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Use of inducible Atg5 deletion and expression cell lines in study of the pro-survival function of autophagy under starvation

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ABSTRACT

At present the role of autophagy in cell death and cell survival remains controversial, partly owning to the contradictory results from the immortalized mouse embryonic fibroblasts (MEFs) with knockout of different autophagy-related genes (Atg). Here we aimed to reexamine the role of autophagy in cell death under starvation and other stress conditions. First, different clones of Atg5 knockout MEFs had different susceptibility to stress-mediated cell death, indicating that it is the clonal variation, rather than the deficiency of Atg5 or autophagy *per se* that determines the susceptibility. Next, we tested two cell lines with inducible Atg5 deletion or expression and demonstrated that cells without Atg5 expression were more sensitive to starvation-induced apoptosis. Finally, we found that chloroquine was only effective in sensitizing starvation-induced cell death in Atg5-expressing cells, but not in Atg5-deficient cells. Such observations thus provide unequivocal evidence supporting the pro-survival function of autophagy under starvation. Moreover, our data demonstrate the usefulness of cells with inducible deletion or expression of Atg in the study of autophagy in cell death and cell survival.

1. Introduction

Autophagy is an intracellular self-degradation process triggered by starvation or other stress stimuli in which cellular proteins and organelles are engulfed within autophagosomes which are eventually degraded by the hydrolytic enzymes in the autolysosomes [1,2]. At present, the biological functions of autophagy have been well studied. Autophagy is known to play important roles in several physiologic processes, including development, differentiation, tissue remodeling, cellular homeostasis, aging, cell survival or death, infection and innate immunity, and pathogenesis [3–5]. More importantly, autophagy is closely involved in many human diseases, including cancer, neurodegenerative diseases and metabolic disorders [1,6,7].

Among the biological functions of autophagy, its involvement in the cell death and cell survival processes appears to be particularly important, but yet highly controversial. On one hand, autophagy has been implicated in the cell death process via (i) promotion of apoptotic cell death and (ii) induction of so called "autophagic cell death" [8]. At present, the cross-talks between autophagy and apoptosis have been well established, while the concept of "autophagic cell death" has been increasingly challenged, especially in the mammalian cells [9,10]. On the other hand, there are mounting reports showing that autophagy is a cytoprotective mechanism that enables the cell to survive unfavorable conditions and prevent cell death [3,10,11]. For instance, mice deficient in Atg3, Atg5, Atg7, Atg9 or Atg16L1 die on the day of birth due to starvation following disruption of the trans-placental nutrient supply [12–16]. Furthermore, autophagy also plays a protective role in non-apoptotic cell death. For example, autophagy can protect apoptosis-deficient cells from necrosis caused by ischemia [17], and play a pro-survival role in zVAD-fmk-induced necroptosis [18].

Looking into the literature, it is believed that part of the controversy regarding the role of autophagy in cell death and cell survival is originated from the studies using mouse embryonic fibroblasts (MEFs) with Atg deletions, a popular model system in study of autophagy *in vitro*. One good example is the inconsistent results whether Atg5 knockout MEFs are more sensitive or resistant to cell death under stress than the Atg5 wild-type MEFs. For instance, an earlier report showed deficiency of Atg5 rendered MEFs more resistant to IFN-γ-induced cell death [19], while subsequent studies from the same group demonstrated that Atg5−/− MEFs were more sensitive to cell death caused by amino acid starvation and tumor necrosis factor-α (TNFα), but more resistant to H_2O_2 [20].

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Moreover, Wang et al. reported that Atg5−/− MEFs responded differently to different stimuli: they were more sensitive to apoptosis induced by the death receptor ligands Fas and TNFα, while they were more resistant to an oxidant menadione and UV [21]. It thus appears that there is inconsistency of Atg5−/− MEFs in response to various cell death stimuli, probably pending on the different systems utilized in those studies.

In our study, we re-evaluated the role of autophagy in cell death and cell survival under stress conditions, especially starvation, using different Atg5 deficient cells, including two cell lines with inducible Atg5 deletion or expression. Data from this study provide unequivocal evidence supporting the pro-survival function of autophagy in response to starvation. In addition, our data demonstrate the usefulness of cells with inducible deletion or expression of Atg in the study of autophagy in cell death and cell survival.

