

ROLE OF AUTOPHAGY IN BCL-2/BCL-XL MEDIATED G0 ARREST

By

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To my loving parents who have, with sacrifice, provided me with the best academic and spiritual education. I am very grateful for their unconditional love, support and prayers. Thank you for instilling in me the desire to strive for the best.

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LISTS OF ABBREVIATIONS

ATG	Autophagy
MEF	Mouse Embryonic Fibroblasts
PI3K	Phosphatidylinositol 3-Kinase
mTOR	Mammalian Target of Rapamycin
LC3	Microtubule-associated protein-1 light chain-3
UVRAG	UV Irradiated Resistance-Associated Gene
IL-3	Interleukin-3
3-MA	3-Methyladenine
GFP	Green Fluorescent Protein
WT	Wild Type
DKO	Double Knockout
ATP	Adenosine Triphosphate
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
DOX	Doxycycline
BrdU	Bromodeoxyuridine
FACS	Flow-Activated Cell Sorter
PY	Pyronin Y
PI	Propidium Iodide

CHAPTER I

INTRODUCTION

The Bcl-2 Family of Proteins

Apoptosis or programmed cell death is necessary to maintain homeostasis in the organism. Some roles for apoptosis include embryo development, tissue homeostasis, developing the immune repertoire and restricting the progress of infections. Defective apoptosis is linked, among others, to autoimmune diseases, neoplastic progression and metastasis, as cells no longer respond to restraining signals ¹.

The Bcl-2 family is the central regulator of programmed cell death ². Bcl-2 was first discovered at the interchromosomal breakpoint t(14:18) in follicular B cell lymphomas ^{3,4}. This family of proteins includes a variety of molecules with both anti and pro-apoptotic functions. Members of this family all share at least one conserved Bcl-2 homology (BH) domain. Three subfamilies have been identified. The anti-apoptosis subfamily is composed of Bcl-2, Bcl-xL, Bcl-w and others; the pro-apoptosis Bax subfamily is composed of Bax, Bak and Bok; and the pro-apoptosis BH3-only subfamily is composed of Bim, Bik, Bad, Bmf, Hrk, Noxa and Puma¹.

Anti-apoptosis Bcl-2 family members guard the mitochondrial membrane integrity to prevent cell death ⁵. For example, Bcl-2 binds the pro-apoptotic protein Bax at the mitochondrial membrane thus inhibiting its cell death function ⁶.

Upon cell death stimulus BH-3 only proteins can bind and activate Bax or Bak. They can also bind and neutralize Bcl-2⁷. Neutralization of Bcl-2 allows Bax to form homo-oligomers that will cause permeabilization of the outer mitochondrial membrane. These events lead to the release of cytochrome C from the mitochondria and the activation of the caspase cascade resulting in apoptosis^{1, 8, 9}.

Bcl2 and Bcl-xL as Cell Cycle Regulators

Although many studies have investigated the pro- and anti-apoptotic functions of the Bcl-2 family of proteins, it has been found that the anti-apoptotic Bcl-2 and Bcl-xL molecules also have a role in the cell cycle¹⁰⁻¹². It has been shown that cells that overexpress either Bcl-2 or Bcl-xL exhibit delayed entry into S-phase from quiescence^{13, 14}.

Previous studies have shown that Bcl-2 mutants that failed to rescue cells from apoptosis also failed to delay cell cycle entry, this observation led to the conclusion that the anti-apoptotic and anti-proliferative functions of Bcl-2 co-segregate¹⁵. Moreover it was shown that cells that overexpress Bad, a pro-apoptotic molecule that binds Bcl-xL and inhibits its anti-apoptotic function, had a shortened interval to S-phase from quiescence. The study suggested that the cell

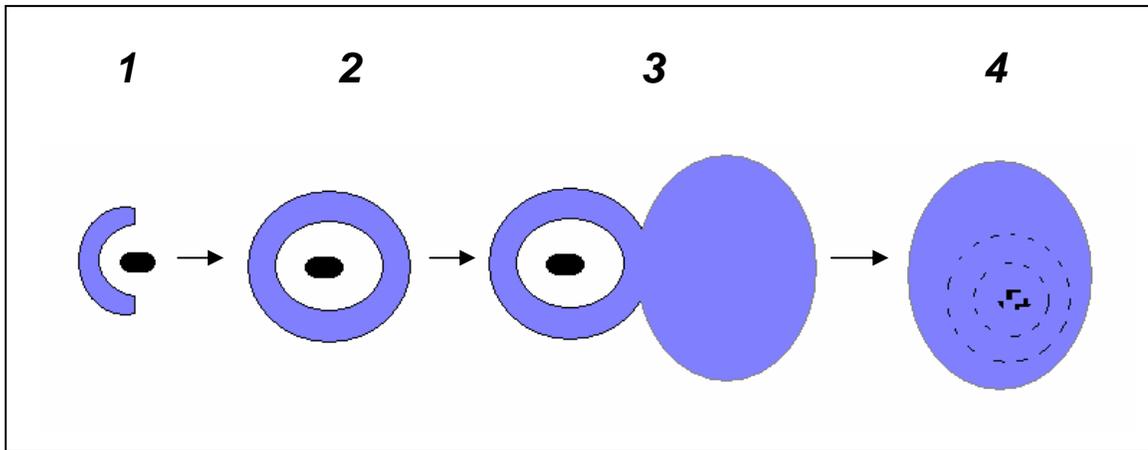


Figure 1. Steps in the process of autophagy. Upon nutrient limitation autophagy is induced. (1) A double membrane bound autophagosome forms and engulfs organelles, proteins and cytosol. (2) The autophagosome is then trafficked to the lysosomes. (3) Fusion between the autophagosome and the lysosome occurs. (4) The contents of the autophagosome are degraded and recycled.

cycle entry delay phenotype is dependent on the anti-apoptotic effect of Bcl-2¹⁵.

Subsequent studies have indicated that the cell cycle entry delay in Bcl-2/Bcl-xL expressing cells is due to the ability of these cells to arrest more efficiently in the G0 phase of the cell cycle¹⁵. Moreover, Bcl-2/Bcl-xL cells display a continuous decrease in cell size and decreased protein content upon serum starvation or contact inhibition-induced arrest¹⁵. Adding to the idea that an enhanced arrest is responsible for the cell cycle entry delay, it has been shown that the cyclin-dependent kinase inhibitor p27 is upregulated upon serum starvation-induced arrest in Bcl-2 and Bcl-xL expressing cells¹³. Bcl-2 and Bcl-xL cell cycle entry delay phenotype is inhibited in the absence of p27.

Autophagy and Cell Cycle Arrest

The characteristic cell cycle arrest phenotype observed in Bcl-2 and Bcl-xL expressing cells resembles the autophagy-induced cell cycle arrest. Autophagy is the digestion of components of the cytoplasm, particularly membrane bound organelles, the end result being the production of amino acids that can then be recycled and reused by the cell. This process is characterized by the formation of double membrane vesicles called autophagosomes that sequester cytosolic constituents and then fuse with lysosomes where enzymes break down the sequestered contents (Fig. 1). Autophagy occurs at basal levels in most tissues and plays an important role in the routine turnover of long lived proteins, damaged organelles and other cytoplasmic components. Autophagy has been implicated in diseases such as cancer, muscular disorders, neurodegeneration and pathogen to host response¹⁶⁻²¹. Autophagy has even been implicated in the process of aging²². Nutrient deprivation, contact inhibition and oxygen limiting conditions are some of the stimuli that cause the upregulation of this process. Autophagy is a self-limited survival strategy and will eventually lead to cell death if it remains constitutively active when the absence of nutrients is persistent²³.

Some studies have shown that upon cell death stimulus inhibition of apoptosis will result in the activation of autophagy also known as type II programmed cell death²⁴⁻²⁸. For example, Bax, Bak doubly deficient mouse embryonic fibroblasts (MEF), which are completely resistant to mitochondrial apoptosis, will undergo autophagy after death stimulation by the topoisomerase II

enzyme inhibitor; etoposide. On the other hand, when Bax or Bak were expressed in these cells and treated with etoposide, apoptosis was induced instead of autophagy. This led to the conclusion that etoposide-induced autophagy is activated in the absence of apoptosis, in this case in the absence of Bax, Bak activity²⁹. Bcl-2 and Bcl-xL overexpression in WT MEF inhibited apoptosis and also activated autophagy after etoposide treatment. On the other hand WT MEF only showed a mild autophagic response and displayed apoptotic cell death after being exposed to etoposide. These studies suggest that overexpression of either Bcl-2 or Bcl-xL not only inhibits apoptosis but results in the enhancement of the autophagic response.

