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Original Article

Beclin-1-independent autophagy mediates programmed cancer cell death through interplays with endoplasmic reticulum and/or mitochondria in colbat chloride-induced hypoxia

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Abstract: Autophagy has dual functions in cell survival and death. However, the effects of autophagy on cancer cell survival or death remain controversial. In this study, we show that Autophagy can mediate programmed cell death (PCD) of cancer cells in responding to cobalt chloride ($CoCl_2$)-induced hypoxia in a Beclin-1-independent but autophagy protein 5 (ATG5)-dependent manner. Although ATG5 is not directly induced by $CoCl_2$, its constitutive expression is essential for $CoCl_2$-induced PCD. The ATG5-mediated autophagic PCD requires interplays with endoplasmic reticulum (ER) and/or mitochondria. In this process, ATG5 plays a central role in regulating ER stress protein CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) and mitochondrial protein second mitochondria derived activator of caspases (Smac). Two pathways for autophagic PCD in cancer cells responding to hypoxia have been identified: ATG5/CHOP/Smac pathway and ATG5/Smac pathway, which are probably dependent on the context of cell lines. The former is more potent than the latter for the induction of PCD at the early stage of hypoxia, although the ultimate efficiency of both pathways is comparable. In addition, both pathways may require ATG5-mediated conversion of LC3-I into LC3-II. Therefore, we have defined two autophagy-mediated pathways for the PCD of cancer cells in hypoxia, which are dependent on ATG5, interplayed with ER and mitochondria and tightly regulated by hypoxic status. The findings provide a new evidence that autophagy may inhibit tumor cell proliferation through trigger of PCD, facilitating the development of novel anti-cancer drugs.

Keywords: Cobalt chloride, autophagy, endoplasmic reticulum stress, mitochondria, programmed cell death

Introduction

Hypoxia is one of the most important pathological features of the solid tumors and the hypoxic responses are mainly mediated through changes in gene profiles regulated by hypoxia inducible factor 1α (HIF-1α) [1]. Cobalt chloride ($CoCl_2$), which stabilized HIF-1α by inhibiting prolyl hydroxylases (PHDs), is an agent widely used *in vitro* to induce cellular responses mediated by hypoxia [2]. Although the underlying mechanisms of such action are unknown, there is evidence that $CoCl_2$ increases the level of reactive oxygen species (ROS), which may serve as signaling molecules in many aspects such as stimulating autophagy [3, 4]. Meanwhile, disturbances in cellular redox regulation caused by $CoCl_2$ also interfere with disulphide bonding in the lumen of the endoplasmic reticulum (ER), leading to protein unfolding and misfolding (ER stress), which activates the unfolded protein response (UPR) [5, 6]. The hypoxic microenvironment stimulated by $CoCl_2$ could effectively induce apoptosis and influence cell proliferation in some tumor cells [7, 8]. The underlying mechanisms of hypoxia in various conditions are complex and unconfirmed.

Autophagy is an evolutionarily conserved catabolic process involving the sequestration and transport of organelles and macromolecules to the lysosomes for degradation. Autophagy is initiated by formation of the phagophore or isolated membrane (vesicle nucleation), which expands (vesicle elongation) and fuses to form a double-membrane vesicle termed autophago-

some [9]. Autophagosomes eventually fuse with lysosomes to degrade their content. The autophagic process requires a set of evolutionarily conserved proteins, most of which are known as autophagy-related (ATG) proteins, functioning at different phases of autophagy formation. Beclin-1 binds to class III phosphatidylinositol 3-kinase (PIK3C3 or Vps34), which forms an initiation complex and promotes autophagosomal membrane nucleation. Autophagosomal elongation then requires 2 ubiquitin like conjugation systems, ATG12-ATG5 and subsequent phosphatidylethanolamine conjugated form of the microtubule associated protein light chain 3 (LC3-II/ATG8-PE) [9]. Autophagy plays a dual role as a tumor suppressor or a tumorigenesis promoter in cancer depending on the contextual microenvironment and stimulation [10]. For example, autophagy is activated in response to starvation, hypoxia and ER stress inducing chemicals such as tunicamycin (Tm) in order to eliminate damaged organelles, protein aggregates, and invading pathogens. In such context, autophagy might initially be triggered to protect the cells by sequestering and degrading the damaged organelles. However, once a certain level of intracellular damage is reached, autophagy might serve to remove the damaged cells from cancer tissues by launching cell death [11]. Therefore, there is not always a cause and effect association between autophagy and cell death.

ER stress is a process of imbalance in ER function and subsequently triggers UPR, which is a tightly orchestrated collection of intracellular signal transduction reactions designed to restore protein homeostasis. Activation of the canonical UPR engages three distinct concerted signaling branches mediated by ER membrane anchored sensors: protein kinase RNA (PKR)-like ER kinase (PERK), Inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6), whose activation are prevented by the interaction of the ER luminal domains with the chaperone protein 78 kDa glucose-regulated protein (GRP78/BiP) [12]. Among UPR target genes, CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP/DDIT3/GADD153), a proapoptotic transcription factor, serves as a downstream component of ER stress pathways, at the convergence of the IRE1, PERK/ATF4 and ATF6 pathways [12]. CHOP expression is low in non-stressed conditions but is markedly increased in response to ER stress, hypoxia and amino acid starvation [13, 14] In prolonged or severe ER stress, the affected cells are comitted to autophagy or cell death mediated by ATF4 and ATF6, as well as activation of the JNK/AP-1/CHOP signaling pathway [11-13].

Apoptosis, one of the best characterized forms of programmed cell death, can be activated through two pathways: The extrinsic pathway (mediated by death receptors) or the intrinsic pathway (mediated by mitochondria). Mitochondrial apoptosis can be induced by several different stimuli like chemotherapy drugs, hypoxia, irradiation, starvation and ER stress through provoking the mitochondrial outer membrane permeabilization (MOMP) and the release of proteins from the intermembrane space, such as cytochrome c and second mitochondria-derived activator of caspases (Smac/DIABLO) [11] Smac promotes caspase-9 activation by binding to inhibitor of apoptosis proteins (IAPs) and removing their inhibitory activity, which in turn induces the activation of downstream effectors caspases-3, -6 and -7 [15].

In this study, we explored the functional link between autophagy, ER stress and mitochondria in hypoxic tumor cells induced by $CoCl_2$, using cervical cancer cell line HeLa and breast cancer cell line MDA-MB-231 as an in vitro cellular model. We have found that $CoCl_2$ induced, Beclin-1-independent but ATG5-dependent autophagy mediates hypoxic PCD of cancer cells in a CHOP-dependent (ATG5/CHOP/Smac) or a CHOP-independent (ATG5/Smac) pathway, probably depending on the context of cell lines targeted. Both pathways appear to be tightly regulated by intracellular status. The findings are of significance in deeply understanding of the mechanisms underlying hypoxic PCD of tumor cells, facilitating development of novel therapeutic strategy for cancers.