2. Materials and methods

2.1. Reagents and antibodies

Propidium iodide (PI, P4170), DAPI (D9542), anti-β-actin antibody (A5441), chloroquine diphosphate (CQ, C6628) and Earles' Balanced Salt Solution (EBSS, E2888) were purchased from Sigma (St. Louis, MO, USA). H_2O_2 and sodium nitroprusside (SNP) were from Calbiochem (San Diego, CA, USA). Anti-caspase 3 antibody (#551150) and anti-p62 antibody (#611038) were obtained from BD Pharmingen (Los Angeles, CA, USA). Antibody against LC3 (AP18029) was from Abgent (San Diego, CA, USA). Anti-Atg5 antibody was purchased from Cosmo Bio Co (Tokyo, Japan). Antibodies against Atg3 (#3415), Atg7 (#2631) and anti poly(ADP-ribose) polymerase-1 (PARP-1) (#9542) were all from Cell Signaling.

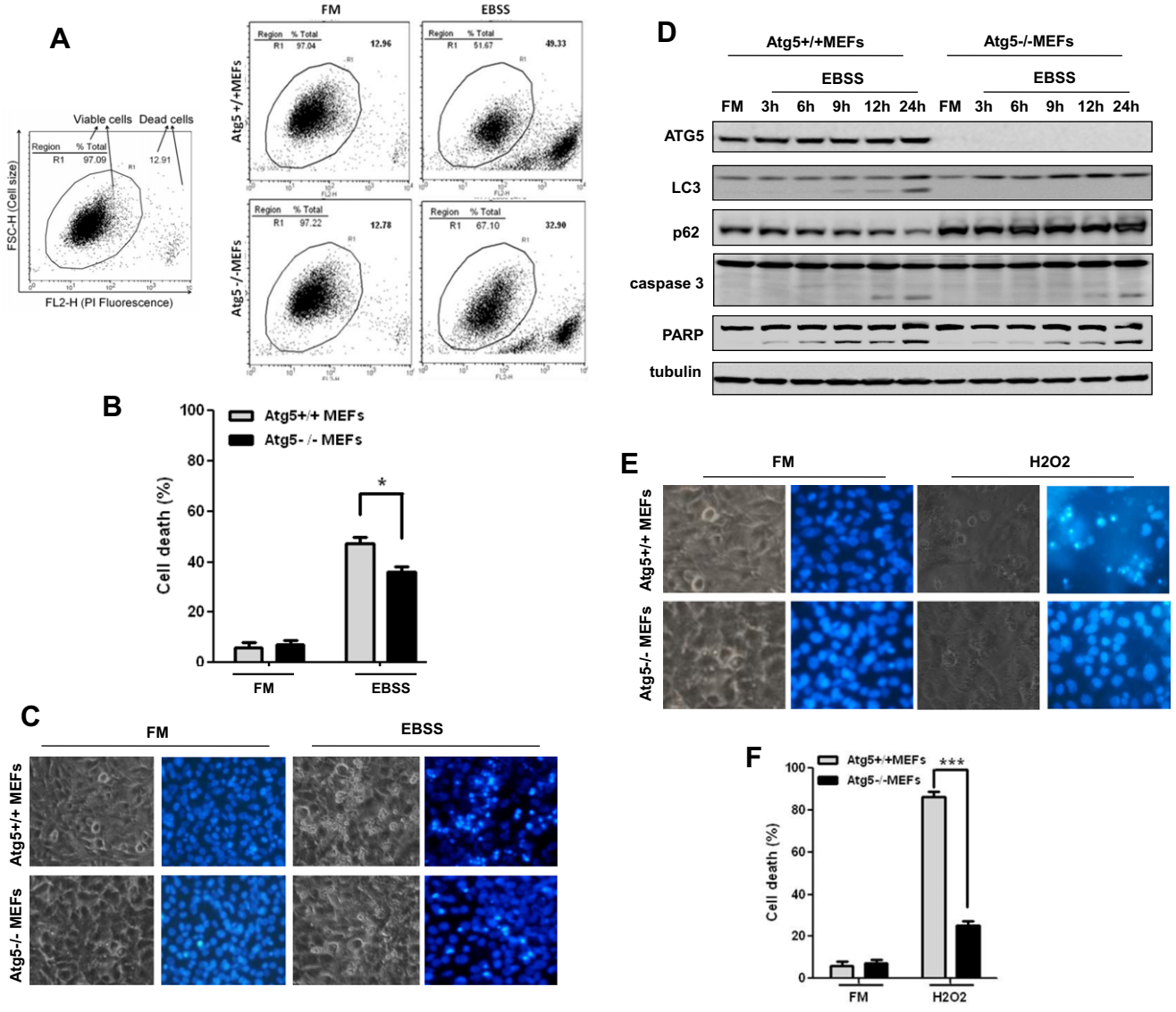

Fig. 1. Atg5 deficient MEFs derived from different clones respond differently to stress-induced apoptosis. (A) Dotplot of PI exclusion test coupled with flow cytometry for cell death. The regions that represent viable and dead cell in the dotplot are