In another study using interleukin-3-(IL-3) dependent *bax* *-/-* *bak* *-/-* bone marrow cells, it was found that after growth factor withdrawal cells activated autophagy. An initial decline in cell size was observed as a result of cell arrest in the G0/G1 phase of the cell cycle. Because during autophagy cells degrade intracellular substrate, the decline in cell size continued and no stable cell size was achieved. Furthermore, these cells displayed a decline in mitochondrial membrane potential and a decline in ATP. The decline in ATP levels was not as great as the decline in glucose transporters, suggesting that ATP levels were being maintained by utilizing alternative substrates, in this case through autophagy³⁰. Inhibition of autophagy during growth factor withdrawal decreased

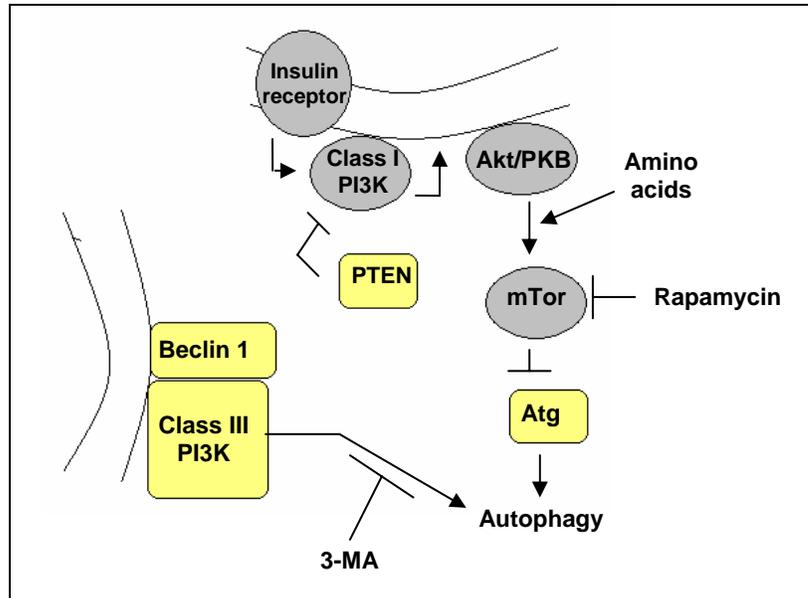


Figure 2. Schematic model of autophagic regulation.

cell viability suggesting that the cell survival was maintained through autophagy. In this study it was also found that growth factor deprivation- induced autophagy, impaired the ability of these cells to proliferate in response to growth factor stimulation. The cell must reverse the catabolic effects of autophagy. Because autophagy degrades organelles and proteins, including those required for cell cycle regulation, it took longer for these cells to re-enter S-phase³⁰.

p27 is required for the cell cycle function of Bcl-xL and Bcl-2¹³. Recent studies demonstrate that ectopic expression of p27 is sufficient to induce autophagy³¹. Moreover, accumulation of p27 permits the survival of cells during growth factor withdrawal through autophagy³¹. Therefore, because Bcl-2 and Bcl-xL can upregulate p27 and because p27 is required for the cell cycle function of these proteins, it is possible that autophagy might be induced during starvation conditions resulting in the delayed cell cycle entry observed after release.

Regulation of Autophagy

The high capacity of autophagy to degrade entire organelles would be harmful if it occurred randomly. Therefore, autophagy is tightly regulated³². Genetic screening in *Saccharomyces cerevisiae* has led to the discovery of autophagy (ATG) genes. ATG genes are important in the regulation, activation and formation of the autophagosome. One such gene is ATG5, which encodes a protein that forms an ubiquitin-like system with ATG12^{33, 34}. ATG5 has been shown to localize to double membranes and cause the elongation of the isolation membrane during the formation of the autophagosome³⁴⁻³⁶. Mouse embryonic stem cells lacking ATG5 showed defects in autophagosome formation resulting in the inhibition of autophagy.

Some players involved in the positive regulation of autophagy are the class III phosphatidylinositol 3-kinase (PI3K), Beclin 1 and UV irradiated resistance-associated gene (UVRAG) (Fig 2). Beclin 1 is the human homolog of *apg6*, first identified in yeast, and has also been shown to be required for autophagy in mammalian cells^{28,4737}. Together, Beclin 1, UVRAG and the class III PI3K form a complex that is thought to be important in mediating the localization of other autophagy proteins to the pre-autophagosomal structure³⁸. Inhibition of the class III PI3K by the use of methyladenine (3-MA) inhibits the formation of autophagosomes³⁹. On the other hand, the Class I PI3K and the mammalian target of Rapamycin (mTOR) have an inhibitory effect on autophagy³⁹⁻⁴¹. Upon external stimuli, such as insulin, the Class I PI3K phosphorylates the

lipid phosphatidylinositol at the cell membrane. Upon phosphorylation of the lipid, various proteins bind it and become activated, thus transmitting the external signal. Rapamycin can reverse the inhibition of autophagy caused by the Class I PI3K, suggesting that mTOR is a downstream target of this kinase. Moreover, although autophagy is sensitive to different kinds of stimuli, most of the signals are transduced to mTOR. In multicellular organisms mTOR is a good regulator of autophagy, because it is also a sensor for amino acids and ATP^{42, 43}.

We decided to investigate whether the enhanced arrest and cell cycle entry delay phenotype observed in Bcl-2 and Bcl-xL expressing cells is in part due to an enhanced autophagic response during serum starvation and contact inhibition induced arrest.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

Mouse embryonic fibroblasts (MEF), M5-7 obtained from Noboru Mizushima (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), *bax*^{-/-} *bak*^{-/-} mouse embryonic fibroblasts kindly provided by Craig Thompson and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) or calf serum and supplemented with 2mM glutamine and 100 U/ml penicillin/streptomycin. Bone marrow FL5.12 cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) with 10% FBS and supplemented with 2mM glutamine, 100 U/ml penicillin/streptomycin and interleukin 3 (IL-3). In m5-7, *atg5* expression was suppressed by the addition of [20ng/ml] doxycycline (Calbiochem) for at least 4 days before experiments were carried out. Inhibition of autophagy in all other cells was performed by treating the cells with the indicated concentrations of 3-MA (Sigma). Bcl-2 and Bcl-xL constructs in pBabe or pWzL were stably introduced in NIH 3T3, FL5.12, m5-7 and *bax*^{-/-} *bak*^{-/-} MEF by retroviral infection and selected with Puromycin or Neomycin. Protein expression was verified by immunoblotting and immunofluorescence.

Fluorescence labeling of DNA and RNA

Cells were collected and resuspended in ice-cold 70% ethanol. Cells were then washed in PBS and resuspended in 2mg/ml pyronin Y (Polysciences, Inc.) and 1.25 mg/ml 7-AAD (BD PharMingen)-containing phosphate buffered saline (PBS) solution. Samples were analyzed by FACS. 7-AAD was measured in FL3 and pyronin Y staining measured in FL2.

Cell cycle analysis

For serum starvation, cells were plated and the next day washed 3 times with PBS and switched to media containing 0.75 % serum. Cells were kept in starvation (serum reduced) media for 3 days, collected at indicated time points and resuspended in Krishan's reagent (0.02 mg/ml RNase A, 0.3% NP-40, 0.1 mg/ml PI, and 0.1% and Na citrate). Samples were analyzed using flow cytometry (FACSCalibur). Cells were stimulated into cell cycle entry by re-addition of 10% serum.

For contact inhibition, 3×10^6 cells were plated in 10cm plates and allowed to reach confluence for 3 days. Cells were collected and processed in the same manner as for serum starvation.

For BrdU incorporation cells were treated with 20uM BrdU for 30 minutes. Cells were then collected and fixed in cold 70% ethanol. After fixation, cells were treated with 4N HCl, neutralized with 0.1M Borax and washed in 0.05% BSA-containing PBS. Anti-BrdU antibody (Becton and Dickson) followed with FITC-conjugated anti-mouse secondary antibody (Sigma) were used in 0.5% BSA and

0.5% Tween-20 containing PBS. Cells were then resuspended in PBS and DNA stained with PI in the presence of RNase A and analyzed by FACS.

Cell death assay

Cells were collected and resuspended in 100mg/ml propidium iodide (PI) in PBS. The cells were then analyzed by flow cytometry. PI positive cells were classified as apoptotic and PI negative cells as live cells.

Western blots and antibodies

Cell lysate electrophoresis was performed using 15% SDS-PAGE. Antibodies against Bcl-xL (H5) and Bcl-2 (C2) were obtained from Santa Cruz Biotechnology and antibodies against p27 (kip1/p27) from BD Transduction Laboratories. Antibodies against LC3 were a kind gift from Noboru Mizushima. Equal protein loading of western blots was verified by blotting for β -actin where indicated.