Materials and methods

Cell lines and culture medium

Human cancer cell lines including breast cancer cell line MDA-MB-231 and cervical cancer cell line HeLa were obtained from the American Type Tissue Culture Collection (ATCC, USA) [16]. The cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum

Table 1. Primer sequences for RT-PCR

Primer ID	GenBank ref. No.	Primer sequence	Size (bp)
BIP-For	NM_005347	5'-TCCTATGTCGCCTTCACTCC-3'	300
BIP-Rev		5'-CTTTCCCAAATAAGCCTCAGC-3'	
ATF4-For	NM_001675	5'-AACCGACAAAGACACCTTCG-3'	360
ATF4-Rev		5'-CCAACAGGGCATCCAAGTC-3'	
ATF6-For	NM_ 007348	5'-CACCGTCTCGTCAGCGTTA-3'	305
ATF6-Rev		5'-GACTCCCAAGGCATCAAATC-3'	
DDIT3-For	NM_001195056	5'-GCACCAAAGCAGCCATAAAC-3'	391
DDIT3-Rev		5'-CTCCTCCTCAGTCAGCCAAG-3'	
BNIP3-For	NM_004052	5'-AGCATGAGTCTGGACGGAGT-3'	302
BNIP3-Rev		5'-CATGACGCTCGTGTTCCTC-3'	
XBP1u-For	NM_005080	5'-AGG AGT TAA GAC AGC GCT TGG GGA TGG AT-3'	206
XBP1u-Rev		5'-CTG AAT CTG AAG AGT CAA TAC CGC CAG AAT-3'	
XBP1s-For	NM_005080	5'-CCTGGTTGCTGAAGAGGAGG-3'	180
XBP1s-Rev		5'-CCATGGGGAGATGTTCTGGAG-3'	
BECN1-For	NM_003766	5'-TGGCACAATCAATAACTTCAGG-3'	320
BECN1-Rev		5'-CAG CGG CTA CCT ACA AAC TTG CTT-3'	
ATG5-For	NM_001286108	5'-AGAGTGTTTATTCGTCGGTTCA-3'	317
ATG5-Rev		5'-GGGCATTGTAGGCTTGACTT-3'	
ACTB-For	NM_001101	5'-AGCGAGCATCCCCCAAAGTT-3'	250
ACTB-Rev		5'-GGGCACGAAGGCTCATCATT-3'	

(FBS) and 100 U/ml penicillin-streptomycin (Gibco, USA). The cells were maintained in a humidified incubator containing 5% CO_2 and air at 37°C.

Cell viability assay

The measurement of viable cells was performed with Cell Counting Kit-8 (Dojin Laboratories, Japan). The cells (3×10^3 cells/well) were initially seeded in 96-well flat-bottomed plates in quintuplicate for 16 h. Then, $CoCl_2$ (Sigma, USA) diluted in phosphate-buffered saline (PBS) was added into the cultures in various concentrations (100, 200, 400 and 800 μM). Viable proliferating cells were detected using the Cell Counting Kit-8 at 12, 24 and 48 h after treatment, according to manufacturer's instruction. Ten μL solution from Cell Counting Kit-8 was added into each well, and the plates were incubated for 2 h in a humidified CO_2 incubator at 37°C. Finally, the OD value for each well was measured on a Synergy HT microplate reader (Bio-Tek, USA) at 450 nm wavelength. Cell viability was calculated with the following formula: percent proliferation (%) = (drug-treated sample-blank)/(untreated sample-blank) ×100%.

Semi-quantitative reverse polymerase chain reaction (RT-PCR)

Total RNA was isolated and purified using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA was solubilized in diethy pyrocarbonate (DEPC) water and RNA concentrations were determined by NanoDrop 2000c spectrophotometer (Thermal scientific, USA). To detect specific mRNA, cDNA was generated from 1 μg of RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). 0.5 μl of cDNA samples was used as the template for PCR amplification of mRNAs of interest with the Premix Taq DNA polymerase (Takara, Japan) using Veriti® 96-Well Thermal Cycler (Applied biosystems, USA). The condition for PCR reactions was used as previously described [17]. PCR products were analyzed on 2% agarose gels containing GelGreen™ nuclear acid staining (Biotium, USA) and image acquisition was made with the ChemiDox™ XRS+ system (BioRad, USA). The housekeeping gene β-actin was used as an internal control. Gene expression was quantified using ImageJ 1.47 V (National Institutes of Health, USA). Primer sequences used for the experiments were listed in **Table 1**.

Western blot analysis

Cells were seeded in 6-well plates and treated with 100, 200 or 400 μM $CoCl_2$ for 24 h. After treatment, cells were lysed in pre-cold Ripa lysis and extraction buffer (Thermal Scientific, USA) containing protease inhibitor cocktail (Roche, USA) on ice. Cell debris was removed by centrifugation at 12000 g for 20 min at 4°C, supernatants were harvested, and protein con-

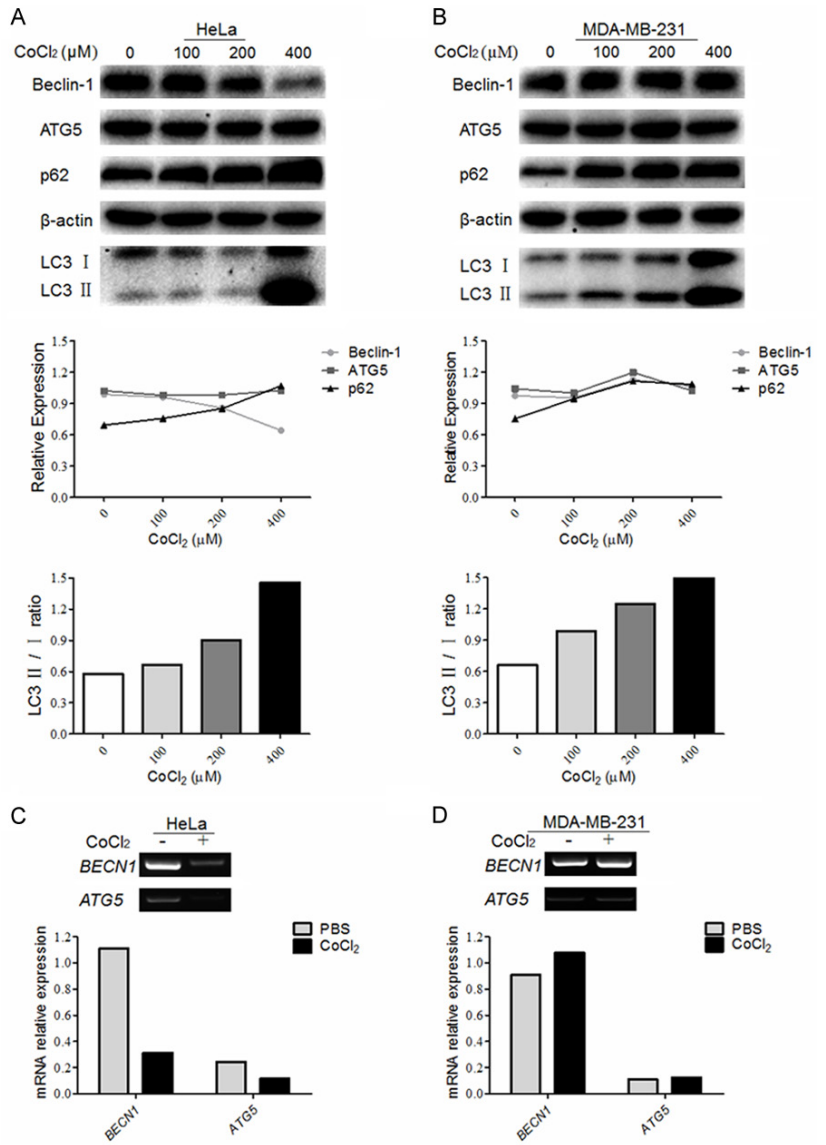

Figure 1. Beclin-1-independent conversion of LC3 I to LC3 II induced by $CoCl_2$. A, B. HeLa cells and MDA-MB-231 cells were treated with $CoCl_2$ (0 (PBS), 100, 200 or 400 μM) for 24 h, and the levels of protein expression were analyzed by western blot using antibodies to Beclin-1, ATG5, p62, LC3 and β-actin, respectively. The band intensity of LC3 II normalized by LC3 I was compared with that in the control. The intensity ratios of LC3 II/I display below LC3 bands. C, D. HeLa cells and MDA-MB-231 cells were treated with 400 μM $CoCl_2$ for 24 h. Relative mRNA expression levels of *BECN1* and *ATG5* were determined by RT-PCR. Curves and histograms represent the quantity of proteins or mRNA from relevant experiments.

centration in the supernatant were determined by the bicinchoninic acid (BCA) assay kit (Beyotime, China). Equal amounts of whole proteins (20 μg) were seperated by 8%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immobilized to 0.45 μM PVDF membranes (Millipore, USA). Then the membranes were blocked with 5% bovine serum albumin (BSA) dissolved in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) plus 0.25% Tween 20 (TBS-Tween, TBST) for 2 h, and incubated with various primary antibodies dissolved in blocking buffer at 4°C overnight. The following primary antibodies were used for Western blot: anti-HIF-1α (Cat. YM0333; Immunoway, USA), anti-SMAC (Cat. GTX61004; GeneTex, USA), anti-cleaved Caspase 3 (Cat. #9664; CST, USA), anti-LC3B (Cat. #3868; CST, USA), anti-Beclin-1 (Cat. #3495; CST, USA), anti-ATG5 (Cat. #12994; CST, USA), anti-P62 (Cat. 18420-1-AP; Proteintech, China), anti-CHOP (Cat. 15204-1-AP; Proteintech, China) and HRP conjucted anti-β-actin (Cat. 70068; Zen Bioscience, China). The secondary HRP conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies (Cat. sc-2004 & sc-2005; Santa Cruze, USA), dissolved in the same solution as primary antibodies were incubated for 2 h at room temperature. The membranes were visualized with Super Signal® West Pico Chemiluminescent Substrate kit (Thermo Scientific, USA), and image acquisition was made with the Chemi-Dox™ XRS+ system. The differences in protein expression among various treatments were determined with the image analysis software Image J, using β-actin as a reference.