shown. Atg5+/+ and Atg5−/− MEFs were treated with control (FM: full medium) or EBSS for 24 h. (B) The percentage of cell death measured in Panel A was presented as means ± SD of three independent experiments ($^{*}p < 0.05$, *t*-test). (C) Cell death measured by morphological changes with DAPI staining. Cells were treated as in panel A and then stained with DAPI for nuclear condensation. (D) Atg5+/+ and Atg5−/− MEFs were treated as stated and cell lysates were analyzed by Western Blotting. (E, F) Atg5+/+ and Atg5−/− MEFs were treated with H_2O_2 (250 μM) for 24 h and cell death was measured as in panels A–C. (G, H) Atg5+/+ and Atg5−/− MEFs were treated with SNP (1000 μM) for 24 h and cell death was measured as in panels A–C. (I) Two additional clones of Atg5+/+ and Atg5−/− MEF were analyzed by Western Blotting to confirm the identity of the cells. (J) Cell death was quantified as described in panel A and B. $^{*}p < 0.05$, $^{***}p < 0.0005$ (Student's *t*-test).

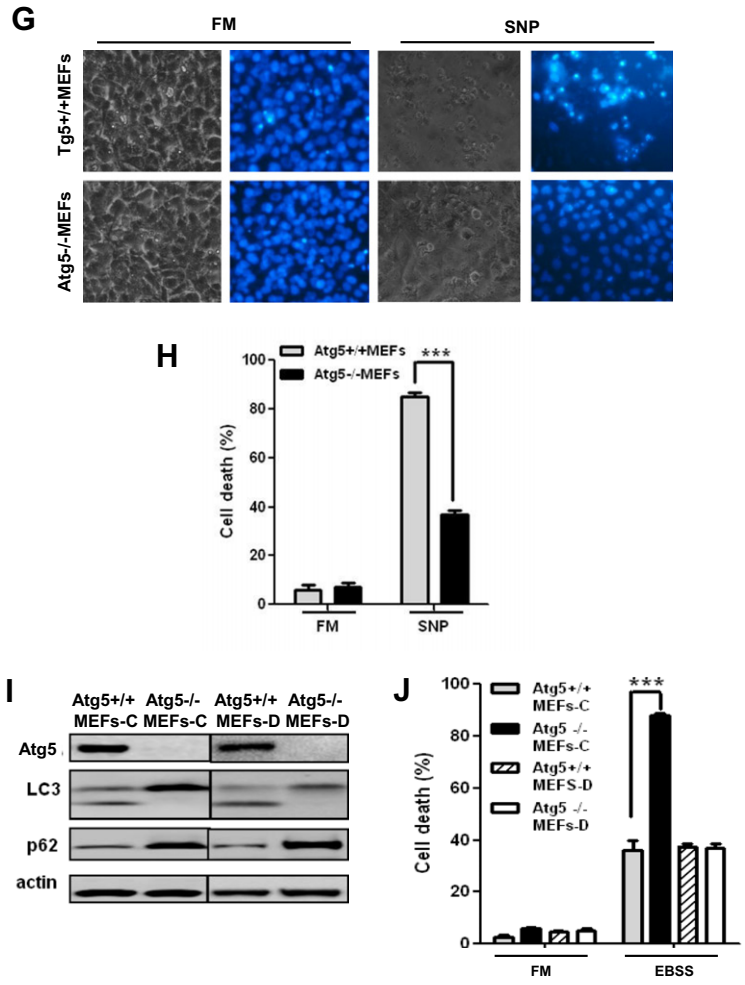

Fig. 1. (*continued*)