CHAPTER III

RESULTS

Intermediate arrest phenotype in *bax*, *bak* double knockout cells

It has been shown that Bcl-2 and Bcl-xL expressing cells display a delay entry into the cell cycle from arrest (Huang 1997). Delayed S-phase entry of Bcl-xL and Bcl2 overexpressing cells is associated with prolonged arrest in G0 rather than in G1 (Janumyan 2003). Transition of cells from G0 to G1 is accompanied by an increase in polyribosomal RNA whereas the DNA content remains the same. Because Bcl-2 protects the cell against cell death many have argued that the arrest phenotype observed is an indirect effect of this protection. In order to address this question we overexpressed Bcl-2 in *bax*, *bak* double knock out (dko) mouse embryonic fibroblasts (MEF) (Fig. 3). Bak and Bax are two pro-apoptotic proteins that are required for mitochondrial mediated programmed cell death. If the anti-apoptotic function of Bcl-2 is responsible for the enhanced G0 arrest, then the same phenotype should be observed in *bax*, *bak* deficient cells. Cells were arrested by contact inhibition and collected after 5 days of arrest and compared to cycling controls. We used pyronin Y (PY) which preferentially stains RNA and propidium iodide (PI) to stain DNA. Cells with 2N DNA content that show low PY suggest an arrest in G0 rather than G1. As expected, Bcl-2 overexpressing wt MEF had a significant decrease (~41%) in RNA content when arrested whereas we did not observe a significant decrease in arrested vector

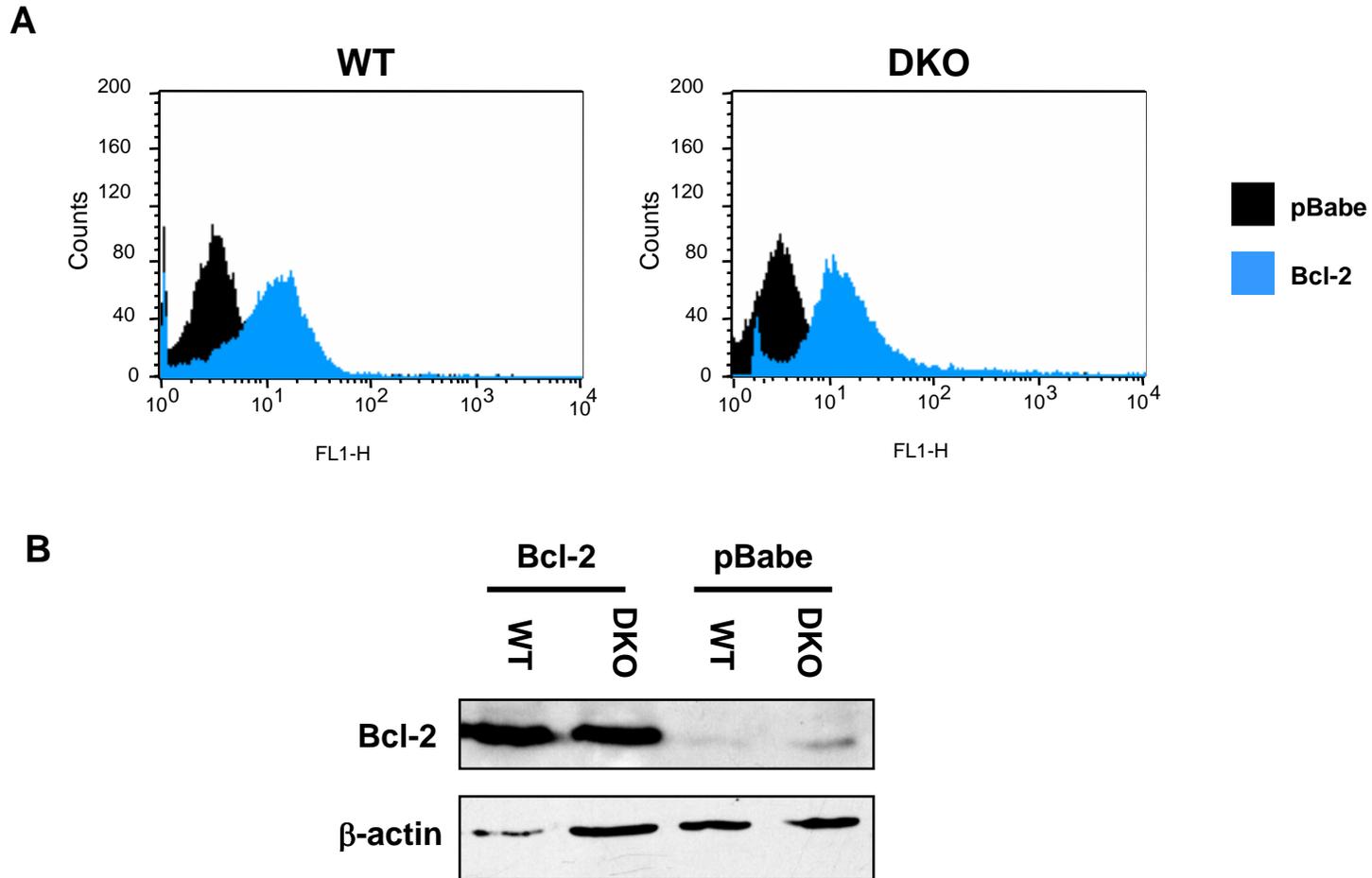


Figure 3. Bcl-2 overexpression in MEF. Wild type and *bax*^{-/-}, *bak*^{-/-} MEFs were retrovirally infected with either vector only (pBabe) or Bcl-2. (A) Bcl-2 overexpression as measured by immunofluorescence using flow cytometry. (B) Bcl-2 expression by Western blot.

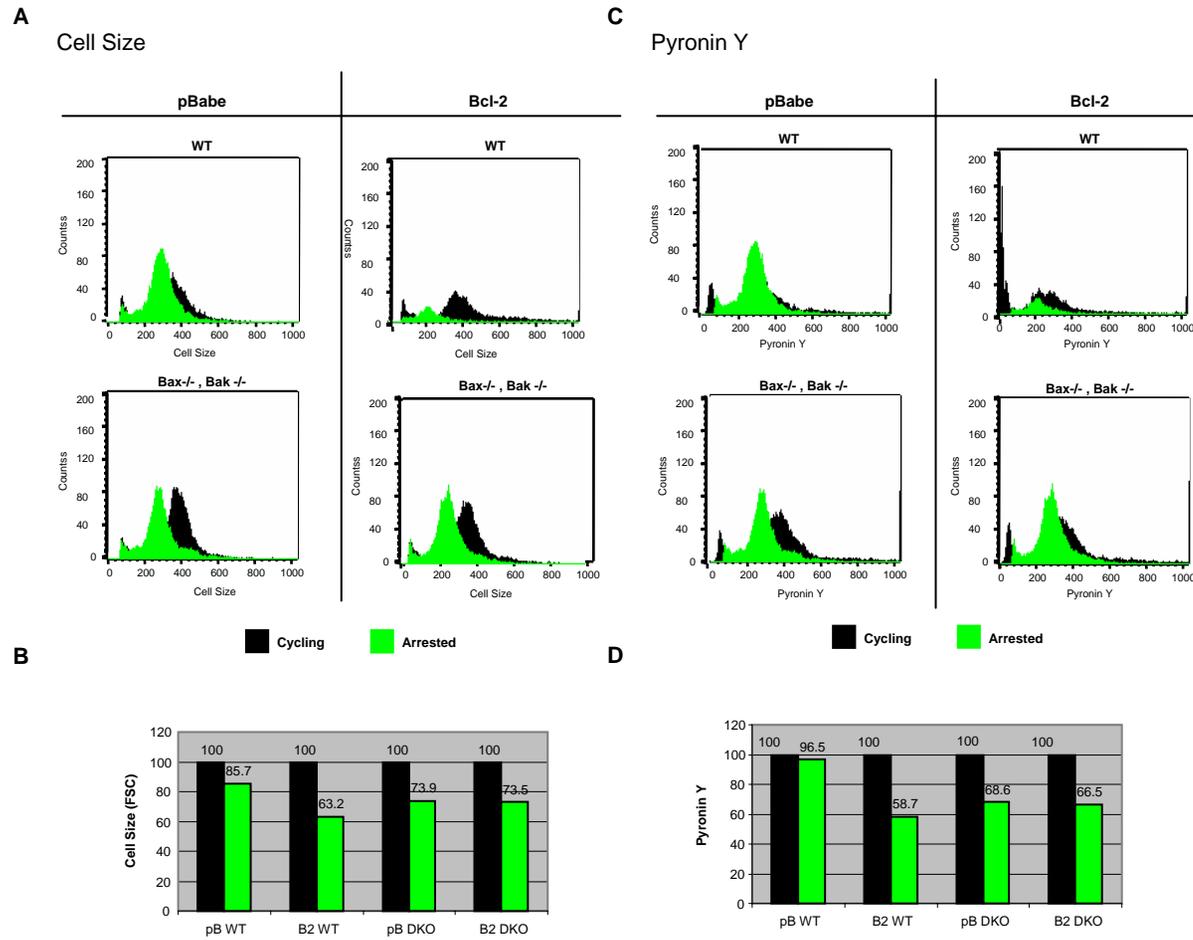


Figure 4. Partial enhanced arrest in *bax*^{-/-} *bak*^{-/-} MEF. Wild type and double knock out mouse embryonic fibroblasts expressing Bcl-2 or pBabe vector were arrested by contact inhibition and collected at day 5. (A) Cells size (Forward scatter) as measured by FACS. (B) Percentages of mean cell size from A. (C) Cells were stained for both pyronin Y and PI for FACS analysis. (D) Percentages of mean pyronin Y from C. These results represent two independent experiments with similar results.