Small interfering RNA (siRNA) assay

Expression of endogenous Beclin-1 (BECN1), ATG5 and CHOP mRNAs was silenced with

chemically modified siRNA (BECN1: 5'-UGUGAAUGGAAUGAGAUUATT-3'; ATG5: 5'-CCAUCAAUCGGAAACUCAUTT-3'; negative control: 5'-UUCUCCGAACGUGUCACGUTT-3' (genepharma, China), CHOP: 5'-GGGAGAACCAGGAAACGGAdTdT-3' (Sigma, USA). After HeLa cells or MDA-MB-231 cells being seeded at 2.4×10^5 per well in 6 well plates overnight, siRNA duplexes (50 pmol) were transfected into the target cell populations using Lipofectamine® 2000 Reagent (Invitrogen, USA) for 4 h according to the manufacturer' instructions. 24 h after transfection, cells were treated with 400 μM $CoCl_2$ for another 24 h. Cell proteins were extracted and the expression levels of genes were assessed by Western blotting.

Statistic analysis

The data are presented as mean values from at least three separate experiments ± S.D. Statistical analysis are performed using GraphPad Prism 5 Software. Statistical comparisons were made by using one-way ANOVA analysis or *t* tests. $P<0.05$ was considered as significant difference, as compared between two groups.

Results

$CoCl_2$ inhibits Beclin-1 expression in HeLa cells but not in MDA-MB-231 cells

To determine whether $CoCl_2$ could induce autophagy in cancer cells, we treated human cervical cancer cell line HeLa and breast cancer cell line MDA-MB-231, respectively, with various concentrations of $CoCl_2$ (0, 100, 200 or 400 μM) for 24 h. As shown in **Figure 1**, Beclin-1 protein expression in the HeLa cells was inhibited by $CoCl_2$ in a dose-dependent manner (**Figure 1A**), but was slightly up-regulated in the MDA-MB-231 cells (**Figure 1B**). There was no significant effect of $CoCl_2$-treatment on ATG5 expression. Consistently, RT-PCR analysis showed that transcription of *BECN1* was significantly inhibited by $CoCl_2$ in the HeLa cells (**Figure 1C**), but not in the MDA-MB-231 cells, in which *BECN1* mRNA was slightly increased (**Figure 1D**). Interestingly, a very low level of ATG5 transcripts was detected in both HeLa and MDA-MB-231 cells without treatment with $CoCl_2$, whereas it was suppressed by $CoCl_2$ in the HeLa cells but not changed in the MDA-MB-231 cells (**Figure 1C**, **1D**), suggesting that ATG5 proteins detected in both cell lines were mainly constitutively expressed through unknown pathways (**Figure 1A**, **1B**). The results indicated that $CoCl_2$ could effect on Beclin-1 expression differentially in the HeLa cells from the MDA-MB-231 cells.

$CoCl_2$ induces Beclin-1-independent but ATG5-dependent autophagy in both HeLa and MDA-MB-231 cells

To determine whether declined expression of Beclin-1, one of key regulators of autophagy formation, effected on macroautophagy formation, we measured the conversion of LC3-I to LC3-II, a hallmark of macroautophagy, in the HeLa cells and MDA-MB-231 cells after treatment with $CoCl_2$. As shown in **Figure 1A** and **1B**, the conversion of LC3-I to LC3-II (LC3 II/I) was not inhibited rather significantly increased in a dose-dependent manner in both HeLa cells and MDA-MB-231 cells. This was not correlated with Beclin-1 expression because Beclin-1 was decreased in the HeLa cells and slightly increased in the MDA-MB-231 cells (**Figure 1A**, **1B**). These results suggested that the $CoCl_2$-induced autophagy formation was independent of Beclin-1 expression in both cell lines. The notion was confirmed by the facts that knockdown of Beclin-1 using si-Beclin-1 did not significantly alter the conversion of LC3-I to LC3-II in both cell lines (**Figure 2A**, **2B**). However, knockdown of constitutively expressed ATG5, one of key regulators of autophagy formation, using si-ATG5 significantly inhibited LC3-I conversion into LC3-II in both cell lines treated with $CoCl_2$. However, inhibition of constitutive expression of ATG5 by si-ATG5 did not affect LC3-I conversion into LC3-II (**Figure 2C**, **2D**). The results suggested that ATG5 could mediate $CoCl_2$-induced autophagy formation, regardless of the differential levels of Beclin-1 in both cell lines. The ATG5-mediated autophagy was functional, because p62, a selective target of autophagy, was significantly up-regulated in both $CoCl_2$-treated cell lines (**Figure 1A**, **1B**). It is interesting that ATG5-mediated autophagy or change of LC3II/I ratio only occurred in the $CoCl_2$-treated cell lines; the base line of autophagy activity was not changed in the untreated cell lines (**Figure 2C**, **2D**; bottom panels).

The $CoCl_2$ induced p62 proteins accumulated in the HeLa cells and MDA-MB-231 cells in a