2.2. Cell culture and treatments

The different clones of Atg5 wild-type (Atg5+/+) and knockout (Atg5−/−) MEFs; and the Tet-off Atg5 expression MEFs (m5–7 cells) were provided by Dr. Noboru Mizushima (Tokyo Medical and Dental University, Japan) [22]. The Atg5+/+ and Atg5−/− iBMKs were from Dr. Eileen White (The Cancer Institute of New Jersey, USA) [23]. All cell lines mentioned above were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, from HyClone) and 1% penicillin–streptomycin (Invitrogen) in a 5% CO2 atmosphere at 37 °C. m5–7 cells were grown in the presence of 10 ng/ml doxycycline (DOX) for 4 day before performing experiments. For starvation, cells were cultured in EBSS for designated periods of time. Treatments with H_2O_2 and SNP were specified in the figure legends.

2.3. Generation of stable Atg5 inducible iBMK cell line (Atg5 Tet-on iBMKs)

Atg5 Tet-on iBMKs were generated using ViraPower™ T-REx™ Lentiviral Expression System (invitrogen) according to manufacturer's protocol. First, establishment of a stable expression tetracycline (Tet) on Atg5−/− iBMKs: (i) co-transfect the 293FT producer cell line with pLenti6/RT and Packaging Mix; (ii) harvest viral supernatant and determine the titer; (iii) add the Lenti6/TR viral supernatant to Atg5−/− iBMKs and use Blasticidin selection to generate a ViraPower™ T-REx™ cell line which stable expressed tetracycline (Tet) on Atg5−/− iBMKs (TetR Atg5−/− iBMKs). Second, generation of Atg5 expression clone: (i) introduce Atg5 gene into the pENTR™/D-TOPO vector to generate an entry vector (pENTR™/D-TOPO-Atg5), for Atg5 amplification, the following specific primers were used (5′-CACCATGACAGATGACAAAGATGTGCTT-3′ and 5′-TCAATCTGTTGGCTGGGGGACA ATGC-3′); (ii) transfer Atg5 gene into the pLenti4/TO/V5-DEST vector to generate destination plasmid (pLenti4/TO/V5-DEST-Atg5) by performing an LR recombination reaction between the pENTR™/D-TOPO-Atg5 vector and the pLenti4/TO/V5-DEST vector. Third, establishment of stable expression of wild type Atg5 gene on TetR Atg5−/− iBMKs: (i) co-transfect the 293FT producer cell line with the pLenti4/TO/V5-DEST-Atg5 and Packaging Mix; (ii) harvest viral supernatant and determine the titer; (iii) add pLenti4/TO/V5-DEST-Atg5 viral supernatant to TetR Atg5−/− iBMKs and use Zeocin selection to generate a Atg5 inducible iBMKs (Atg5 Tet-on iBMKs); (iv) check Atg5 gene expression by Tet addition using Western Blot.

2.4. Detection of cell death/cell viability

In this study, two approaches were used to detect cell death quantitatively and qualitatively, including (i) PI live cell exclusion assay coupled with flow cytometry, (ii) morphological changes with DAPI staining examined under a fluorescent microscope, and (iii) Western Blots for PARP and caspase-cleavages, all have been established and described previously [24,25].

2.5. Statistics

The quantitative data are expressed as mean ± standard deviation (SD) from at least 3 independent experiments. The significance level was set at $p < 0.05$ for each analysis using Student's *t*-test.