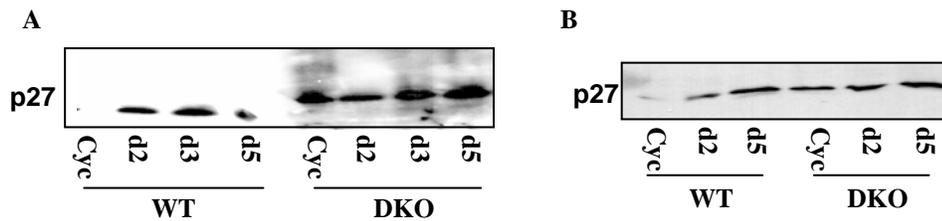


Figure 5. p27 upregulation in cycling *Bax*^{-/-}, *Bak*^{-/-} MEFs. Western blot of protein extracts from cycling (Cyc) and arrested cells at the indicated time points (d =days of arrest). (A) Cells were arrested by serum starvation (0.75% FBS containing media). (B) Cells were arrested by contact inhibition.

only expressing wt (Fig. 4C and D). On the other hand, when arrested, vector only *bax*^{-/-} *bak*^{-/-} MEF had a decrease of approximately 31% in RNA content suggesting that protection from apoptosis alone can result in an intermediate arrest phenotype between wt MEF and Bcl-2 expressing cells. No added effect was observed by overexpressing Bcl-2 in *bax*^{-/-} *bak*^{-/-} MEF. This result suggests that protection from cell death by Bcl-2 overexpression is in part responsible for the enhanced arrest but Bax and Bak are required for the complete enhanced arrest phenotype to occur. This result is in agreement with previous findings where the expression of pro-apoptotic Bad together with Bcl-xL resulted in an intermediate arrest phenotype where PY content only decreased partially during serum starvation¹⁵.

Another parameter used to identify cells in G0 is cell size, where cells arrested in G0 are smaller than cells in G1. As expected, arrested Bcl-2 expressing wild type MEF decreased significantly (~37%) in cell size. On the other hand, cell size in vector only wild type MEF did not decreased significantly

(~14%) when arrested (Fig. 4A and B). Again we saw that arrested *bax*^{-/-} *bak*^{-/-} MEF had an intermediate arrest phenotype and decrease more in cell size than arrested vector only wt. Also, Bcl-2 overexpression in *bax*, *bak* deficient cells had no added effect in decreasing cell size. The results obtained for RNA content and cell size suggest that inhibiting apoptosis is enough to cause an intermediate arrest phenotype. It has been reported that many cells will undergo autophagy when the apoptotic response fails subsequently leading to cell cycle arrest and lower RNA content. Interestingly, we saw that in *bax*^{-/-} *bak*^{-/-} cells p27 levels were already upregulated in cycling cells (Fig 5.) when compared to the wild types. As mentioned earlier, studies have shown that ectopic expression of p27 is sufficient to induce autophagy. We decided to investigate whether autophagy is responsible for the enhanced cell cycle arrest phenotype observed in Bcl-2 and Bcl-xL overexpressing cells.

3-MA has no effect on the ability of Bcl-2 to enhance arrest

Autophagy is known to be inhibited by PI3 kinase inhibitors such as 3-methyladenine (3-MA). We first determined the concentration of 3-MA that would result in less toxicity to our wt MEF (Fig. 6A and B). We exposed wt MEF to different concentrations of 3-MA during serum starvation conditions (0.75% FBS containing media) and assayed for cell death by PI exclusion/FACS. We decided to use 5mM 3-MA in our experiments. As expected, we saw that 3-MA had no toxicity in *bax*^{-/-} *bak*^{-/-} cells. Next we asked whether treating the Bcl-2 expressing cells with 3-MA during arrest would result in inhibition of enhanced arrest. We

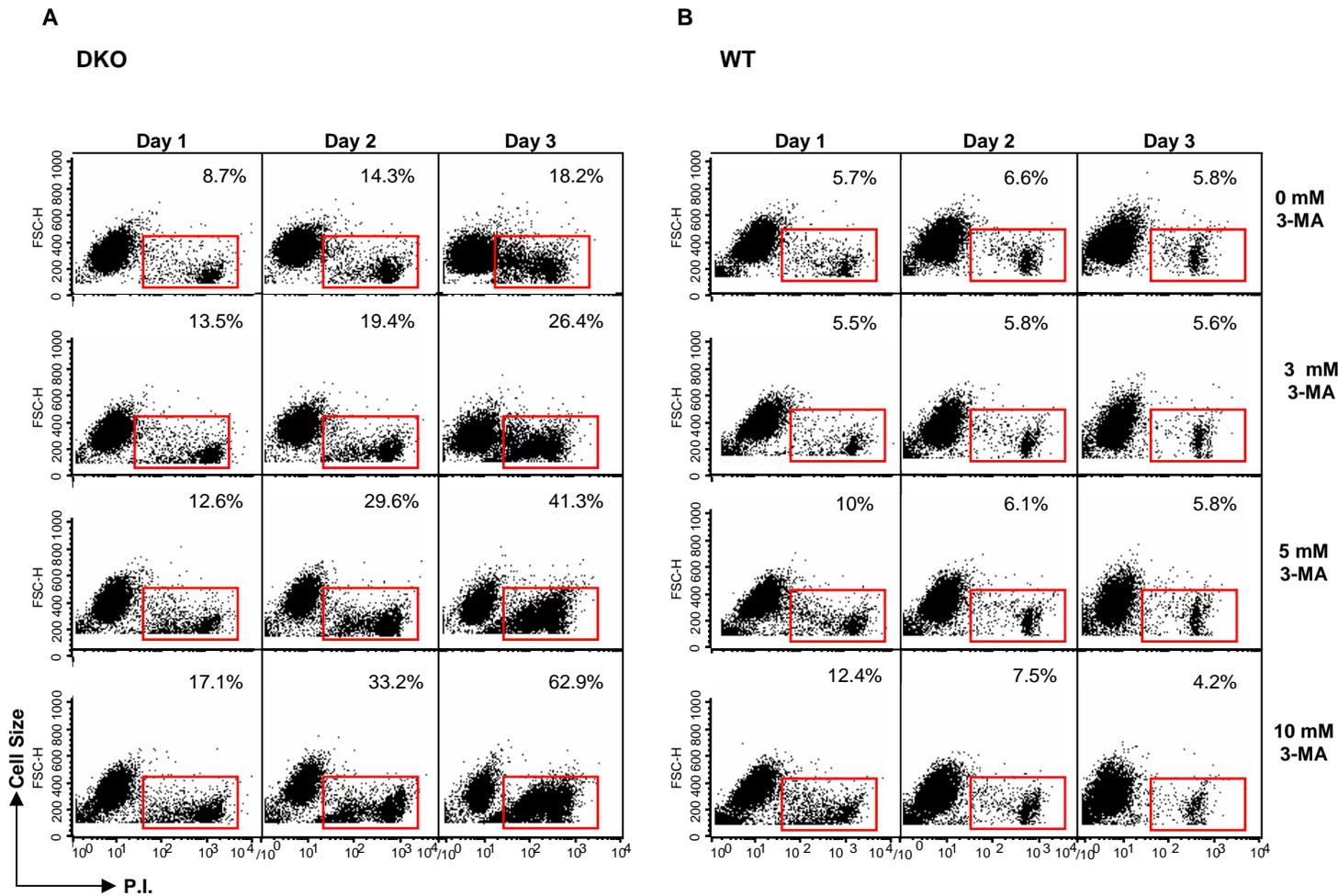


Figure 6. 3-MA toxicity in MEF. The best 3-MA concentration to be used was assayed by exposing the cells to different concentrations of 3-MA and measuring cell death by PI exclusion at the indicated time points. Cells were exposed to 3-MA during starvation conditions (0.75% FBS- containing media). (A) Wild type MEFs. (B) *bax*^{-/-} *bak*^{-/-} MEF.