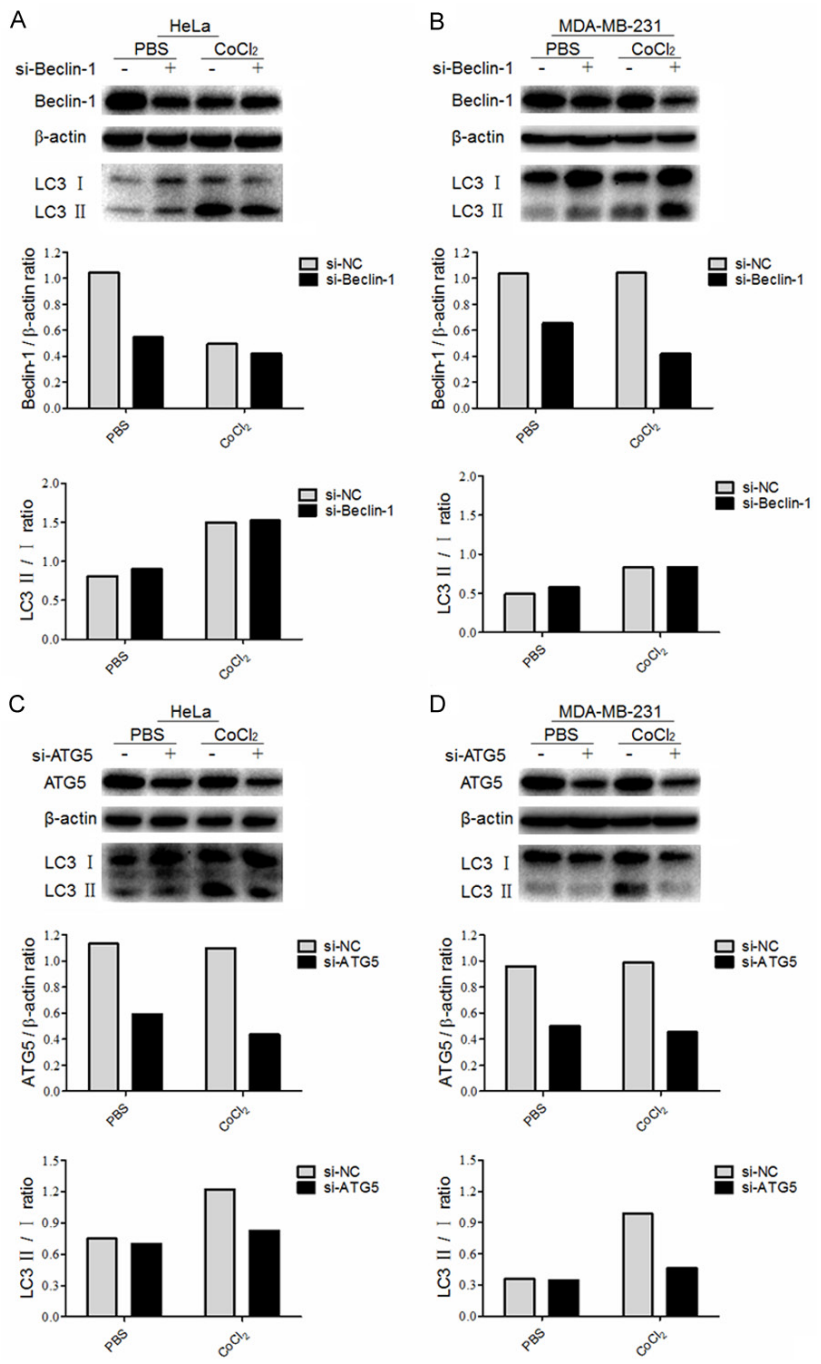

Figure 2. The effects of knockdown of Beclin-1 and ATG5 on autophagy formation in hypoxic cancer cells. The HeLa cells and MDA-MB-231 cells were transfected with control siRNA Beclin 1 siRNA (A, B) or ATG5 siRNA (C, D) for 24 h and then treated with $CoCl_2$ (400 μM) for another 24 h. Levels of protein expression were measured by western blot analysis using antibodies to Beclin-1, ATG5, LC3 and β-actin. The band intensity of LC3 II normalized by LC3 I and the ratios of LC3 II to LC3 I were compared with that of the control. (A, B) The effects of knocking down Beclin-1 by Beclin-1-specific siRNA on autophagy formation; (C, D) The effects of knocking down ATG5 by ATG5-specific siRNA on autophagy formation.

dose-dependent manner. The p62 is involved in cargo recruitment for autophagic degradation. It has been reported that p62 up-regulation, and at least transient increase, has been observed in some situations where autophagy flux occurs [18]. Taken together, the $CoCl_2$ induced-autophagy is independent of Beclin-1, but highly dependent on ATG5.

ER stress markers are differentially induced by $CoCl_2$ between HeLa cells and MDA-MB-231 cells

Since severe hypoxia may trigger ER stress [5, 19], we hypothesized that $CoCl_2$ could induce ER stress. Thus, we investigated the effects of $CoCl_2$ on the transcriptional expressions of ER stress markers in HeLa cells and MDA-MB-231 cells, including the products of genes *HSPA5/GRP-78*, *ATF4*, *ATF6*, *DDIT3* and *XBP1*. As shown in **Figure 3**, among the markers, BiP/GRP78, an endoplasmic reticulum (ER) chaperone binding protein, and ATF4, a DNA binding protein, were not significantly altered in both cell lines after treatment with $CoCl_2$. However, the mRNAs of *CHOP/DDIT3* were prominently altered after treatment with $CoCl_2$ for 24 or 48 hr, The *DDIT3* mRNAs increased by about 2 folds in both HeLa cells and MDA-MB-231 cells, as compared to the untreated counterparts, respectively. However, the constitutive levels of the *DDIT3* mRNAs were about 4~5 times higher in the HeLa cells than in the MDA-MB-231 cells

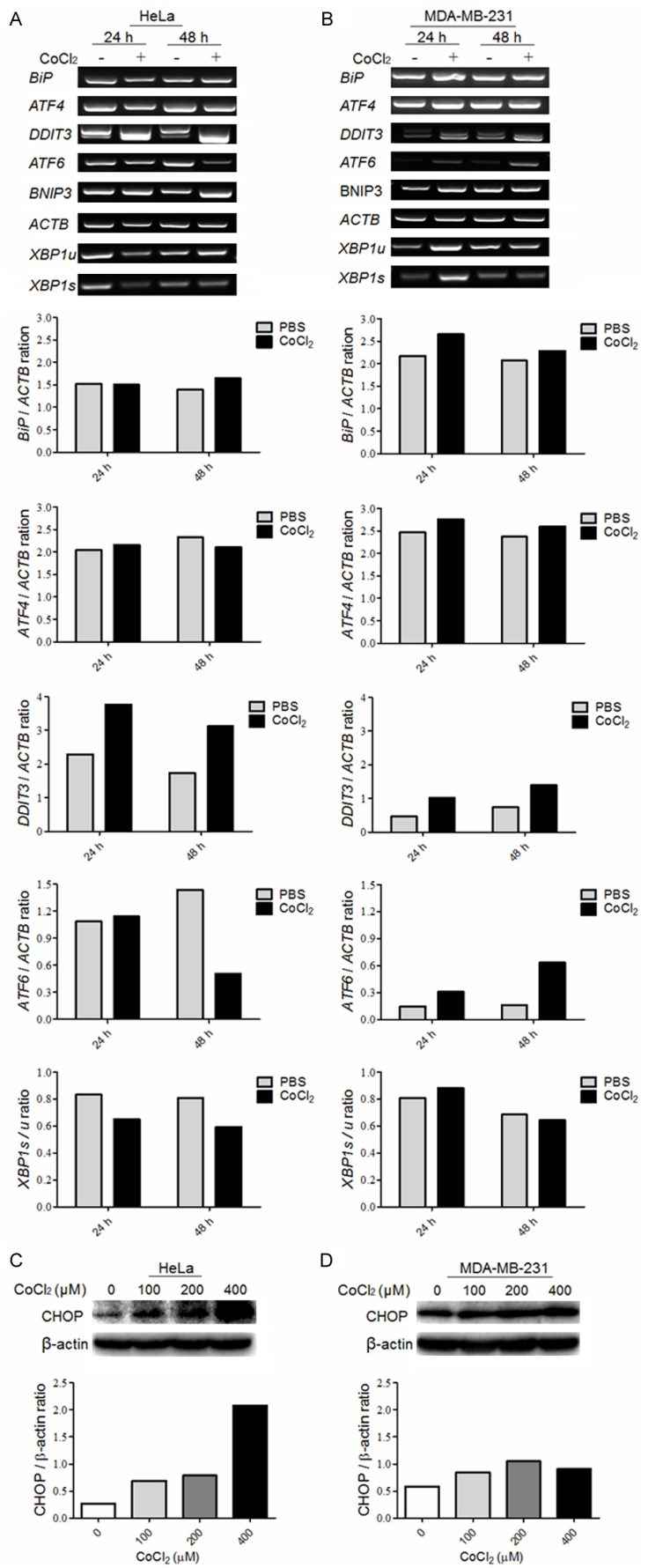

Figure 3. ER stress marker expression induced by $CoCl_2$ was differentially expressed in the HeLa cells from MDA-MB-231 cells. Hela and MDA-MB-231 cells were treated with 400 μM $CoCl_2$ for 24 and 48 h. Relative mRNA expression levels of Bip, ATF4, DDIT3, ATF6, XBP1u and XBP1s were determined by RT-PCR. BNIP3 was used as a positive control (A, B). The proteins expression of CHOP and β-actin was analyzed by Western blot in HeLa cells and MDA-MB-231 cell lines exposed to $CoCl_2$ (100, 200 or 400 μM) 24 h after treatment (C, D).

