods and materials

2.1. Reagents and cell culture

Rottlerin, 3-methyladenine, Akt1/2 inhibitors, puromycin, rapamycin, and phenazine methosulfate were from Sigma–Aldrich Corp. (St. Louis, MO). Anti-human LC3, Atg7, Beclin-1, PKC-δ, Bak, Bcl-2, Bcl-X_L, Bax, cIAP-1, Akt, pAkt, mTOR, pmTOR and XIAP were from Cell Signaling Technology (Danvers, MA).

Human pancreatic CSCs (CD44+/CD24+/ESA+) were characterized and described previously [20]. CSCs were grown in DMEM culture medium with 1% N2 Supplement (Invitrogen, Grand Island, NY), 2% B27 Supplement (Invitrogen), 20 ng/ml human platelet growth factor (Sigma–Aldrich), 100 ng/ml epidermal growth factor (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.2. Western blot analysis

After drug treatment, whole cell lysates were extracted using RIPA lysis buffer containing 1× protease inhibitor cocktail. Cell lysates were loaded and separated on 10.0% Tris–HCl gel. Proteins from the gel were transferred on polyvinylidene difluoride (PVDF) membranes and subsequently blocked in blocking buffer [5% nonfat dry milk in 1× Tris buffer saline] and probed with primary antibody at 4 °C for overnight. After washing, the blots were incubated with secondary antibody conjugated with horseradish peroxidase at a dilution of 1:5000 for 1 h at room temperature. Membranes were developed using chemiluminescence kit.

2.3. Electron microscopy

To demonstrate the induction of autophagy in ROT-treated cells morphologically, cells treated with or without ROT (0.5, 1 and 2 μM) for 24 h were harvested by trypsinization, washed and fixed in 2.0% glutaraldehyde in 0.1 M phosphate buffer, then post-fixed in 1% osmium tetroxide buffer. After dehydration in a graded series of ethanol, the cells were embedded in spur resin. Thin sections (60 nm) were cut on an Ultramicrotome. The sectioned grids were stained with saturated solutions of uranyl acetate and lead citrate. The sections were examined by electron microscope.

2.4. Lentiviral particle production and PKC-δ, Atg7 and Beclin-1 transduction

PKC-δ shRNA, *Atg7* shRNA and Beclin-1 shRNA were obtained from Open Biosystems (Lafayette, CO). Lentivirus particles were produced by triple transfection of HEK 293T cells. Packaging 293T cells were plated in 10-cm plates at a cell density of 5×10^6 a day prior to transfection. Transfection of packaging cells and infection of pancreatic CSCs were carried out using standard protocols [26] with some modifications. In brief, 293T cells were transfected with 8 μg of plasmid and 4 μg of lentiviral vector using lipid transfection (Lipofectamine-2000) according to the manufacturer's protocol. Viral supernatants were collected and concentrated by adding PEG-it virus precipitation solution (SBI System Biosciences, Mountain View, CA). Pancreatic CSCs stably expressing PKC-δ, *Atg7* and Beclin-1 were generated.

2.5. Cell viability analysis

Cell viability was determined by the XTT assay as we described elsewhere [27].

2.6. Measurement of apoptosis

The apoptosis was measured as described [20].

2.7. Immunocytochemistry for Beclin-1 expression

Pancreatic CSCs were grown on fibronectin-coated coverslips (Beckton Dickinson, Bedford, MA) in the absence or presence of ROT (0.5, 1 and 2 μM) for 24 h. Subsequently, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in 1× PBS, washed and blocked in 10% normal goat serum. After washing with PBS, cells were stained with Beclin-1 primary antibodies (1:50) for 16 h at 4 °C and washed with PBS. Afterwards, cells were incubated with fluorescently labeled secondary antibody (1:200) along with DAPI (1 mg ml^{-1}) for 1 h at room temperature. Finally, coverslips were washed and mounted. Isotype-specific negative controls were included with each staining. Stained cells were mounted and visualized under a fluorescent microscope.

2.8. Statistical analysis

Results were representative of multiple experiments and expressed as means ± S.E. Statistical analysis was performed with an analysis of variance (ANOVA) followed by the Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. ROT decreased cell viability, induced apoptosis and caused vacuolation in pancreatic CSCs

The serum free medium (nutrient deprivation) causes stress to cells and triggers autophagy so that cell can survive [28]. To investigate the cytotoxic effect of ROT on the human pancreatic CSCs, we treated CSCs with different concentrations of ROT (0.5, 1 and 2 μM) for various time points (12, 24 and 48 h). ROT inhibited cell viability in a time- and dose-dependent manner (Fig. 1A). While the treatment with 0.5 μM ROT had little effect, treatments with 1 or 2 μM ROT for 48 h significantly inhibited cell viability. Since ROT inhibited cell viability in pancreatic CSCs, we next measured induction of apoptosis by ROT (Fig. 1B). ROT induced apoptosis in pancreatic CSCs in a dose-dependent manner. Moreover, the pancreatic CSCs treated with ROT showed morphological features of cytoplasmic vacuole accumulation when cultured in the presence (CM; complete medium) or absence of serum (SFM; serum free medium, autophagy stimulant). ROT increased more number of vacuoles formation in the cytoplasm of pancreatic CSCs under SFM than those in CM (Fig. 1C).

3.2. Induction of autophagy in ROT-treated pancreatic CSCs

LC3, the mammalian equivalent of yeast *Atg8*, is one method that can be used to monitor autophagy [29]. A hallmark of mammalian autophagy is the conversion of LC3-I to LC3-II via proteolytic cleavage and lipidation. This modification of LC3 is essential for the formation of autophagosomes and for the completion of macroautophagy [30]. To verify whether LC3 (autophagosomes) is redistributed after ROT treatment, we observed the CSCs after transfection of pEGFP-LC3 (Fig. 2A). Cells were cultured in both CM and SFM conditions, treated with or without ROT and subjected to immunofluorescence for visualization of LC3-II. Our results indicated that serum deprivation induced more autophagy than complete medium (CM). ROT-induced autophagy was enhanced in SFM than that in CM (Fig. 2A). 3-Methyladenine (3-MA), an inhibitor of the enzyme phosphatidylinositol 3-kinase class III, is essential for the autophagic process [31]. The autophagy inducing potential of ROT was partially reverted with 3-MA (Fig. 2A), indicating that inhibition of PI3K class III reduced the number of cells undergoing autophagy. We next counted and graded CSCs based on abundance of LC3-II positive staining. The number of LC3-II positive CSCs and severity