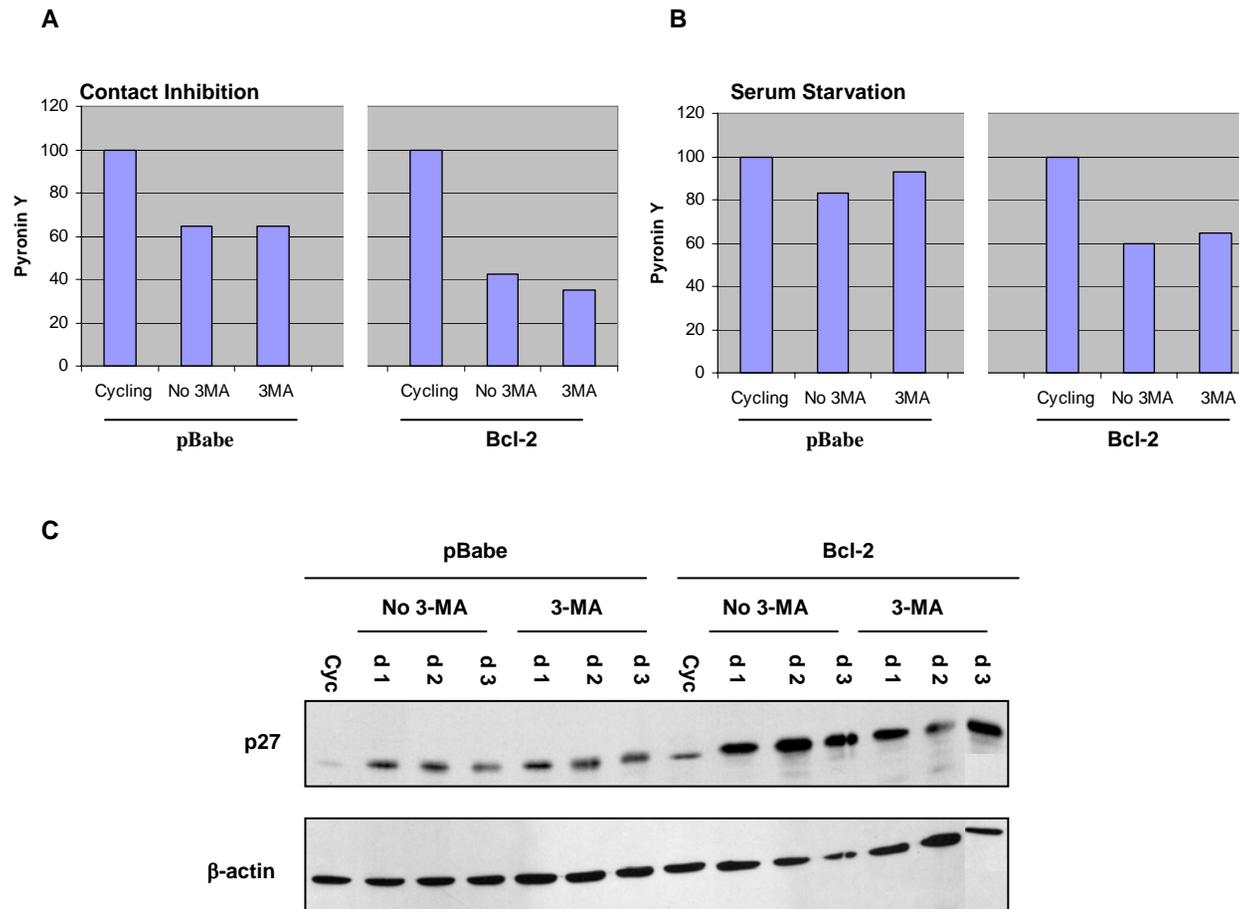


Figure 7. 3-MA treatment has no effect on Bcl-2 induced enhanced arrest. Wild type MEF expressing Bcl-2 or vector only were arrested in the presence of 5mM 3-MA and stained with both Pyronin Y and 7-AAD for FACS analysis. 100% = Mean PY fluorescence for cycling cells. (A) Cells were contact inhibited for 10 days. (B) Cells were serum starved by culturing them for 3 days in 0.75% FBS-containing media (starvation media). (C) Western Blot analysis of cells collected at the indicated time points during starvation, where d=days of arrest and cyc=cycling cells). Although contact inhibition was performed once, serum starvation results represent 3 independent experiments where no effect was seen in 3-MA treated cells.

treated vector only and Bcl-2 wt MEF with 5mM of 3-MA during arrest by either serum starvation or contact inhibition, and looked at cell size and RNA content. As shown, (Fig. 7A and B) there was no significant difference in RNA content when the cells were treated with 3-MA. We also looked at cell size by Flow Cytometry, and again there was no difference between 3-MA treated cells and untreated control. These results were true for both contact inhibition and serum starvation. The same experiment was performed using Bcl-2 and vector only expressing *bax* *-/-* *bak* *-/-* MEF and we found that 3-MA treatment during arrest had no effect (data not shown).

We also looked at p27 upregulation during arrest (Fig. 7C). As expected p27 levels were upregulated during arrest and p27 was higher in our Bcl-2 expressing cells. On the other hand we observed that there was no difference in 3-MA treated cells and untreated controls.

3-MA treatment inhibits the ability of Bcl-xL to enhance arrest in G0/G1

Next we decided to see whether this was true in another system. We had previously described Bcl-2 and Bcl-xL enhanced arrest phenotype in NIH 3T3 cells. After looking at 3-MA toxicity in wild type 3T3 cells (Fig. 8A) we decided to use 5mM 3-MA during the entire period of serum starvation in our experiments. NIH 3T3 cells expressing either Bcl-2 or vector alone were arrested by serum starvation and then induced to re-enter the cell cycle by serum stimulation. S-phase entry was measured by propidium iodide (PI) staining of the DNA content. As expected, vector alone control began S-phase entry at 14h after serum