3. Results

3.1. Atg5 deficient MEFs derived from different clones had different susceptibility to starvation-induced apoptosis

Immortalized Atg5−/− MEFs have been widely used in the study of autophagy, with contradictory results whether the Atg5−/− MEFs are more sensitive or resistant to cell death comparing to the Atg5+/+ MEFs [20,21]. In this study, we first subjected the Atg5−/− MEFs to starvation (by culturing cells in EBSS) and then examined their responses to cell death. It was indeed surprising to see that the Atg5−/− MEFs were more resistant than the Atg5+/+ MEFs to starvation-induced cell death measured by the PI-exclusion test coupled with flow cytometry (Fig. 1A and B). Similar results were also found when cell death was assessed by morphological changes and with DAPI staining (Fig. 1C). The identity of the two cell lines were confirmed by Western Blot showing the absence of Atg5 protein, lack of LC3-II and accumulation of p62 protein in Atg5−/− MEFs (Fig. 1D). Nuclear condensation by DAPI staining (Fig. 1C) and evident cleavage of caspase 3 and PARP were detected in Atg5+/+ MEFs (Fig. 1D), indicating that cells die by apoptosis. In addition, Atg5−/− MEFs were also found to be more resistant than the Atg5+/+ MEFs under other treatments, including H_2O_2 (Fig. 1E and F), SNP (Fig. 1G and H) and TNFα (data not shown).

Since the above data are not consistent with the common understanding for the pro-survival function of autophagy under stress especially under starvation, we then tested two other clones of Atg5−/− MEFs provided by Dr. Mizushima's laboratory together with their respective paired Atg5+/+ MEFs. The identity of these two pairs (clone C and D) were shown in Fig. 1I by the absence of the Atg5 protein and enhanced p62 protein level. It is indeed intriguingly to note that the Atg5−/− MEFs from clone C was highly sensitive to starvation-induced cell death, while no obvious difference in cell death between Atg5+/+ and Atg5−/−MEFs in clone D (Fig. 1J). Therefore, the different sensitivity of the 3 different clones of Atg5−/− MEFs to starvation-induced cell death implies that such difference is unlikely due to the absence of Atg5 or autophagy *per se*, and it is possible that the clonal variation occurred during the establishment of immortalized MEFs determines the cellular responses to cell death stimuli such as starvation.

3.2. Inducible deletion of Atg5 enhances starvation-induced cell death using the Atg5 Tet-off MEFs (m5–7)

To eliminate the clonal variation occurred during the establishment of immortalized MEFs, we used one inducible cell line (m5–7) established by Dr. N Mizushima's laboratory in which Atg5 expression was completely suppressed with the addition of doxycycline hydrochloride (DOX) [22]. As shown in Fig. 2A, addition of DOX for 4 days completely eliminated the expression of Atg5, and thus also prevented the conversation of LC3-I to LC3-II, leading to accumulation of p62 protein. Notably, the percentage of dead cells in starved m5–7 with DOX addition was significantly higher that the cells without DOX (Fig. 2B). Similar results were also found when cell death was assessed by other methods such as morphological changes and Western Blot for apoptotic markers (data not shown).

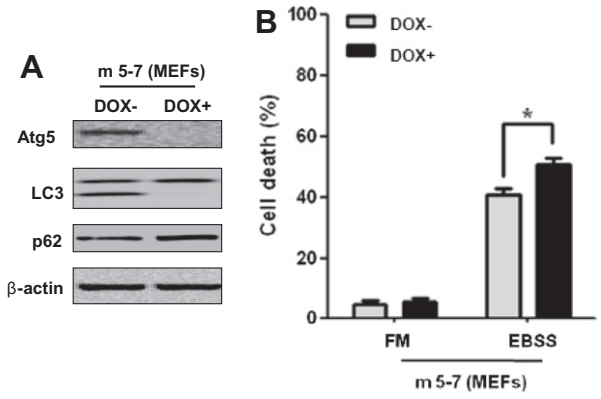

Fig. 2. Inducible deletion of Atg5 enhances starvation-induced cell death using the Atg5 Tet-off MEFs (m5–7). (A) Identification of Atg5 Tet-off MEFs (m5–7). After cells were treated with or without DOX (10 ng/ml) for 4 days, cell lysates of m5–7 cells were analyzed by Western Blotting. (B) m5–7 cells were treated with full medium or EBSS for 24 h. Cell death was measured using the same methods as described in Fig. 1A and presented as means ± SD of three independent experiments. $^*p < 0.05$ (Student's *t*-test).