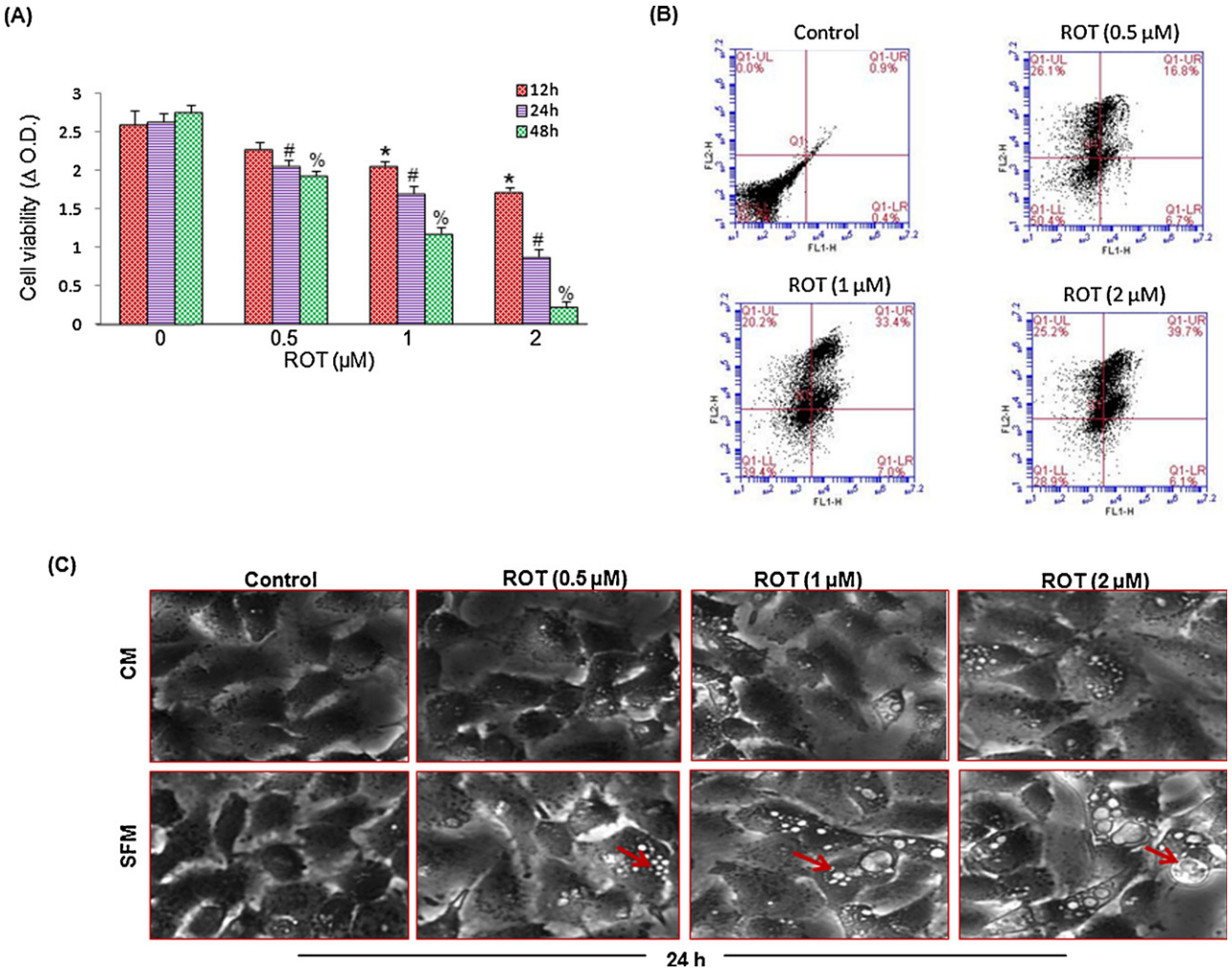

Fig. 1. Rottlerin (ROT) inhibited cell viability, and induced apoptosis and cytoplasmic vacuolation in pancreatic cancer stem cells (CSCs). (A) Cells were grown in complete medium and treated with or without ROT (0.5, 1 and 2 μM) for different time periods. Cell viability was determined by XTT assay as described in Section 2. The results are expressed as mean ± S.E. ($n = 4$). ★, # and %, significantly different from respective control ($P < 0.05$). Red color, 12 h; violet, 24 h; and green, 48 h. (B) Pancreatic CSCs were treated with ROT (0–2 μM) for 48 h, and the apoptosis was measured by annexin/PI staining. (C) Cells were cultured in complete medium and treated with the different concentrations of ROT (0.5, 1 and 2 μM) for 24 h. Representative images were obtained by phase contrast microscopy. Magnification, 200×. Red arrows indicate cytoplasmic vacuoles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