(**Figure 3A**, **3B**). In addition, the transcripts of *ATF6* in the HeLa cells were also remarkably higher than in the MDA-MB-231 cells. They were decreased in the HeLa cells but increased in MDA-MB-231 cells after treatment with $CoCl_2$ for 48 hr. The ratio of spliced *XBP1* to unspliced *XBP1*, which reflected a part of ER stress response, was slightly reduced in the HeLa cells but not in the MDA-MB-231 cells after treatment with $CoCl_2$ (**Figure 3A**, **3B**). *BNIP3*, one of HIF-1α target genes were used as a positive control of hypoxic responses. These results indicated that among the tested stress markers, DDIT3/CHOP mRNAs were remarkably up-regulated in both HeLa and MDA-MB-231 cells after exposure to $CoCl_2$, although the levels of constitutive expression between two cell lines were different. Therefore, we analyzed CHOP protein levels in both HeLa and MDA-MB-231 cells using Western blotting after treatment with various concentrations (0, 100, 200 or 400 μM) of $CoCl_2$ for 24 h. The level of CHOP proteins was up-regulated in both cell lines with the increased concentrations of $CoCl_2$. However, the range of dose-response was different between HeLa cells (**Figure 3C**) and MDA-MB-231 cells (**Figure 3A**, **3D**), The level of CHOP proteins in the HeLa cells continued to be up-regulated,

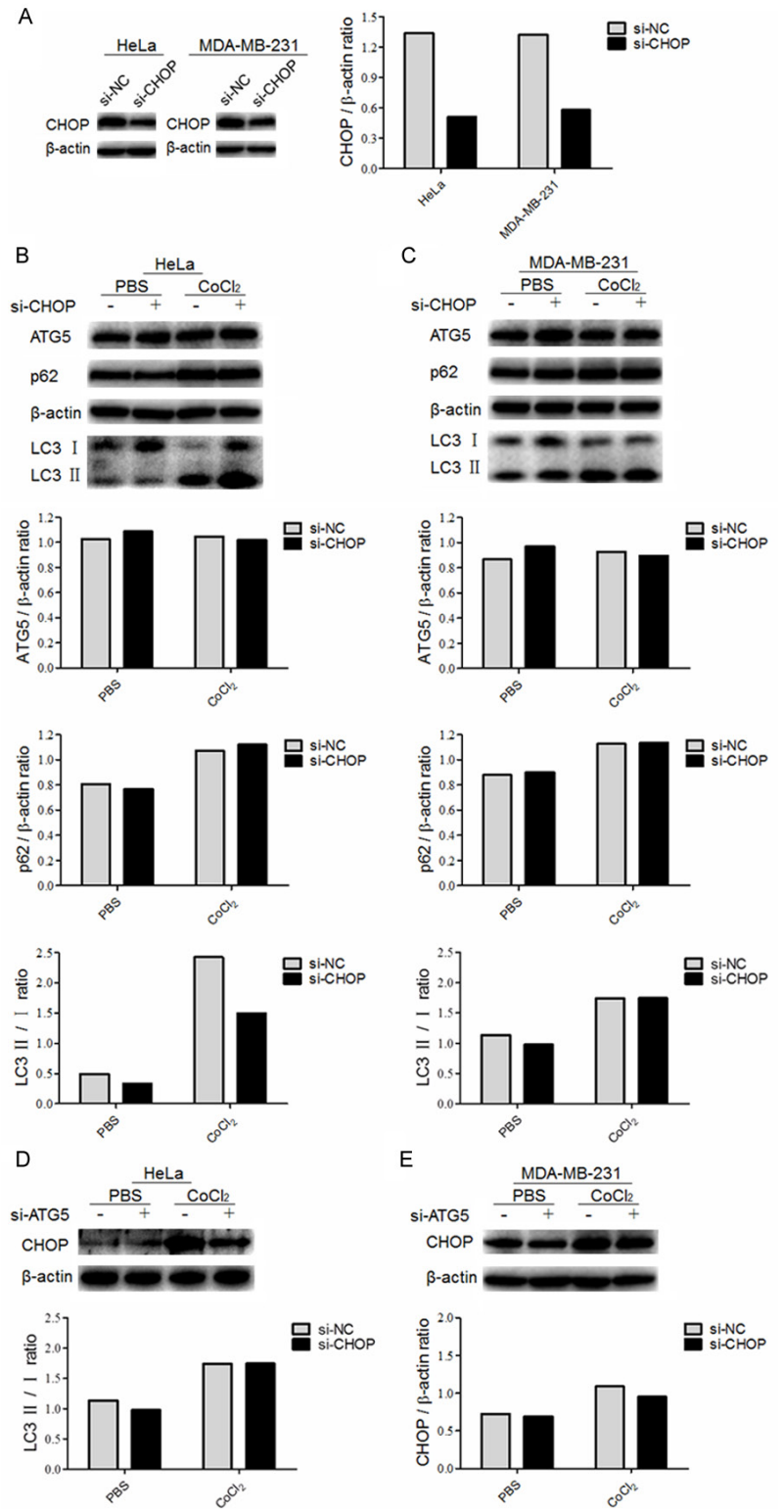

Figure 4. CHOP is required for $CoCl_2$-induced autophagy formation in HeLa cells but not in MDA-MB-231 cells. A. CHOP siRNA suppressed CHOP expression: HeLa cells and MDA-MB-231 cells were transfected with CHOP siRNA or control siRNA (si-NC) for 48 hrs and CHOP protein expression was analyzed by Western blot. B, C. CHOP is required for autophagy formation in HeLa cells but not in MDA-MB-231 cells in responding to $CoCl_2$: HeLa cells and MDA-MB-231 cells were transfected with si-NC or CHOP siRNA for 24 h and then treated with $CoCl_2$ (400 μM) or vehicles (PBS) for another 24 h. The cells were harvested and the levels of protein expression were measured by western blot analysis using antibodies against CHOP, ATG5, LC3, p62 or β-actin. The band intensity of LC3 II was normalized by LC3 I and the ratios of LC3 II to LC3 I was compared with that of the control. D, E. ATG5 is required for CHOP expression in HeLa cells: HeLa cells and MDA-MB-231 cells were transfected with control siRNA or ATG5 siRNA for 24 h and then treated with $CoCl_2$ (400 μM) for another 24 h. Levels of protein expression were measured by western blot analysis using antibodies to CHOP and β-actin.

whereas it was not altered in the MDA-MB-231 cells when they were exposure to 400 μM $CoCl_2$. The results confirmed that $CoCl_2$-triggered ER stress was different in mechanistic pathway between HeLa cells and MDA-MB-231 cells, reflecting differential intracellular milieu between the cell lines.

ATG5/CHOP pathway mediates autophagy formation in the HeLa cells but not in the MDA-MB-231 cells

Since ER stress is one of the signaling pathways involved in regulation of autophagy, we hypothesized that the ER stress-associated proteins CHOP might play an important role in $CoCl_2$-induced autophagy. To this end, HeLa cells and MDA-MB-231 cells were treated with or without $CoCl_2$ (400 μM) and transfected with CHOP siRNA to eliminate CHOP expression. The protein levels of CHOP, ATG5, p62 and LC3 II/I were assessed by