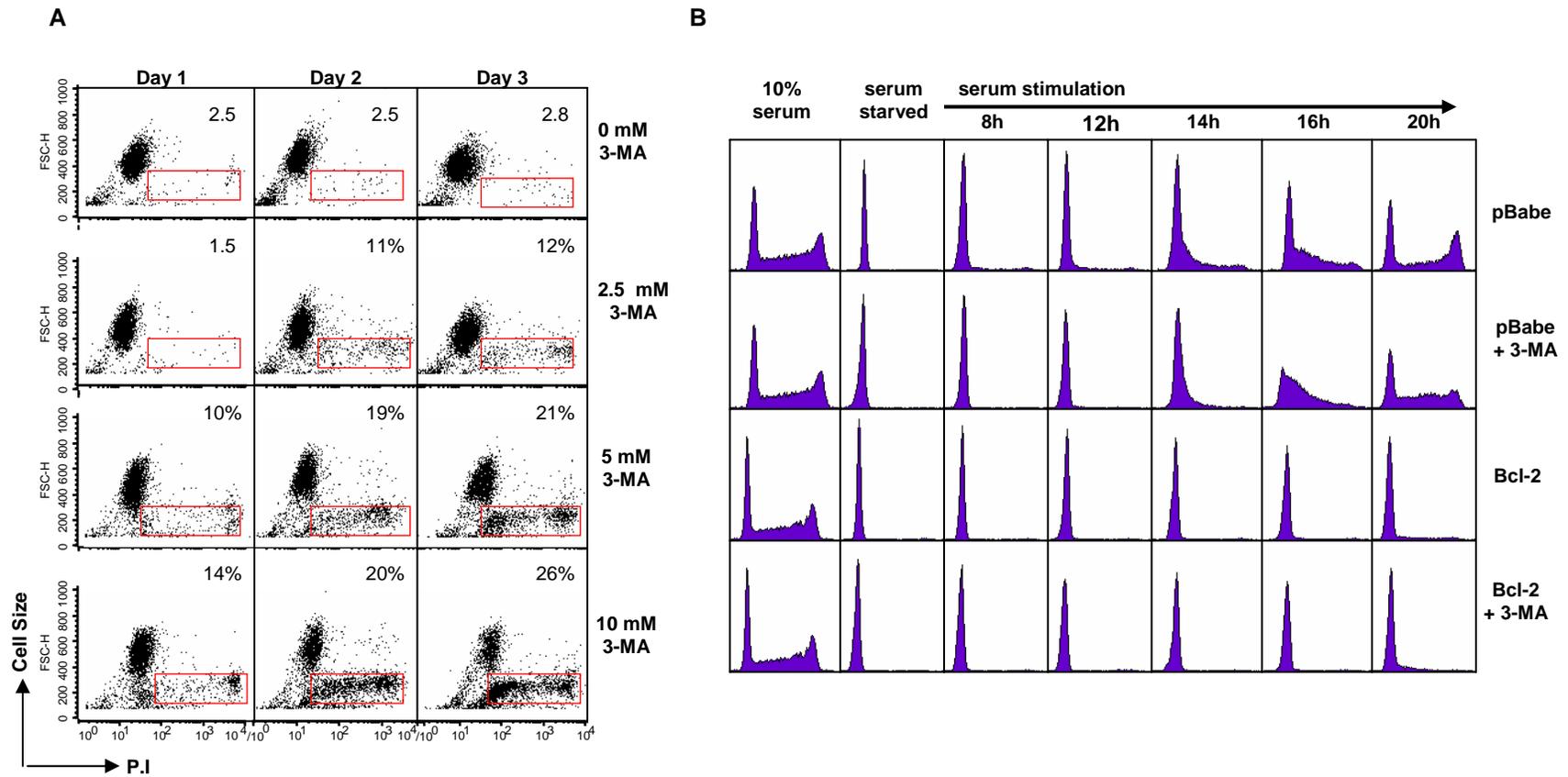


Figure 8. Bcl-2 expressing 3T3 can arrest in the presence of 3-MA during serum starvation. (A) To determine the concentration of 3-MA to be used, wild type 3T3 were cultured in starvation media (0.75% FBS) using different concentrations of 3-MA and cell death assayed at the indicated time points. Cell death was assayed by PI exclusion and flow cytometry. (B) Bcl-2 and vector only 3T3 cells were harvested before serum starvation (10% serum) and after 3 days of serum starvation in 0.75% containing media in the presence of 5mM 3-MA. Cell cycle profiles were obtained by PI/FACS analysis

stimulation whereas Bcl-2 expressing cells were still arrested even after 20h of serum re-addition. No difference between 3-MA treated and untreated Bcl-2 cells was observed as both groups arrested and were delayed in cell cycle entry when compared to vector alone (Fig. 8B).

Because no effect was found in the timing of cell cycle entry between 3-MA treated and untreated cells we decided to examine cells only during arrest. During arrest the number of cells in S and G2/M phase diminishes significantly and cells exit to G0/G1. We arrested Bcl-2, Bcl-xL and vector only expressing cells by serum withdrawal in the presence or absence of 3-MA and measured the percentage of cells in S and G2/M by PI staining of DNA content. As expected, during arrest conditions, the number of cells in S and G2/M in untreated Bcl-2 and Bcl-xL decreased approximately 85% (Fig. 9A and B), compared to a 60% decrease in the vector alone. The decrease in S and G2/M cells in 3-MA treated Bcl-2 cells and vector only cells was very similar to that of untreated cells, although there was a greater decrease in our 3-MA treated vector alone cells. To our surprise, arrested Bcl-xL expressing cells had only a 60% decrease in the number of cells in S and G2/M phase when treated with 3-MA. In contrast untreated cells displayed a decrease of 85%. The 60% decrease in the number of cells in S and G2/M phase in 3-MA treated Bcl-xL cells was comparable to the 60% decrease in the untreated vector alone cells. This finding suggests that 3-MA treatment during arrest inhibits the ability of Bcl-xL to enhance arrest in the G0/G1 phase of the cell cycle.

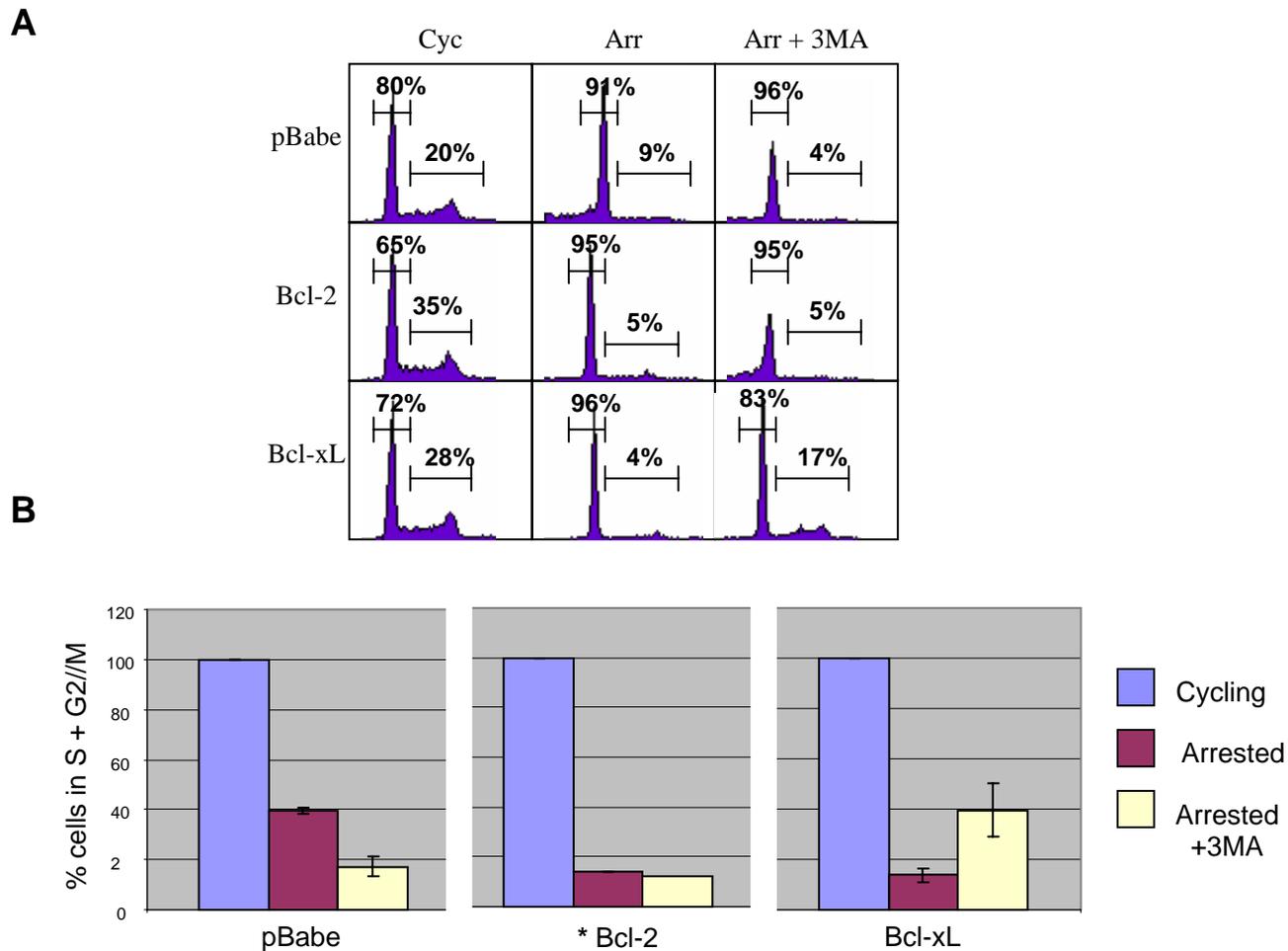


Figure 9. Bcl-xL enhanced arrest in G1/G0 is inhibited by 3-MA treatment during arrest in 3T3 cells. Bcl-xL, Bcl-2 and vector only (pBabe) expressing 3T3 were arrested by serum starvation (0.75% FBS containing media) for 3 days in the presence of 5mM 3-MA or without 3-MA. Cells were collected before arrest (Cyc=cycling) and after arrest (Arr). **(A)** Cell cycle profiles were obtained by PI DNA staining and analyzed by flow cytometry. **(B)** Shows percentage of cells in the S and G2/M phases from A. 100%= % of cells in S and G2/M from cycling cells. pBabe and Bcl-xL graphs represent data from 3 independent experiments. *Bcl-2 graph represents results from 2 independent experiments.