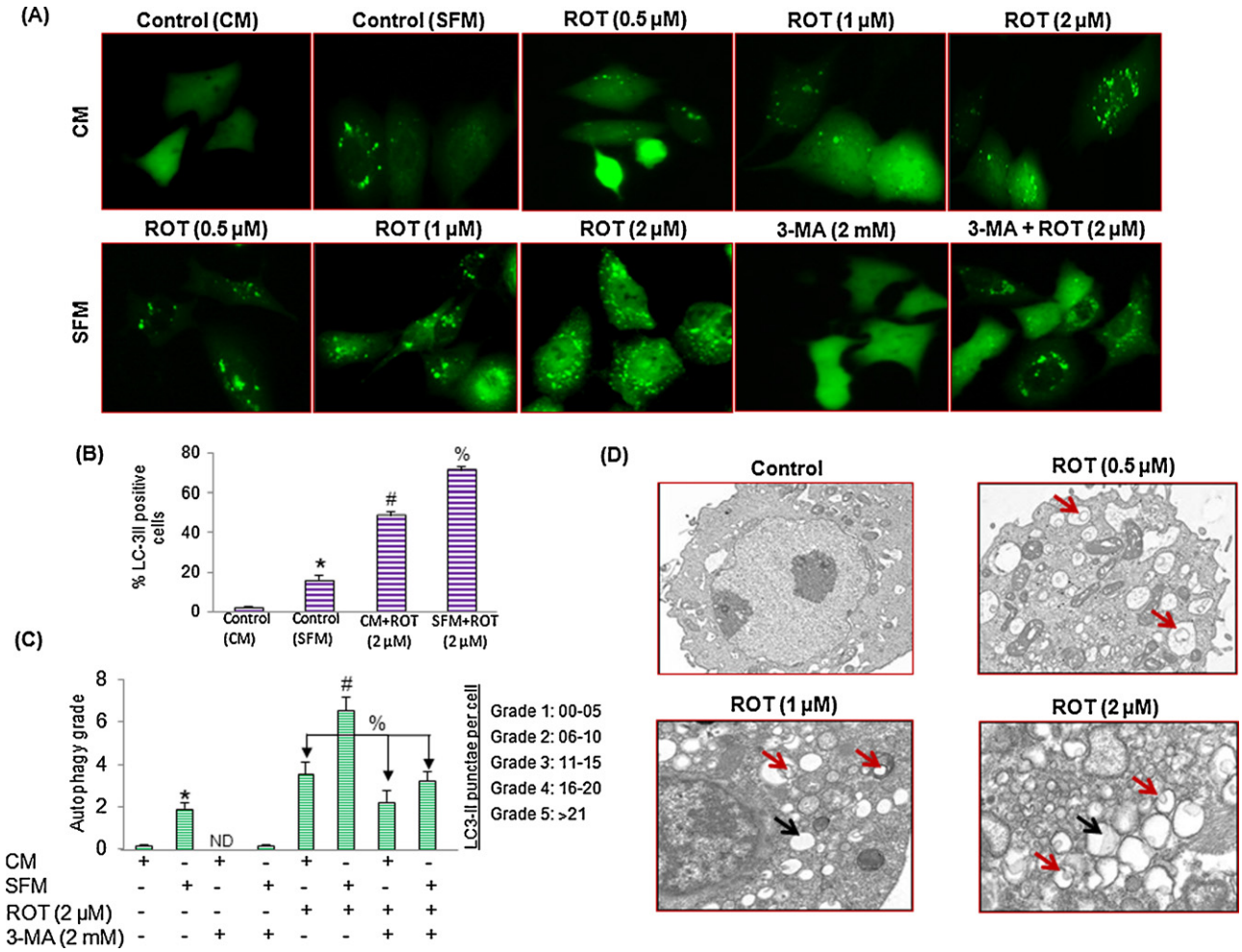

Fig. 2. Autophagic cell death induced by ROT in pancreatic CSCs. (A) Redistribution of GFP-LC3. Pancreatic CSCs were stably transfected with a pEGFP-LC3 fusion construct and cultured either in complete serum media (CM) or serum free media (SFM), and treated with or without 0.5, 1.0 and 2.0 μM ROT in the presence or absence of autophagy inhibitor 3-methyladenine (3-MA) for 24 h. Cells were visualized under a fluorescence microscope to examine the expression of LC3-II. (B) Quantitation of autophagic cells based on % GFP-LC-3-positive cells with GFP-LC3 dots. Quantitation represents at least 100 cells counted and scored per treatment. (C) Autophagic grade based on the number of cells with punctate pattern. Data are representative of triplicate experiments. The results are expressed as mean ± S.E. ($n = 3$). ★, # and %, significantly different from respective control ($P < 0.05$). (D) Electron microscopy shows the ultrastructure of pancreatic CSCs treated with different concentrations of ROT in complete medium for 24 h. Arrows indicate autophagosomes including residual digested material (red arrow) and empty vacuoles (black arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of autophagic response per cell (number of autophagosomes present per cell) was increased following ROT treatment at 24 h, and regardless of serum (Fig. 2B and C).

To examine whether cell vacuolation induced by ROT is related to autophagy, pancreatic CSCs were treated with ROT (0.5, 1 and 2 μM) for 24 h, and the ultrastructure of the cells was analyzed by electron microscopy. Numerous autophagic vacuoles containing lamellar structures or residual digested material and empty vacuoles were observed in the pancreatic CSCs when treated with 1 and 2 μM of ROT, indicating that ROT not only increased the number of vacuoles, but also increased the number of mature autophagosomes formed per cell (Fig. 2D).

3.3. Molecular evidence for autophagy induction by ROT

To determine if ROT regulates autophagy at 24 h, we first examined the levels of LC3-II, which is an LC3-phosphatidyl-ethanolamine conjugate and a promising autophagosomal marker [32]. ROT induced an increase in the lipidated form of LC3 (LC3-II) at 24 h, further evidence for the induction of autophagy at early stage (Fig. 3A). However, ROT-induced conversion of LC-3I to LC-3II was not observed at 48 and 72 h.

We next measured the expression of autophagy-related proteins LC-3, Atg-7, Beclin-1, Bcl-2 and Bcl-X_L in CSCs treated with ROT under both conditions (CM, SFM). ROT treatment of CSCs resulted in a decrease in LC3-I protein and a concomitant increase in LC3-II in both CM and SFM (Fig. 3B). In addition, the levels of *Atg7* and Beclin-1 expression were gradually increased following the ROT treatment. These results indicate that ROT stimulated not only the conversion of a fraction of LC3-I into LC3-II but also caused the accumulation of *Atg7* and Beclin-1 proteins. The cellular levels of Bcl-2 and Bcl-X_L proteins were significantly decreased after the treatments with ROT for 24 h (Fig. 3B). The accumulation of *Atg7* and Beclin-1 proteins may be mediated by the reduction in Bcl-2 and Bcl-X_L expression.

To assess how the pro-apoptotic effect of ROT was linked to the autophagy signal, we used 3-MA. Treatment of CSCs with 3-MA inhibited ROT-induced conversion of LC-3, and induction of *Atg-1* and Beclin-1 (Fig. 3C), suggesting that ROT has potential to induce autophagy in CSCs. To confirm the role of Beclin-1, we next examined the expression of Beclin-1 in presence or absence of ROT in CSCs by fluorescence microscopy (Fig. 3D). ROT increased expression of Beclin-1 in CSCs. However, the expression was higher with 2 μM ROT.