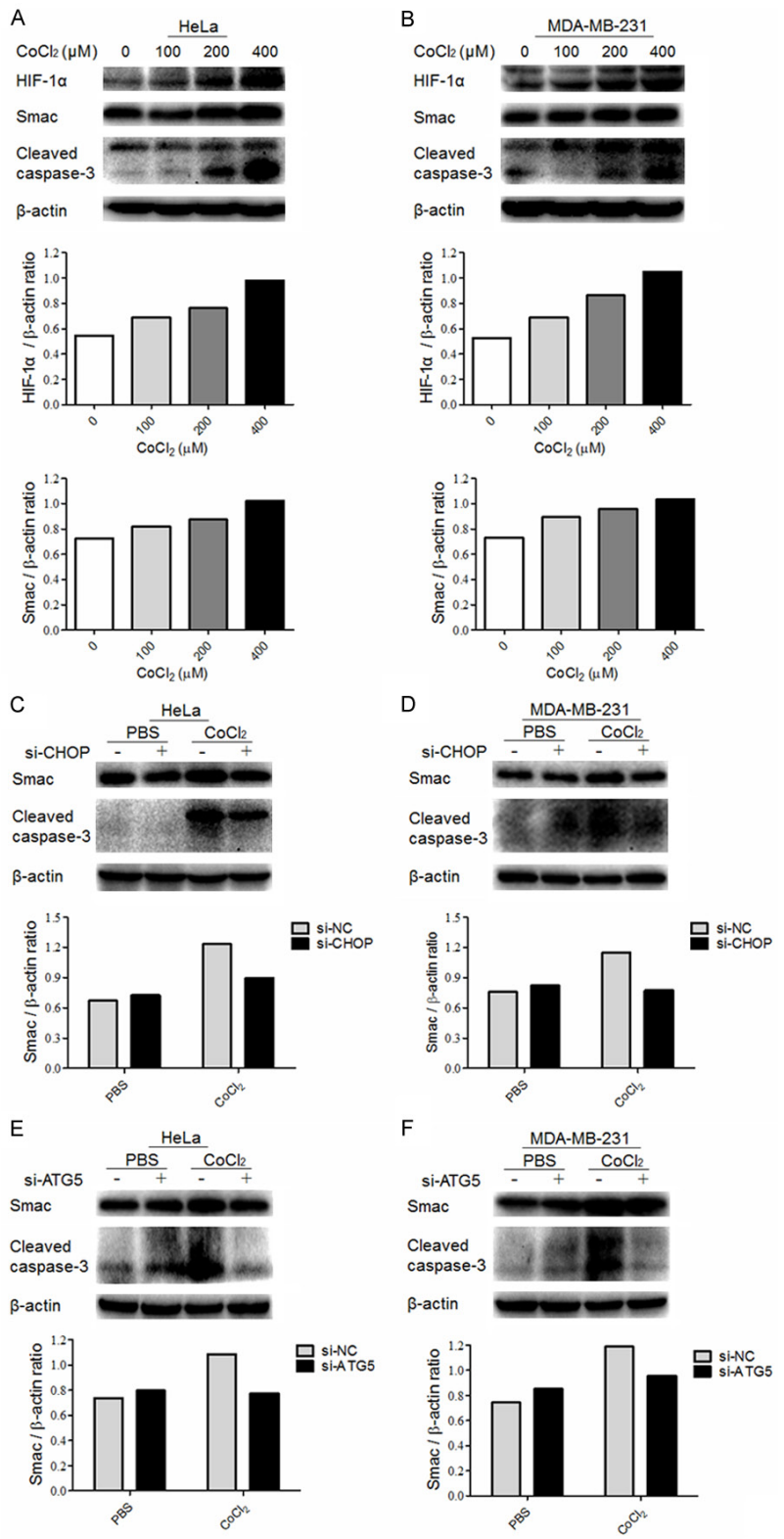

Figure 5. Mechanistic links among autophagy, ER and mitochondria in the hypoxic cancer cells. A, B. HIF-1α expression in cancer cells induced by $CoCl_2$: HeLa and MDA-MB-231 cells were treated with $CoCl_2$ (100, 200 or 400 μM) for 24 h. Levels of protein expression were analyzed by western blot using antibodies to HIF-1α, Smac, cleaved caspas-3 or β actin. C, D. Association of ER stress with mitochondrial stress: HeLa and MDA-MB-231 cells were transfected with control siRNA, CHOP siRNAs for 24 h and then treated with $CoCl_2$ (400 μM) for another 24 h. Levels of protein expression were measured by western blot analysis using antibodies to Smac, cleaved caspase-3 or β actin. E, F. Association of autophagy with mitochondrial stress: HeLa and MDA-MB-231 cells were transfected with control siRNA or ATG5 siRNA for 24 h and then treated with $CoCl_2$ (400 μM) for another 24 h. Levels of protein expression were measured by western blot analysis using antibodies to Smac, cleaved caspase-3 or β actin.

Western blot after the cells were treated for 24 h (**Figure 4**). The results demonstrated that the knockdown of CHOP mRNA remarkably suppressed CHOP protein expression equally in the HeLa cells and MDA-MB-231 cells (**Figure 4A**). However, the levels of ATG5 and p62 were not altered in both cell lines, and the ratio of LC3 II/I was decreased only in the HeLa cells (**Figure 4B**). The results suggested that CHOP expression was associated with autophagy formation in the HeLa cells but not in the MDA-MB-231 cells (**Figure 4B**, **4C**). In the absence of $CoCl_2$, knockdown of CHOP caused slightly increased levels of ATG5 in the HeLa cells and MDA-MB-231 cells, slight decrease of the ratio of LC3 II/I (**Figure 4B**, **4C**).

To determine whether $CoCl_2$-induced CHOP upregulation was autophagy-dependent in HeLa cells, we knocked down ATG5 with ATG5 siRNA in the cells treated with $CoCl_2$ (400 μM). As a result, CHOP expression was remarkably

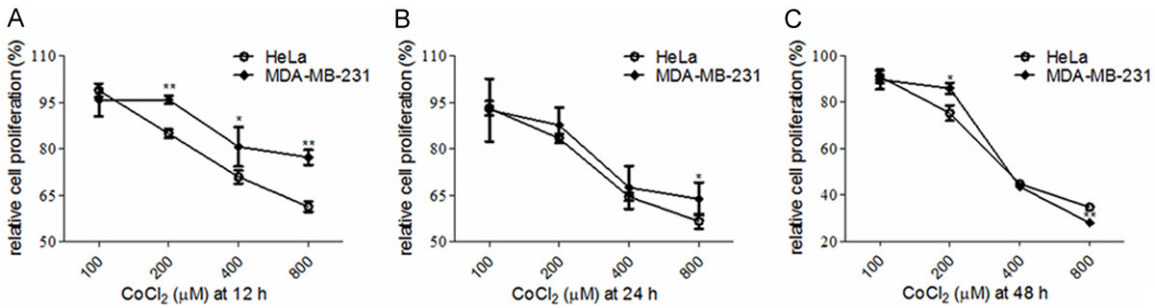

Figure 6. ATG5/CHOP/Smac pathway was more potent than ATG5/Smac pathway in the induction of programmed cancer cell death in hypoxia. HeLa and MDA-MB-231 cells were treated with PBS or indicated concentrations of $CoCl_2$ (100, 200, 400 or 800 μM) and OD values were measured at 12 (A), 24 (B) and 48 h (C) by a cck-8 assay, respectively. Representative results of three independent experiments are expressed as percentage of cck-8 reduction, relative to NT control. The viability of the $CoCl_2$-treated cells at 0 h is regarded as 100%; *P<0.05 and **P<0.01 when compared between the groups of HeLa cells and MDA-MB-231 cells.

inhibited (**Figure 4D**), but not in the MDA-MB-231 cells (**Figure 4E**) confirming that $CoCl_2$-iduced CHOP expression in ER was autophagy-dependent in the HeLa cells but not in the MDA-MB-231 cells.

Taken together, ATG5 could promote CHOP expression in HeLa cells rather than in MDA-MB-231 cells, whereas the CHOP in turn promoted the conversion of LC3-I to LC3-II in the HeLa cells. In other word, $CoCl_2$-induced autophagy formation is CHOP-dependent in HeLa cells but CHOP-independent in MDA-MB-231 cells.