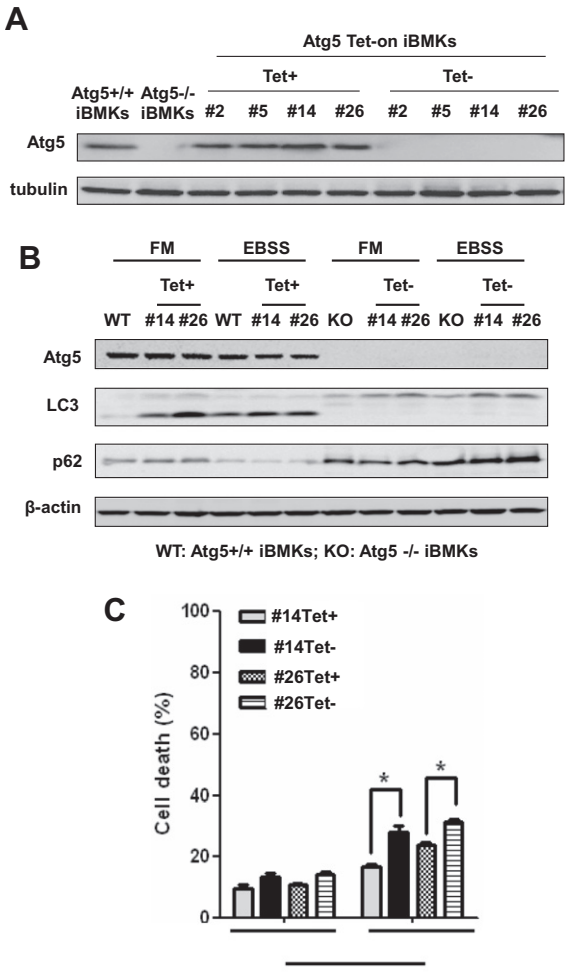

Fig. 3. Inducible expression of Atg5 protects against starvation-induced cell death using the Atg5 Tet-on iBMKs. (A) Atg5 Tet-on iBMKs were generated as described in Materials and Methods. Cell lysates from different Atg5 Tet-on iBMKs clones (#2, #5, #14 and #26) were analyzed by Western Blot after treatment with or without Tet (100 ng/ml) for 4 days. (B) Detection of starvation-induced autophagy in Atg5 Tet-on iBMKs. Cell lysates were analyzed by Western Blotting after cells were treated as designated. (C) Atg5 Tet-on iBMKs in response to starvation. Cell death was measured using the same methods as described in Fig. 1A and presented as means ± SD of three independent experiments. $^*p < 0.05$ (Student's *t*-test).