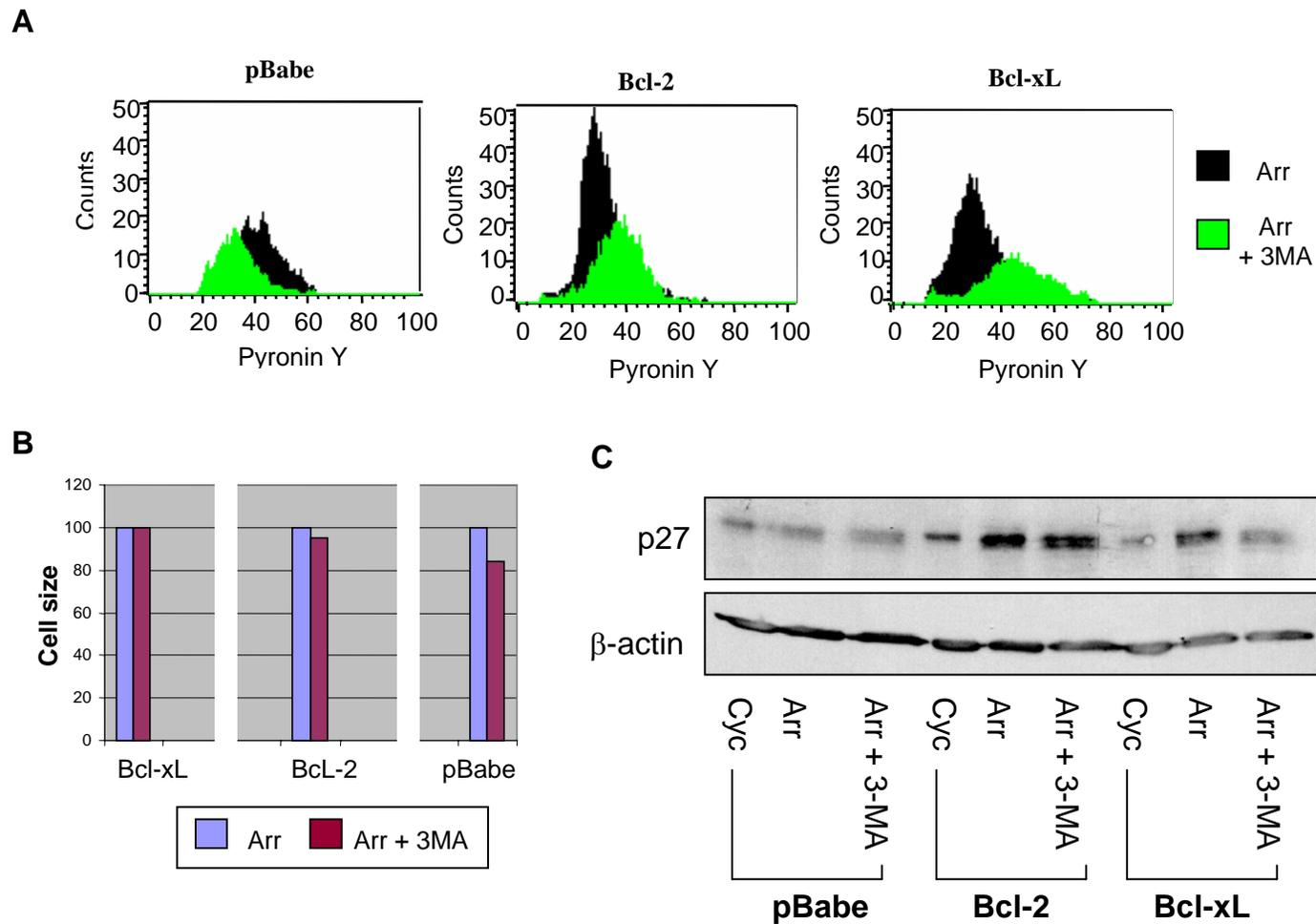


Figure 10. RNA content increase and p27 down regulation in 3-MA treated Bcl-xL cells during arrest. Bcl-xL, Bcl-2 and vector only (pBabe) expressing 3T3 were arrested (Arr) by serum starvation (0.75% FBS containing media) for 3 days in the presence of 5mM 3-MA or without 3-MA. **(A)** Cells were stained with both Pyronin Y and 7-AAD and analyzed by flow cytometry. Shows only arrested cells with 3-MA (Arr + 3-MA) or without 3-MA (Arr). **(B)** Cell size by forward scatter (FSC). Shows only arrested cells with or without 3-MA. 100% = Mean FSC from cycling. **(C)** Western blot analysis for p27. Compares cells before arrest (Cycling) and at day 3 of arrest with or without 3-MA. WB data represents two independent experiments.

We next investigated whether the effect observed in 3-MA treated Bcl-xL cells was due to the inability of the cells to arrest in G₀. To address this question we measured the RNA content by PY staining by gating on cells with 2N DNA content. As shown in figure 10A, arrested vector only cells treated with 3-MA showed no significant differences but a slight decrease in PY content when compared to untreated control. In contrast, a significant increase in the amount of PY was observed in arrested Bcl-xL cells that were treated with 3-MA when compared to untreated Bcl-xL controls. Bcl-2 expressing cells displayed a slight increase in the amount of PY that was not as significant as the one observed in Bcl-xL cells. Bcl-xL cells that arrest in the presence of 3-MA appear to be at G₁ or S-phase rather than at G₀. These results are consistent with the cell cycle profile phenotype described earlier. Using flow cytometry we looked at forward scatter (FSC) and measured cell size on arrested cells with 2N DNA content. We found that there was no significant change in cell size between 3-MA treated and untreated cells during arrest (Fig. 10B). We also looked at p27 expression (Fig. 10C) upon arrest and found that in 3-MA treated Bcl-xL cells p27 appeared to have decreased.

To confirm that the inhibition of enhanced G₀ arrest by 3-MA in Bcl-xL expressing cells was consistent and not a cell line specific event we decided to look at the effect of autophagy inhibition in Bcl-2 and Bcl-xL overexpressing interleukin (IL-3)-dependent FL5.12 bone marrow cells. We arrested these cells by IL-3 withdrawal and then stimulated cell cycle entry by re-addition of IL-3 into the media. During IL-3 withdrawal induced arrest we inhibited autophagy with

5mM 3-MA. Control cells began S-phase entry by 10h while Bcl-2 expressing cells remained arrested until 22h as expected (Fig. 11A). Inhibition of autophagy during arrest in Bcl-2 cells did not have a marked effect in the cell cycle entry kinetics. Both, Bcl-2 and 3-MA treated Bcl-2 cells arrested after IL-3 withdrawal and displayed a delayed entry into S-phase. These results are consistent with what we saw in Bcl-2 expressing 3T3 cells described earlier. We then looked at Bcl-xL overexpressing FL5.12 cells (Fig. 11B). Again, cells were arrested by IL-3 withdrawal and as stated earlier our control cells began cell cycle entry by 10h. On the other hand Bcl-xL delayed cell cycle entry until 22h after growth factor stimulation. Consistent with our 3T3 results, Bcl-xL cells treated with 3-MA did not show enhanced arrest in G0 and the cell cycle profile showed increased number of cells in S-phase during arrest conditions. This experiment was repeated twice with consistent results. In contrast to what we saw earlier in 3T3 cells, arrest induced p27 upregulation in Bcl-xL cells remained unaltered in the presence of 3-MA (Fig 11C).

Because 3-MA is a PI3 kinase inhibitor it does not specifically inhibit the Class III PI3 kinase responsible for inducing autophagy but both the Class I and Class III PI3 kinases. It is also possible that 3-MA might suppress other growth factor signaling pathways. Because serum starvation will signal the cell to arrest, inhibition of the class I PI3 kinase and other growth factor pathways should not be a problem in our experiments. Even so, we decided to further study autophagy using m5-7 cells, a cell line with tetracycline-regulated autophagy⁴⁴.