Mechanistic links among autophagy, ER and mitochondria in cancer cells in the hypoxic milieu

Since $CoCl_2$ can induce oxidative stress in mitochondria [3], we investigated whether $CoCl_2$-induced autophagy formation and ER stress pathways crossed with mitochondrial stress. HeLa cells and MDA-MB-231 cells were incubated with increasing doses of $CoCl_2$ (0, 100, 200 and 400 μM) for 24 h, and HIF-1α, which is a key transcription factor playing pivotal roles in hypoxia, Smac and cleaved caspase-3 were analyzed, respectively, by Western blot. As shown in **Figure 5A** and **5B**, in both cell lines, HIF-1α, Smac and cleaved caspase-3 was significantly up-regulated in a dose-dependent manner. The levels of HIF-1α, Smac and cleaved caspase-3 reached to the peak at the dose of 400 μM. To determine whether ATG5 and $CoCl_2$-induced CHOP effected on Smac expression in mitochondria, both HeLa cells and MDA-MB-231 cells were transfected with CHOP siRNA (si-CHOP) in the presence or absence of 400 μM $CoCl_2$ for 48 h. As a result, the expression of Smac was down regulated in both cell lines when exposed to $CoCl_2$. Consistently, activation of caspas-3 was also partially inhibited (**Figure 5C**, **5D**). To determine whether ATG5 is required for the activation of Smac, we transfected the Hela cells and MDA-MB-231 cells with ATG5 siRNAs (si-ATG5) in the presence or absence of 400 μM $CoCl_2$ for 48 h. As a result, Smac and the cleaved caspase-3 were significantly decreased in both HeLa and MDA-MB-231 cells treated with $CoCl_2$ (**Figure 5E**, **5F**). Importantly, compared to partial inhibition in the si-CHOP transfected cells (**Figure 5C**, **5D**), activation of caspas-3 was almost completely inhibited in the si-ATG5 transfected cells. The results suggested that hypoxia-induced programmed cell death (PCD) was biased to the ATG5-meidated. Collectively, $CoCl_2$ induced hypoxia caused autophagic cell death linking with ER stress and mitochondrial stress (ATG5/CHOP/Smac pathway) in HeLa cells, but only with mitochondrial stress (ATG5/Smac pathway) in MDA-MB-231 cells.

ATG5/CHOP/Smac pathway was more potent than ATG5/Smac pathway in the induction of programmed cell death at the early stage of hypoxia

Since autophagy has dual roles in cell survival and cell death [10] and ATG5/CHOP/Smac pathway and ATG5/Smac pathway were differentially activated, respectively, in HeLa cells and MDA-MB-231 cells (**Figure 5**), we investigated the status of programmed cell death

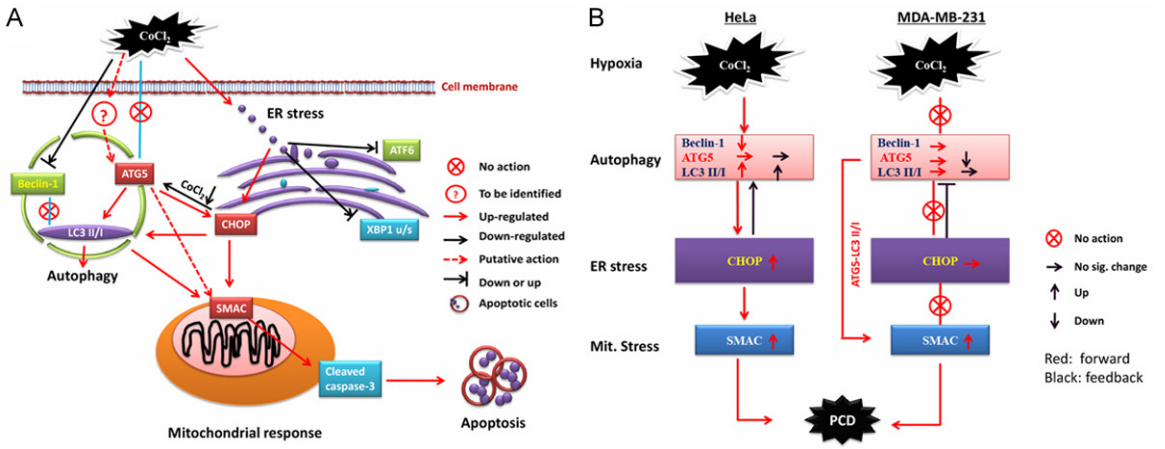

Figure 7. Schematic Model for ATG5/CHOP/Smac pathway and ATG5/Smac pathway in cancer cells responding to hypoxia. A. Interactions between autophagy, ER stress and mitochondrial response in cancer cells responding to hypoxia induced by $CoCl_2$: Although Beclin-1 can be down or up-regulated in responding to the $CoCl_2$-induced hypoxia, it does not participate in autophagy formation. In contrast, ATG5 does not significantly respond to the hypoxia in transcription, it critically participate autophagy formation in the hypoxia. There are two pathways for ATG5 participating the induction of programmed cell death of cancer, each pathway have a subpathway by passing LC3II formation. Pathway A, autophagy, ER and mitochondrial responses: (a1) ATG5/CHOP/LC3II/Smac; (a2) ATG5/CHOP/SMAC; pathway B, autophagy and mitochondrial responses: (b1) ATG5/LC3II/SMAC; and (b2) ATG5/SMAC. B. Comparison of the ATG5/CHOP/SMAC pathway in the HeLa cells with the ATG5/SMAC pathway in the MDA-MB-231 cells: In the HeLa cells, ATG5 up-regulates CHOP, which subsequently up-regulates SMAC; while in the MDA-MB-231 cells, ATG5 induces LC3 II/I, leading to activation of SMAC. In addition, ATG5-induced CHOP in the HeLa cells also promotes LC3II, forming a positive loop to strengthen PCD through activation of SMAC/caspase-3 pathway, whereas CHOP inhibits ATG5 in the MDA-MB-231 cells in the absence of $CoCl_2$. ATG5: Autophagy protein 5; ATF4: activating transcription factor 4 (tax-responsive enhancer element B67); XBP1 u/s: X-box binding protein 1 unspliced/spliced; LC3/ MAP1LC3A: Microtubule-associated proteins 1A/1B light chain 3A; a light chain of the microtubule-associated protein 1; CHOP/DDIT3: DNA-damage inducible transcript 3; SMAC/ DIABLO: second mitochondria-derived activator of caspases; ER: endoplasmic reticulum; Mit. Stress: mitochondrial stress; PCD: programmed cell death.

(PCD) in HeLa cells and MDA-MB-231 cells after exposed to $CoCl_2$. The HeLa cells and MDA-MB-231 cells were incubated with increasing doses of $CoCl_2$ (100, 200, 400 or 800 μM) for 12 h (**Figure 6A**), 24 h (**Figure 6B**) and 48 h (**Figure 6C**), respectively. The cell viability was measured using a CCK-8 kit. We found that $CoCl_2$ significantly inhibited the proliferation of HeLa and MDA-MB-231 cells when the cells were exposed to more than 400 μM $CoCl_2$. In other word, the cell viability decreased dramatically in a dose-dependent and time-dependent manner. However, the percentage of viable cells was significantly lower in the HeLa cells than in the MDA-MB-231 cells at 12 h of treatment (**Figure 6A**). The difference was reduced at 24 and 48 h after treatment (**Figure 6B**, **6C**). The results suggested that $CoCl_2$ could effectively induce PCD in both cell lines through either ATG5/CHOP/Smac pathway or ATG5/Smac pathway. However, ATG5/Smac pathway (in the MDA-MB-231 cells) was significantly less potent than ATG5/CHOP/Smac pathway (in the HeLa cells) for the induction of PCD at the early stage of hypoxia induced by $CoCl_2$, because the cell viability in the HeLa cells was significantly lower than that in the MDA-MB-231 cells at initial 12 h of treatment with $CoCl_2$ (**Figure 6A**). The reduced PCD in the MDA-MB-231 cells was associated with reduced amounts of the cleaved caspase-3 in the MDA-MD-231 cells compared to that in the HeLa cells (**Figure 5A**, **5B**). Therefore, ATG5/CHOP/Smac pathway was distinct from ATG5/Smac pathway with regard to the kinetics of PCD induced by $CoCl_2$.