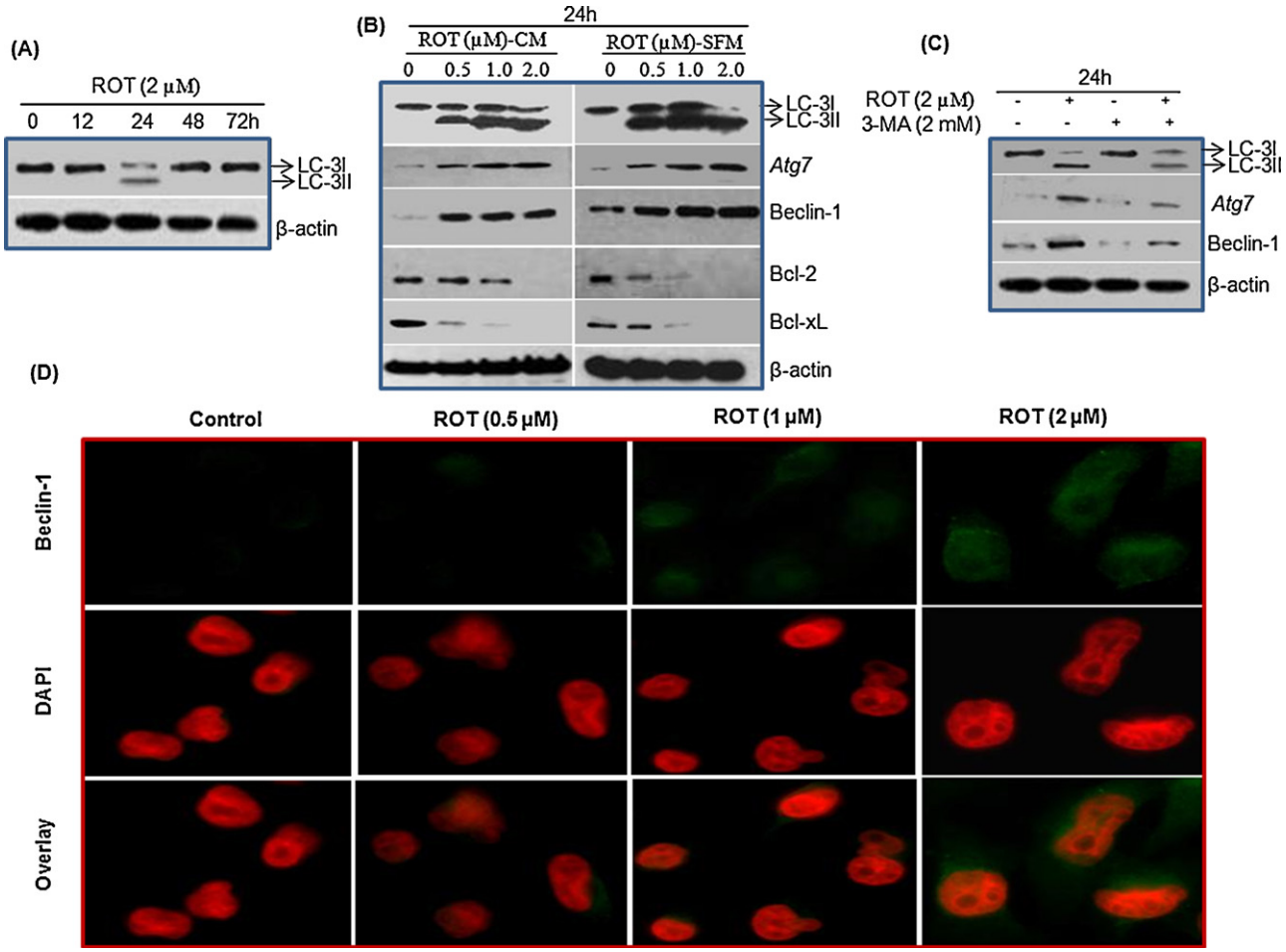

Fig. 3. ROT regulates autophagy-related genes in pancreatic CSCs. (A) Conversion from LC-3I to LC-3II by ROT. Pancreatic CSCs were grown in complete medium and treated with ROT (2 μM) for 0, 12, 24, 48 and 72 h; the cells were lysed and cellular proteins were then separated in SDS–polyacrylamide gels, after which they were transferred onto nitrocellulose membranes. The membrane was then probed with anti-LC3 antibody or β-actin antibody. (B) Pancreatic CSCs were grown either in complete medium (CM) or and serum free medium (SFM) and treated with ROT (0, 0.5, 1 and 2 μM) for 24 h. The Western blot analysis was performed to measure the expression of LC3, *Atg7*, Beclin-1, Bcl-2, and β-actin. (C) Pancreatic CSCs were pre-incubated with 3-MA (2 mM) for 2 h, followed by treatment with ROT (2 μM) in complete medium for 24 h. The Western blot analysis was performed to measure the expression of LC-3, Atg7 and Beclin-1. β-Actin was used as a loading control. (D) ROT induces expression and nuclear localization of Beclin-1 in human pancreatic CSCs. Cells were seeded on fibronectin-coated coverslips and treated with different concentrations of ROT (0.5, 1 and 2 μM) in complete medium for 24 h. Subsequently, cells were fixed with 4% paraformaldehyde, blocked with 10% normal goat serum and stained with anti-Beclin-1 antibody (1:200) for 16 h at 4 °C and washed with PBS. Afterwards, cells were incubated with fluorescently labeled secondary antibody (1:200) along with DAPI (1 μg/ml) for 1 h at room temperature and cells were mounted and visualized under a fluorescent microscope.

3.4. ROT induced apoptotic cell death via PKC-δ-independent pathway

PKC-δ is a potent inhibitor of autophagy in pancreatic cancer cell lines [33]. We examined the effect of ROT on induction of autophagy in pancreatic CSCs by inhibiting the expression of PKC-δ by shRNA. First, we confirmed that PKC-δ protein levels in CSCs transduced with PKC-δ shRNA by the Western blot analysis. PKC-δ shRNA inhibited the expression of PKC-δ protein in CSCs (Fig. 4A, upper left panel).

We next examined whether inhibition of PKC-δ modulate ROT-induced autophagy (Fig. 4A, upper right panel and bottom). Pancreatic CSCs transduced with scrambled shRNA and PKC-δ shRNA were treated with different concentrations of ROT (0.5, 1 and 2 μM) for 24 h, and the formation of autophagosomes was examined by fluorescent microscopy and quantified. ROT induced the formation of autophagosomes in CSCs/PKC-δ scrambled cells. The inhibition of PKC-δ expression by PKC-δ shRNA enhanced ROT-induced autophagosomes formation.

Since PKC-δ shRNA enhanced ROT-induced autophagy, we next examined the effects of overexpression of PKC-δ on ROT-induced autophagy (Fig. 4B). We overexpressed PKC-δ in pancreatic CSCs as demonstrated by the Western blot analysis (insert Fig. 4B). ROT induced autophagy in CSCs transfected with empty vector. By comparison, overexpression of PKC-δ inhibited ROT-induced autophagy. However, PKC-δ did not completely block ROT-induced autophagy, suggesting other pathway may mediate ROT-induced autophagy.

To molecularly confirm the induction of autophagy, we measured the expression of autophagy-related proteins such as LC3-II, *Atg7* and Beclin-1 in scrambled shRNA and sh-PKC-δ CSCs (Fig. 4C). Noteworthy, ROT treatment of CSCs resulted in an increase in LC3-II, *Atg7* and Beclin-1 proteins in both scrambled and sh-PKC-δ CSCs. These results indicate that the autophagy-inducing potential of ROT was PKC-δ-independent.