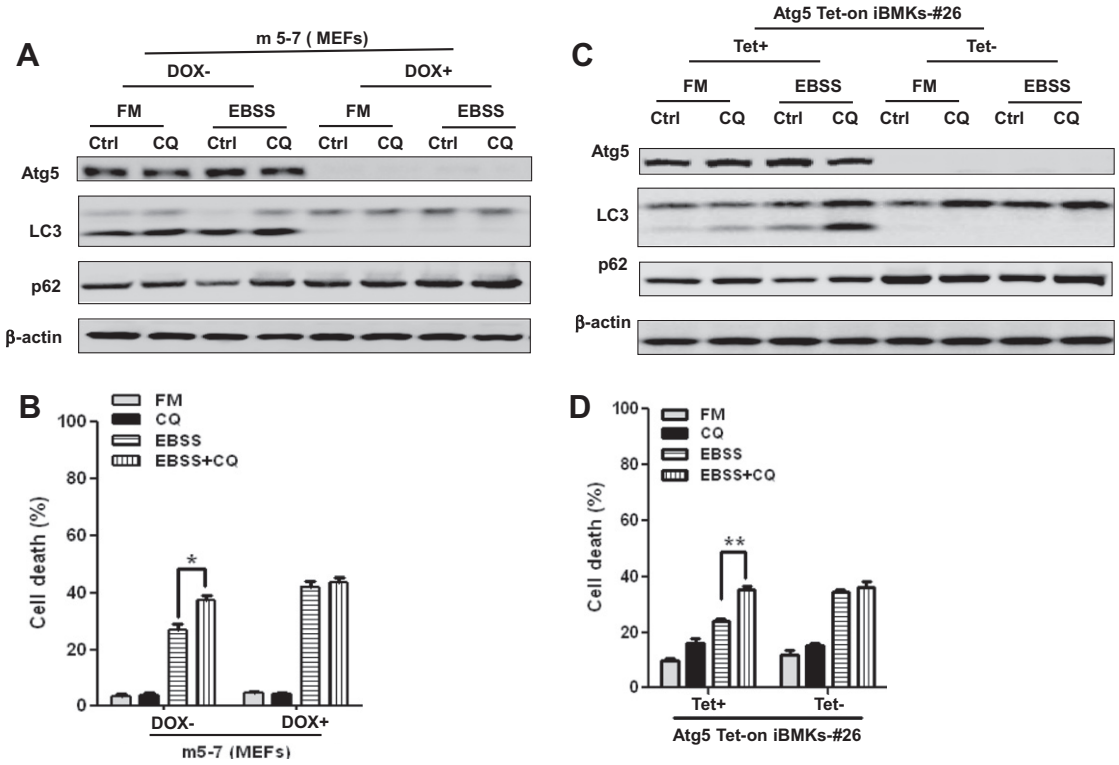

Fig. 4. CQ is only effective in sensitizing starvation-induced cell death in cells with Atg5 expression, but not in Atg5 deficient MEFs and iBMKs. (A and C) Blockage of autophagic flux by CQ in both m5–7 cells and Atg5 Tet-on iBMKs, respectively. Cells were treated with EBSS in the presence or absence of CQ (20 μM) for the indicated period of time. Autophagy flux level was examined using Western Bloting. (B, D) The sensitization effect of CQ on starvation-induced cell death in m5–7 cells and Atg5 Tet-on iBMKs, respectively. Cell death was measured using the same methods as described in Fig. 1A and presented as means ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.005$ (Student's *t*-test).

3.3. Inducible expression of Atg5 protects against starvation-induced cell death using the Atg5 Tet-on iBMK

In order to further confirm the findings from m5–7 MEFs, we went onto establish stable cell lines in which Atg5 was conditionally induced in Atg5−/− iBMKs. As shown in Fig. 3A, Atg5 expression was completely restored with Tet. As shown in Fig. 3B, the addition of Tet markedly increased LC3-II and reduced p62 protein level. Subsequently, the restored Atg5 expression made the iBMKs more resistant to starvation-induced cell death (Fig. 3C) in both clones selected. Such observations from Tet-on iBMKs are indeed consistent with the data from the inducible m5–7 cells described earlier.

3.4. CQ is only effective in sensitizing starvation-induce cell death in cells with Atg5 expression, but not in Atg5 deficient MEFs and iBMKs

To further test the pro-survival function of autophagy in response to starvation, here we used CQ to block autophagy through neutralizing the lysosomal pH [26,27]. As shown in Fig. 4A and C, CQ blocked the autophagic flux under starvation in cells with Atg5 expression (in m5–7 MEFs without DOX and in Tet-on iBMKs with Tet), but not in those cells without Atg5 expression (in m5–7 MEFs with DOX and Tet-on iBMKs without Tet). While CQ alone is not cytotoxic to both cells cultured in full medium, addition of CQ was able to enhance cell death induced by starvation in cells with Atg5 expression (m5–7 MEFs without DOX (Fig. 4B) and Tet-on iBMKs with Tet (Fig. 4D), but not in cells without Atg5 expression (m5–7 MEFs with DOX (Fig. 4B) and Tet-on iBMKs without Tet (Fig. 4D). Notably, m5–7 MEFs were found to have higher basal LC3-II level in the control cells (Figs. 2A and 4A). Such observations thus suggest that the sensitization effect of CQ on starvation-induced cell death is Atg5 or autophagy-dependent and autophagy plays a pro-survival role in cells under starvation.