Discussion

Autophagy has dual roles in cell survival and cell death. Especially, its role in tumorigenesis remains controversial. In this study, we have defined two distinct pathways for autophagic death of hypoxic cancer cells: ATG5/CHOP/Smac pathway and ATG5/Smac pathways. We demonstrated that $CoCl_2$ induced autophagic death of both cervical cancer cells (HeLa) and breast cancer cells (MDA-MB-231) through

ATG5-triggered mitochondrial responses characterized by releasing Smac and activating caspase-3. However, ER stress (CHOP) involved in the autophagy formation was found only in the HeLa cells rather than in the MDA-MB-231 cells (**Figure 4B**, **4C**), although CHOP in both HeLa and MDA-MB-231 cells could up-regulate Smac (**Figure 5C**, **5D**). Therefore, CHOP participated in autophagic cell death in HeLa cells but not in MDA-MB-231 cells. The ATG5/CHOP/Smac pathway is more potent than ATG5/Smac pathway at the initial stage of apoptosis (**Figures 5**, **6**). Furthermore, the $CoCl_2$ induced autophagic cell death is independent of Beclin-1. These findings indicate that ATG5 and Smac are essential for PCD of cancer cells induced by $CoCl_2$-mediated hypoxia, whereas CHOP may be compensable in some cancer cells such as MDA-MB-231 cells. Therefore, the interplay between autophagy, ER and mitochondria may be involved in hypoxic cell death in tumors. This is the first report that $CoCl_2$-mediated hypoxia induces autophagic cell death in a Beclin-1-independent and ATG5-dependent pathway through activating ER and/or mitochondria (**Figure 7A**).

Recently, non-canonical pathways, which lead to autophagosomal degradation through variants of the canonical pathway, have been described where the formation of the double-membrane autophagosome may occur in the absence of some of the key autophagy proteins such as Beclin-1 [20]. This autophagosome is possibly derived from a pre-existing membrane (ER, plasma membrane, mitochondria, ER-mitochondria contact site or Golgi) [9, 20, 21]. Of the alternative pathway, Beclin-1-independent autophagy can be induced by different stresses [20, 22, 23]. For example, As_2O_3 treatment can generate reactive oxygen species (ROS), a form of oxidative stress, which can induce Beclin-1-independent autophagy [22]. Specifically, ROS can oxidize the cysteine protease, ATG4, involved in initiating the conjugation of LC3-I to autophagosomal membranes and its consequent release from the autophagophore membrane [4]. As demonstrated in this study, $CoCl_2$-induced autophagy is also Beclin-1-independent. This may be related to the ability of $CoCl_2$ inducing ROS [3, 4]. We have observed that Beclin-1 in HeLa cells decreased upon treatment with $CoCl_2$ in a dose-dependent manner, whereas it was not apparently altered in MDA-MB-231 cells (**Figure 1A**, **1B**). However, down-regulation of Beclin-1 in the HeLa cells did not reduce LC3 conversion (**Figure 1A**). Also, in contrast to knockdown of ATG5, siRNA targeting Beclin-1 did not suppress autophagy formation in $CoCl_2$-treated cervical and breast cancer cells (**Figure 2A**, **2B**). Thus, it can be reached that $CoCl_2$ induces autophagy in a Beclin-1-independent manner in both cancer cell lines. Considering that $CoCl_2$ increases the level of ROS [3], we speculate that $CoCl_2$ could induce a Beclin-1-independent autophagic pathway through generation of ROS and activation of ROS related signaling pathways. Further investigations are warranted to elucidate the molecular mechanism by which the upstream of ATG5-ATG12 and LC3 conversion are regulated, contributing to $CoCl_2$-induced Beclin-1-independent autophagy.

In this study, we have observed substantial differences in gene profiles (**Figure 3A**, **3B**) and regulation patterns (**Figure 4A**, **4B**) in responding to $CoCl_2$-induced ER stress between the HeLa cells and MDA-MB-231 cells. These observations suggest the complexity and heterogeneity of the responses to hypoxia induced by $CoCl_2$ in cancer cells. However, the accumulation of CHOP in both cancer cells treated with $CoCl_2$ was obvious (**Figure 3C**, **3D**), providing direct evidence for an ER UPR response. CHOP plays a convergent role in the UPR and it has been identified as one of the most important mediators of ER stress-induced apoptotic proteins [13, 24, 25]. Also, CHOP is one of the potent autophagy inducers [26, 27], as we observed in this study (**Figures 4**, **7**). While ATG5 could induce CHOP expression, the induced CHOP in turn promoted the conversion of LC3-I to LC3-II (**Figures 4A**, **7**). These results suggest that CHOP is an important feedback regulator for autophagy. However, CHOP in HeLa cells but not in MDA-MB-231 cells participates in ATG5-mediated autophagic cell death, although CHOP was also up-regulated mildly in the MDA-MB-231 cells (**Figure 3D**). Blocking CHOP using CHOP-specific siRNA could alleviate autophagy and mitochondrial apoptosis (reducing Smac and cleaved caspase-3) in $CoCl_2$-treated HeLa cells but not MDA-MB-231 cells (**Figures 4B**, **4C**, **5C**), suggesting whether CHOP play a positive feedback role in autophagy formation is determined by intracellular milieu of targeted cells (**Figure 7B**).

Autophagy and ER stress have been considered two essential mechanisms to promote cell survival or apoptosis, and activation of ER stress along with induction of autophagy have been observed in cancer cells in response to different stimuli [9, 26, 28, 29]. Severe hypoxia (<0.1% O_2) -induced maintenance of autophagy is attained by the transcriptional up-regulation of ATG5 and LC3 by ATF4/CHOP [26, 28]. However, our results showed that $CoCl_2$ did not alter the protein expression of ATG5, even though Beclin-1 mRNA and protein expression were decreased in HeLa cells (**Figure 1A**, **1C**). The driver force of constitutive expression ATG5 remains elusive in both HeLa cells and MDA-MB-231 cells. Although CHOP can promote LC3-I conversion into LC3-II in hypoxia, its ability to inhibit ATG5 in normxia might be more important for maintenance of homeostasis of cancer cells. It appears that CHOP might play a differential role in regulation of autophagy in the status of hypoxia versus normxia, which is critical for the maintenance of balance between cell survival and apoptosis. ER stress-mediated CHOP expression in normxia might rescue the cells from PCD via inhibiting ATG5 expression, whereas it might promote PCD through enhancing LC3 conversion in hypoxia (**Figure 4**). Therefore, the consequence of interactions between autophagy and ER stress may determine the fate of a stressed cell. If the interaction leads to activation of mitochondria, the cells may undergo PCD (ATG5/CHOP/Smac pathway); if the interaction is not involved in activation of mitochondria, autophagy might function toward cell survival. In addition, should autophagy directly interact with mitochondria, PCD might be predominantly triggered. Further experiments are warranted to verify the hypothesis.

Signaling pathways determine whether cells undergo autophagy, ER stress and/or mitochondrial apoptosis are complex. It is likely that multiple mechanisms, involved in ER stress, alternative autophagy and mitochondrial apoptosis, operate to inhibit the growth of cancer cells. The fact that ATG5/CHOP/Smac pathway and ATG5/Smac pathway exist, respectively, in the HeLa cells and MDA-MB-231 cells explains the complexity of interplay between autophagy, ER and mitochondria in response to cellular stresses. In hypoxia, autophagic cancer cell death is at least mediated by two pathways: direct and indirect pathways. In the ATG5/CHOP/Smac pathway, autophagy indirectly interacts with mitochondria, in which CHOP may serve as a bridge between autophagy and mitochondria, while in ATG5/Smac pathway, autophagy directly interacts with mitochondria. In other word, Smac can be upregulated by ATG5 or CHOP-mediated autophagy. A pathway used by hypoxic cells for PCD is dependent on their intracellular status (**Figure 7B**).

In summary, we have defined two pathways for autophagic cell death in hypoxic cancer cells, providing an in vitro model for further investigation of the molecular networks between the autophagy, ER and mitochondria in responding to hypoxia-induced cellular stresses, and a novel strategy for the development of effective anti-cancer drug.

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Disclosure of conflict of interest

None.

Abbreviations

$CoCl_2$, Cobalt chloride; ER, endoplasmic reticulum; CHOP, C/EBP homologous protein; ATG, Autophagy-related gene; Smac/DIABLO, second mitochondria derived activator of caspases; LC3, microtubule-associated protein 1 light chain 3; UPR, unfolded protein response; PCD, programmed cell death.

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