PKC-δ is involved in cell migration and apoptosis in various cell types [34]. Although ROT was originally identified as a specific inhibitor of PKC-δ and was shown to have anti-carcinogenic properties [35], it also act in a PKC-δ-independent manner [23]. To confirm whether the PKC-δ is related to ROT-induced apoptotic cell death, we used flow cytometry. ROT did not significantly induce apoptosis in scrambled shRNA and sh-PKC-δ cells at 12 and

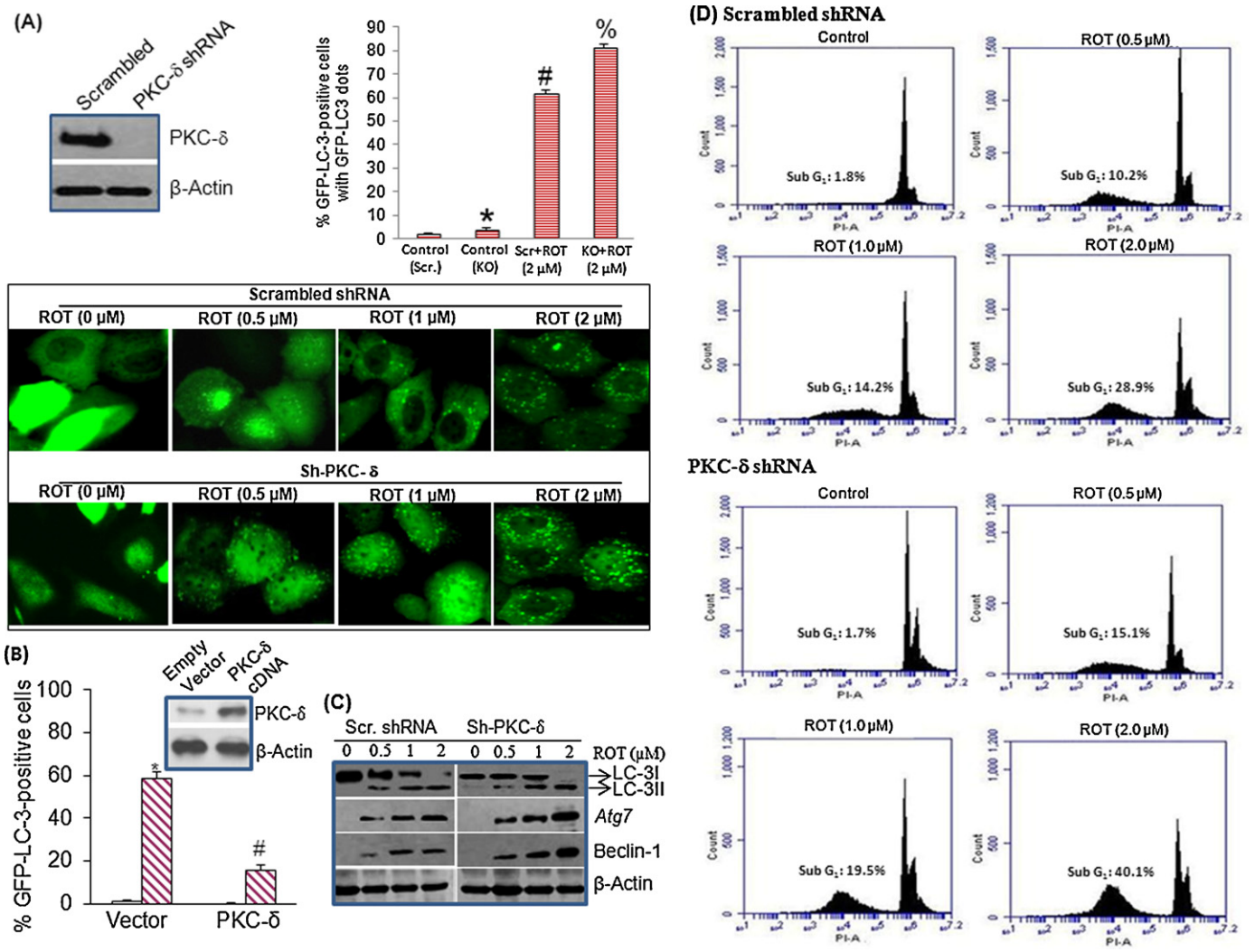

Fig. 4. Role of PKC-δ in ROT-induced autophagy and apoptosis. (A) Upper left panel: pancreatic CSCs were stably transduced with PKC-δ-targeted shRNA. Cell lysates were prepared and the expression of PKC-δ and β-actin was measured by the Western blot analysis. Upper right panel: quantification of autophagic CSCs. Stably transduced pancreatic CSCs with scrambled shRNA and sh-PKC-δ were transfected with a pEGFP-LC3 fusion construct. These CSCs were treated with ROT (0, 0.5, 1 and 2 μM) for 24 h. GFP-LC3-positive cells were quantified. The results are expressed as mean ± S.E. ($n = 3$). ★, # and %, significantly different from respective control ($P < 0.05$). Lower panel, expression of pEGFP-tagged LC3. Stably transduced pancreatic CSCs were treated with ROT as described above, and subjected to fluorescence microscopy. (B) PKC-δ blocks ROT-induced autophagy. Pancreatic CSCs expressing empty vector or PKC-δ were co-transfected with pEGFP-tagged LC3 and treated with ROT (2 μM) for 24 h. GFP-LC3 positive cells were counted as described above. Data are the means of triplicate experiments. Insert shows the overexpression of PKC-δ by the Western blot analysis. (C) Scrambled shRNA and sh-PKC-δ pancreatic CSCs were treated with or without ROT (0.5, 1 and 2 μM) in complete medium for 24 h. The Western blot analysis was performed to measure the expression of LC-3, *Atg7*, Beclin-1 and β-actin. (D) Scrambled shRNA and sh-PKC-δ pancreatic CSCs were treated with ROT (0.5, 1 and 2 μM) for 48 h, and apoptosis was measured by PI staining followed by flow cytometry. Data are the means of triplicate experiments.

24 h (data not shown), but significantly induced apoptotic cell death at 48 h (Fig. 4D). PKC-δ inhibition by shRNA enhanced ROT-induced apoptosis.

3.5. ROT induced apoptotic cell death via inhibition of PI3K/Akt/mTOR pathway and activation of caspase-3 and -9

PI3K/Akt/mTOR signaling pathway is well-known pathway involved in the regulation of cell cycle, cellular transformation, cell growth, and tumorigenesis [36]. To investigate the upstream inhibition of mTOR by ROT, we examined Ser473 phosphorylation of Akt. As shown in Fig. 5A, treatment with ROT decreased the levels of phosphorylated Akt and mTOR in pancreatic CSCs. These data suggest that ROT induces autophagy by inhibiting PI3K/Akt/mTOR pathway.