4. Discussion

So far most of the studies examining the role of autophagy were conducted *in vitro* in cultured cells, including MEFs with deletions of various Atgs. One intriguing finding from our study is that the original clone of Atg5−/− MEFs were largely resistant to starvation-induced cell death (Fig. 1), which is contradictory to the well-established fact that autophagy is a pro-survival mechanism and also at odds with an earlier report that the Atg5−/− MEFs were resistant to amino acid starvation [20]. Since Atg5 cleavage product has been reported to directly promote apoptosis through an autophagy-independent mechanism [28], one possible explanation for the resistance of Atg5−/− to starvation may be due to an inability of the Atg5−/− cells to generate an Atg5 cleavage product. Nevertheless, no Atg5 cleavage fragment was detected in starved Atg5+/+ MEFs (data not shown), suggesting that the observed resistant to starvation-induced apoptosis is unlikely due to the loss of the direct pro-apoptotic effect of Atg5. Our data that different clones of Atg5−/− MEFs possess different susceptibility to different stress factors including starvation (Fig. 1), thus made us believe that the immortalized MEFs with Atg5 deletion are not an ideal system for evaluating the role of autophagy in cell death. In fact, our findings could explain some of the inconsistent findings related to the role of autophagy in cell death in the literature. Varied sensitivity of cells to death stimuli caused by clonal variation has also been reported by other studies. For example, TRAIL sensitivities were variable ranging from as high as 35% apoptosis to less than 5% apoptosis over 24 h in different clones derived from PC3 cells

(the human metastatic, androgen cell line) [29]. Similarly, A549 subclones were found to be either susceptible or resistant to cytotoxicity by TNF-α plus actinomycin D [30].

As discussed above, clonal variation is a particular problem in generation of immortalized cell lines which directly affects the outcome of cell death. To avoid genetic difference between wild type and knockout Atg5 MEFs caused by clonal variation, Atg5 inducible MEFs (m5–7) [22], an cell line with DOX-regulated Atg5 expression generated from a single clone based on Atg5−/− MEFs, were used to test our hypothesis. The major advantage of this system is to avoid the potential clonal variation. In order to further confirm our findings, we utilized the Atg5−/− iBMK cells to establish another Tet-on inducible stable line. Earlier studies have shown that Atg5−/− iBMK are more sensitive to cell death under metabolic stress [23]. Consistently, in this study inducible expression of Atg5 offered significant protection against starvation-induced cell death (Fig. 4), thus supports the notion that autophagy is an important pro-survival mechanism in response to starvation.

Finally we confirmed the pro-survival function of autophagy under starvation by suppression of autophagy using CQ, as CQ is only effective in sensitizing starvation-induced cell death in cells with Atg5 expression (m5–7 cells without DOX and Tet-on iBMKs with Tet), but not in cells without Atg5 expression. CQ is known to suppress autophagy by neutralizing lysosomal pH and then blocking the degradation stage of autophagy [26,27]. Intriguingly, some reports showed that CQ induces autophagic cell death in human dermal fibroblasts [31] and human glioblastoma cells [32]. However, in these two studies, cell death was featured with an increased level of autophagic vacuoles (autophagosomes) and LC3 immunostaining. It is argued that the increase of autophagic markers in the dying cells treated with CQ is most probably caused by the suppression of the lysosomal function, leading to blockage of autophagic flux. Therefore, autophagy should not be convicted as a killer in CQ-induced cell death [9]. The differential effects of CQ on the two inducible cell lines with or without Atg5 expression thus further establish the pro-survival function of autophagy in response to starvation.

Taken together, in this study we provide unequivocal evidence supporting the pro-survival function of autophagy in response to starvation. In addition, data from our study also demonstrate that: (1) the immortalized MEFs with deletion of Atgs may have significant limitations in examining the pro-survival role of autophagy, due to clonal variation; (2) stable cell lines with inducible Atg deletion or expression have the advantage of eliminating such clonal variation and thus offer a good system to examine the function of autophagy and Atgs, especially in determining the pro-death or pro-survival function.

Acknowledgments

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