Next, we performed experiments to confirm whether ROT-induced cell death is associated through the PI3K/Akt pathway at 48 h. Here, we used wild type Akt (WT-Akt), myristoylated Akt (myr-Akt) and dominant negative Akt (DN-Akt) which have been previously described [37]. Human pancreatic CSCs were transfected with WT-Akt, myr-Akt, and DN-Akt and treated with ROT for 48 h (Fig. 5B). ROT induced cell death in CSCs transfected with empty vector. Overexpression of WT-Akt and myr-AKT inhibited ROT-induced cell death. Interestingly, overexpression of DN-Akt enhanced ROT-induced cell death, indicating the involvement of Akt pathway in ROT-induced cell death. We next used the pharmacological approach to inhibit Akt. As expected, ROT induced cell death in the absence of Akt1/2 inhibitor. Interestingly, Akt1/2 inhibitor enhanced ROT-induced cell death, suggesting ROT induced cell death by inhibiting Akt in pancreatic CSCs. Several lines of evidences support the hypothesis that resistance to rapamycin results from a positive feedback loop from mTOR/Akt, resulting in enhancement of Akt phosphorylation at Ser 473 [38]. Because ROT-induced cell death was associated with inhibition of Akt pathway, we next examined the effects of mTOR inhibitor rapamycin on ROT-induced cell death. ROT induced cell death in

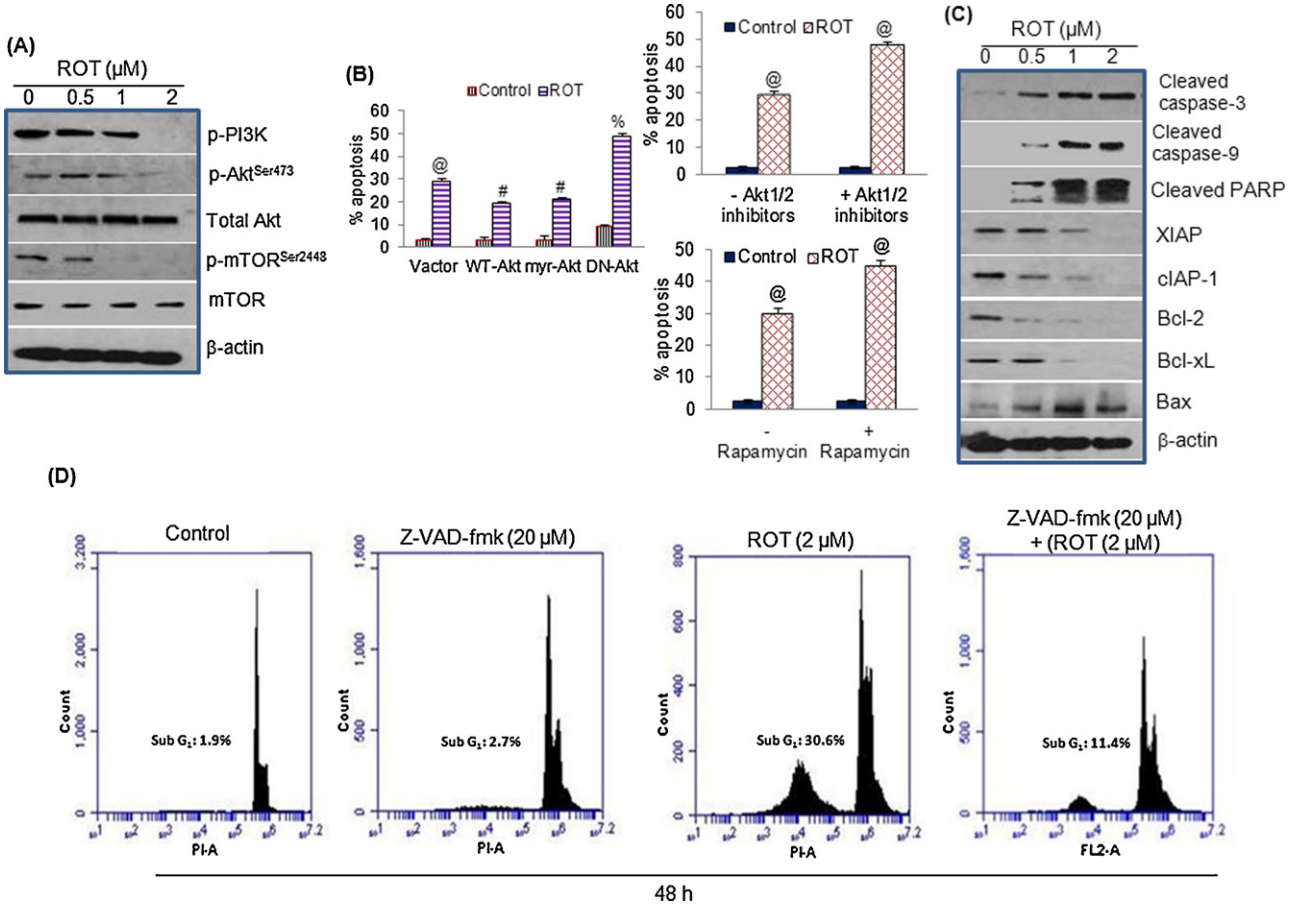

Fig. 5. ROT induced apoptosis through inhibition of PI3K/Akt/mTOR pathway and activation of caspases. (A) Pancreatic CSCs were incubated in the absence or presence of ROT (0.5, 1 and 2 μM) in complete medium for 48 h. Cells were harvested and lysed for the detection of phospho-PI3K, phospho-Akt^{Ser473}, Akt, phospho-mTOR^{Ser2448}, and mTOR by immunoblotting. β-Actin was used as a loading control. (B) Left panel: cells were transfected with control plasmid DNA or a plasmid encoding wild type Akt (WT-Akt), constitutively active Akt (myr-Akt), and dominant negative Akt (DN-Akt) and then treated with 2 μM ROT for 48 h. Annexin-PI staining was subsequently performed to assess total apoptosis using a flow cytometry. The results are expressed as mean ± S.E. ($n = 3$). @, #, and %, significantly different from respective control ($P < 0.05$). Right panel: cells were treated with 2 μM ROT, 10 μM Akt1/2 inhibitor, 1 μM rapamycin, 2 μM ROT with 10 μM Akt1/2 inhibitor or 2 μM ROT with 1 μM rapamycin for 48 h in complete medium and apoptosis (Annexin-PI staining) was measured. The results are expressed as mean ± S.E. ($n = 3$). @, significantly different from respective controls ($P < 0.05$). (C) Cells were treated with different concentrations of ROT (0.5, 1 and 2 μM) for 48 h, and the Western blot analysis was performed to measure the expression of cleaved caspase-3, cleaved caspase-9, cleaved PARP, XIAP, cIAP-1, Bcl-2, Bcl-X_L and Bax. β-Actin was used as a loading control. (D) ROT induces caspase-dependent apoptosis. Pancreatic CSCs were pretreated with 20 μM z-VAD-fmk for 2 h, followed by treatment with ROT (2 μM) for additional 48 h, and apoptosis was determined by PI-staining through flow cytometry. Data are the means of triplicate experiments.

the absence of rapamycin. However, ROT and rapamycin showed an additive effect on the enhancement of cell death compared to the single treatment alone. These data suggest that ROT induces cell death through inhibition of PI3K/Akt/mTOR pathway.

To gain further insight into the mechanism by which ROT induces cell death, we examined the effects of ROT on the expression of apoptosis-related proteins (Fig. 5C). Treatment of pancreatic CSCs with ROT resulted in cleavage of caspase-3, caspase-9 and poly (ADP-ribose) polymerase (PARP), which is a downstream target of the activated caspase-3 [39]. Furthermore, the levels of IAP family proteins, such as XIAP and cIAP-1, which bind to caspases and lead to their inactivation [39], were downregulated by ROT treatment. Moreover, the cellular levels of anti-apoptotic Bcl-2 and Bcl-X_L proteins were significantly decreased, whereas pro-apoptotic Bax level was increased in response to ROT, indicating ROT induced cell death in CSCs due to an increase in the relative ratio of Bax/Bcl-2 (and/or Bcl-X_L) expression.

In order to assess whether ROT-induced cell death occurred due to caspase activation, we used a pan caspase inhibitor z-VAD-fmk (Fig. 5D). ROT induced cell death in pancreatic CSCs. z-VAD-fmk had no effect on apoptosis. The pretreatment of CSCs with z-VAD-fmk inhibited ROT-induced apoptosis, suggesting the involvement of caspase(s) in ROT-induced cell death.

3.6. Inhibition of Atg7 or Beclin-1 by shRNA suppressed autophagy and restored the sensitivity of pancreatic CSCs to ROT

To investigate the function of ROT-induced autophagy in pancreatic CSCs, we inhibited autophagy by suppressing the expression of *Atg7* or Beclin-1 by shRNA. As shown in Fig. 6A, the protein levels of *Atg7* and Beclin-1 were significantly decreased after transduction of CSCs with sh-*Atg7* and sh-Beclin-1, respectively. We next examined whether inhibition of Atg7 or Beclin-1 by shRNA suppressed ROT-induced conversion of LC3-I to LC3-II in CSCs (Fig. 6B). Inhibition of Atg7 or Beclin-1 by shRNA blocked ROT-induced conversion of LC3-I to LC3-II. These data suggest that Atg7 and Beclin-1 are involved in ROT-induced autophagy. We next quantified the autophagy grade in these transduced CSCs treated with ROT (Fig. 6C). The number of LC-3II positive cells and severity of autophagic response per cell was increased following ROT treatment at 24 h in scrambled cells, whereas ROT did not induce autophagy in both sh-*Atg7* and sh-Beclin-1 cells.

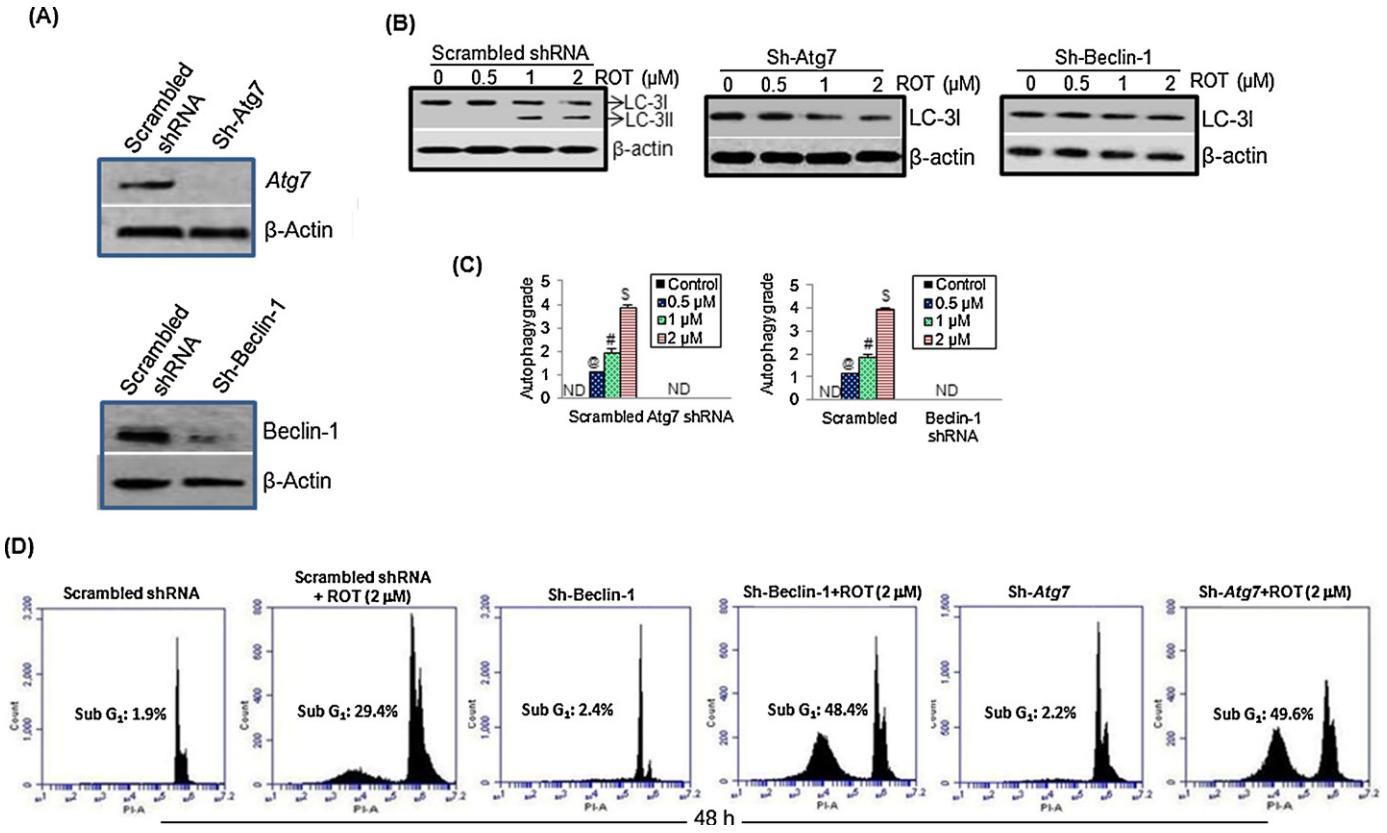

Fig. 6. Inhibition of autophagy by sh-*Atg7* and sh-Beclin-1 accelerated apoptotic potential of ROT. (A) Pancreatic CSCs were stably transduced with sh-*Atg7* or sh-Beclin-1-targeted lentiviral particles. Cell lysates were prepared and the expression of *Atg7*, Beclin-1 and β-actin was assessed by Western blot analysis. (B) Scramble shRNA, sh-*Atg7* and sh-Beclin-1 cells were treated with or without ROT (0.5, 1 and 2 μM) for 24 h. Immunoblotting was performed using antibody against LC-3 or β-actin. (C) Knockdown of *Atg7* and Beclin-1 inhibited autophagy. Scrambled shRNA, sh-*Atg7* and sh-Beclin-1 pancreatic CSCs were seeded on fibronectin-coated coverslips and treated with or without ROT (0.5, 1 and 2 μM) in complete medium for 24 h. Subsequently, cells were fixed with 4% paraformaldehyde, blocked in 10% normal goat serum and stained with LC-3 primary antibody (1:200) for 16 h at 4 °C. Afterwards, cells were incubated with fluorescently labeled secondary antibody (1:200) for 1 h at room temperature, mounted and visualized for the expression of LC-3II (punctate structure) under a fluorescent microscope. Quantification represents at least 100 cells counted and scored per treatment. The results are expressed as mean ± S.E. ($n = 3$). @, # and $, significantly different from respective control ($P < 0.05$). ND, not detected. (D) Cells without *Atg7* and Beclin-1 expression are more susceptible to ROT-induced apoptosis. Scrambled shRNA, sh-*Atg7* and sh-Beclin-1 cells were treated with ROT (2 μM) for 48 h and examined for apoptosis. PI staining was subsequently performed to assess apoptosis by flow cytometry. Data are the means of triplicate experiments.

We next examined the effects of inhibiting Atg7 and Beclin-1 on ROT-induced apoptosis (Fig. 6D). ROT induced 29.4% apoptosis in CSCs at 48 h. By comparison, inhibition of Atg-7 or Beclin-1 by shRNA enhanced ROT-induced apoptosis in CSCs. These data suggest that inhibition of autophagy can enhance ROT-induced cell death in pancreatic CSCs.

4. Discussion

In this study, we showed that ROT induced early autophagy as a survival strategy against late apoptosis through PKC-δ-independent, but dependent on PI3K/Akt/mTOR cascade in human pancreatic CSCs. The CSC death was associated with the presence of autophagic vacuoles in the cytoplasm. Interestingly, ROT-treated cells did not undergo cell death at 24 h, while at late time points (48 h) showed significant cell death. ROT induced autophagy at 24 h, as evident by formation of autophagosomes and conversion of LC-3I to LC-3II form. Overall, our data suggest that ROT-induced early autophagy may act as a survival mechanism against late cell death in pancreatic CSCs.

Autophagy is a conserved dynamic process in which intracellular membrane structures sequester proteins and organelles, which are finally delivered to lysosomes for bulk degradation and ATP generation to maintain basal cellular bioenergetics [40]. Whereas the above situations envision autophagy as a survival mechanism, autophagy can also lead to cell death under some circumstances [41]. In this study, ROT was found to cause autophagy, including formation of autophagosomes, redistribution of LC3 and induction of autophagy related proteins including *Atg7* and Beclin-1 at 24 h. Bcl-2 family proteins are potential inhibitor of Beclin-1 [42]. ROT significantly inhibited Bcl-2 and Bcl-X_L expression, and induced *Atg-7* and Beclin-1. Furthermore, 3-MA inhibited ROT-induced conversion of LC-3I to LC-3II, and expression of autophagy-related proteins *Atg7* and Beclin-1 at 24 h. These results indicate that ROT induces autophagy at an early stage in pancreatic CSCs.

Beclin-1 was originally discovered as a Bcl-2 interacting protein and was one of the first human proteins shown to be indispensable for autophagy [43]. Another autophagic gene *Atg7* is responsible for autophagosome biogenesis [44]. Both genes are monoallelically deleted in 50–75% of cases of human sporadic breast, ovarian and prostate cancers [44]. Our data demonstrate that down-regulation of *Atg7* and Beclin-1 by shRNA inhibited autophagy in pancreatic CSCs. Although it is debatable whether *Atg7* and Beclin-1 inhibit the autophagosome biogenesis, both genes are still used as inhibitors to study autophagic flux [45]. Our study also demonstrates that gene silencing of Atg7 and Beclin1, or cotreatment of the CSCs with 3-MA inhibited the ROT-induced autophagy. Therefore, ROT-induced autophagy may play some role as a protective mechanism against apoptosis.

Apoptosis is a key tumor suppressor mechanism that is blocked in the majority of human cancers, owing to the over-activation of the PI3K/Akt/mTOR pathway [25]. Activation of PI3K/Akt/mTOR pathway regulates transcription factors which modulate distinct sets of genes involved in cell cycle, apoptosis, oxidative stress and DNA repair [25]. Treatment of CSCs with ROT decreased the levels of phosphorylated Akt and mTOR. Furthermore, downregulation of constitutively active Akt or mTOR rendered pancreatic CSCs sensitive to ROT. ROT induced significant apoptosis in pancreatic CSCs at 48 h by inhibiting phosphorylation of Akt and mTOR, and expression of Bcl-2, Bcl-X_L cIAP1 and XIAP, up-regulation of Bax, and activation of caspase-3 and -9. Therefore, we concluded that the ROT-induced apoptosis is also dependent on the PI3K/Akt/mTOR pathway.

To evaluate whether these effects of ROT are related to PKC-δ, we determined the autophagy and apoptosis using PKC-δ shRNA. In our results, the induction of autophagic cell death was detected after transfection of PKC-δ shRNA as revealed by formation of autophagosomes, conversion of LC-3I to LC-3II, and expression of *Atg7* and Beclin-1. Moreover, ROT induced apoptosis in CSCs/PKC-δ shRNA cells to the same degree when compared to scrambled cells. Similarly, recent studies have shown that ROT can exert its biological effects through PKC-δ independent manner [46,47]. These observations suggest that ROT can induce autophagy leading to apoptosis in a PKC-δ independent manner.

In conclusion, our results indicate that ROT causes early autophagy and late apoptosis through inhibition of PI3K/Akt/mTOR pathway in human pancreatic CSCs. Moreover, the precise mechanisms underlying the role of autophagy in ROT-induced cell death remain to be studied. The present study also suggests that autophagy at early stage may act as a survival mechanism against late apoptosis. Thus, inhibition of autophagy by the potent drugs or genetic means (e.g. inhibiting the expression of *Atg7* and Beclin-1) may enhance the apoptosis-inducing potential of ROT in highly therapy-resistant human pancreatic CSCs.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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