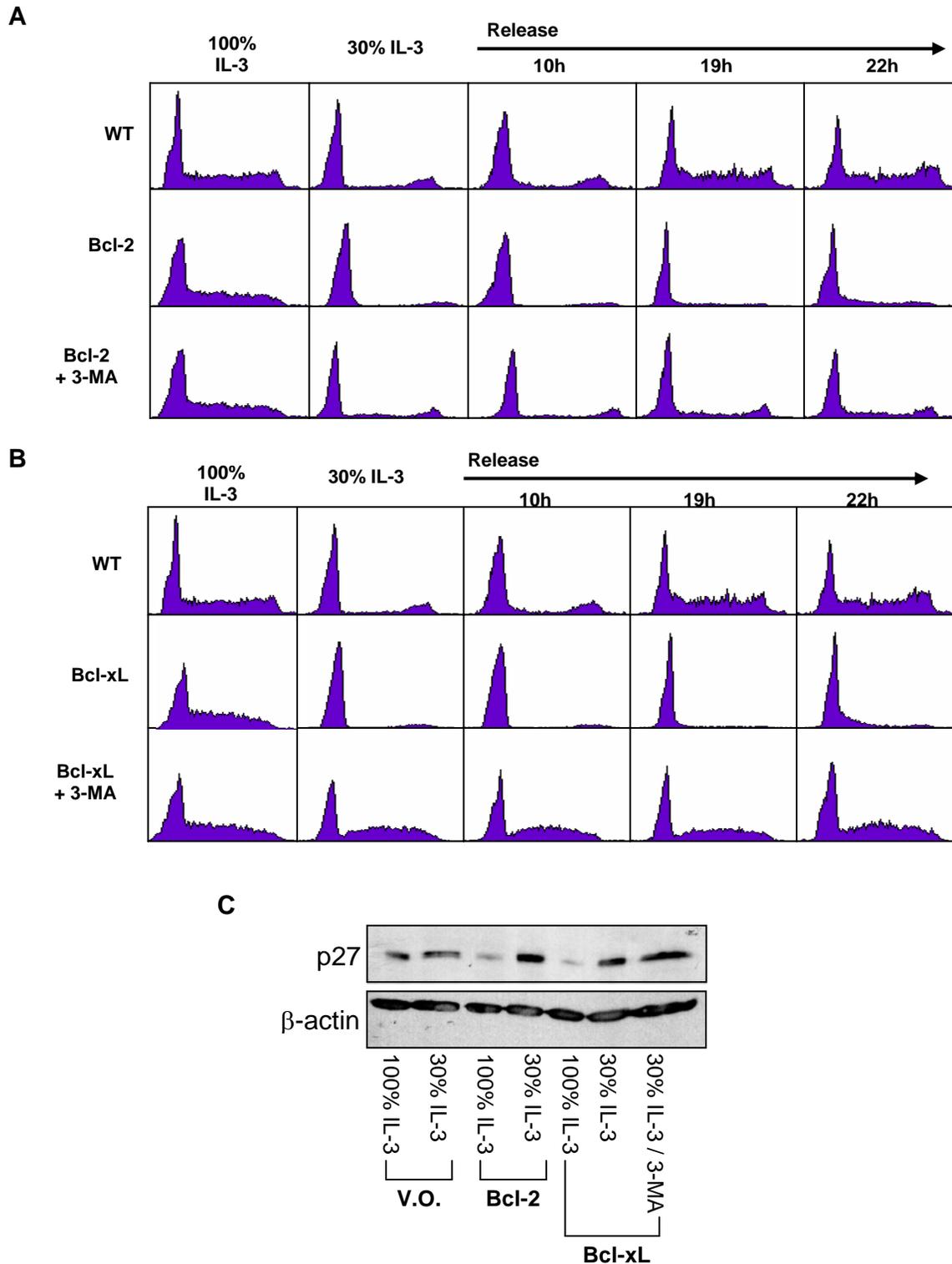


Figure 11. Arrest in G1/G0 is inhibited by 3-MA in Bcl-xL expressing cells. Bcl-xL, Bcl-2 and wild type (WT) FL5.12 cells were arrested by IL-3 withdrawal (30% IL-3-containing media) for 4 days with or without 5 mM 3-MA. Cell cycle profiles were obtained by PI /FACS analysis. Cells were collected before IL-3 withdrawal (cycling / 100% IL-3), during arrest (30% IL-3) and after re-addition (release) of IL-3 at the indicated time points (h = hours after IL-3 stimulation). (A) Bcl-2 vs. wt cells. (B) Bcl-xL vs. wt cells. (C) Western blot analysis for A and B.

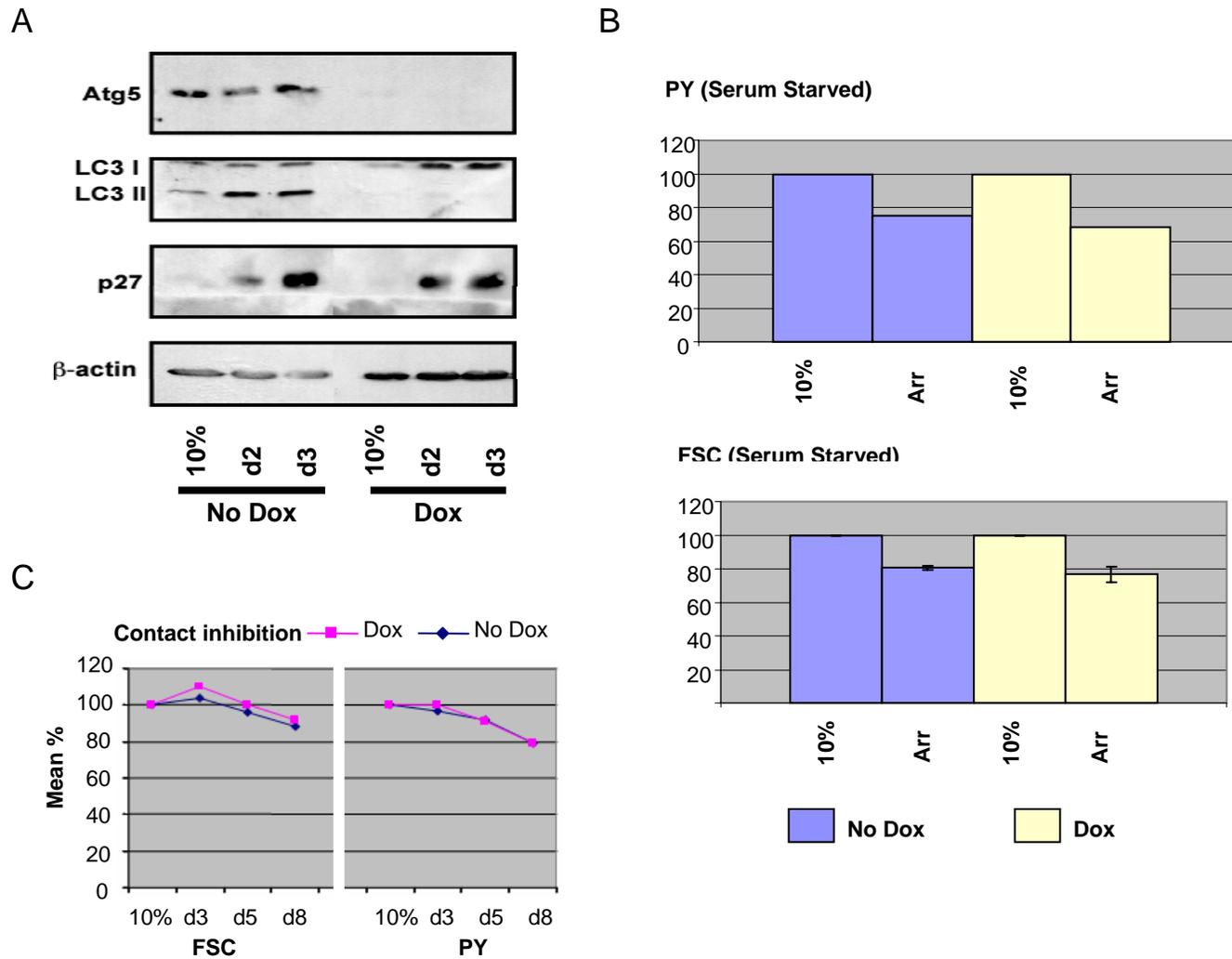


Figure 12. Inhibition of autophagy in m5-7 cells. Cells were arrested by either serum starvation (0.75% FBS containing media) or by contact inhibition in the presence or absence of Dox. Cells were collected before arrest (10%) and at the indicated days (d) during arrest. Cells were stained with both 7-AAD and Pyronin Y (PY) and analyzed by FACS. Cell size was assayed by measuring the forward scatter (FSC). (A) Western blot analysis of cells arrested by serum starvation (SS). (B) Cells were serum starved for 3 days. PY data is representative of 2 independent experiments. Cell size data is representative of 3 independent experiments. 100% = Mean PY or FSC for cycling cells. (C) Contact inhibition induced arrest. 100% = Mean PY or FSC for cycling cells.

The m5-7 cells are mouse fibroblasts in which expression of Atg5 is regulated by tetracycline or doxycycline (Dox). In the presence of Dox, Atg5 expression is suppressed and Dox removal allows its expression (Fig. 12A). Because Atg5 is required for the elongation step during the formation of the autophagosome, its suppression results in the inhibition of autophagy. First we determined whether inhibition of autophagy during arrest had any effect on cell cycle arrest in these cells. To confirm that autophagy was inhibited, we assayed for the microtubule-associated protein-1 light chain-3 (LC3) by Western blot. LC3 is a cytosolic protein (LC3-I) that colocalizes to the membrane of autophagosomes (LC3-II) upon induction of autophagy⁴⁵. This change in localization is accompanied by conversion of the LC3-I to the LC3-II form resulting in a different molecular weight^{46, 47}. As seen in figure 12A upon serum starvation LC3-I was converted to LC3-II suggesting that autophagy was being induced. On the other hand, in the presence of Dox, Atg5 was suppressed and the LC3-II band can no longer be observed suggesting that autophagy has been inhibited effectively. We then arrested m5-7 cells in the presence of Dox by either serum starvation or by contact inhibition (Fig. 12B and C), and assayed for RNA content and cell size. During serum starvation and contact inhibition there was a decrease in both cell size and RNA content in the presence and in the absence of autophagy. Inhibition of autophagy in m5-7 cells during arrest had no effect on their ability to arrest, consistent with results where vector only expressing cells were treated with 3-MA.

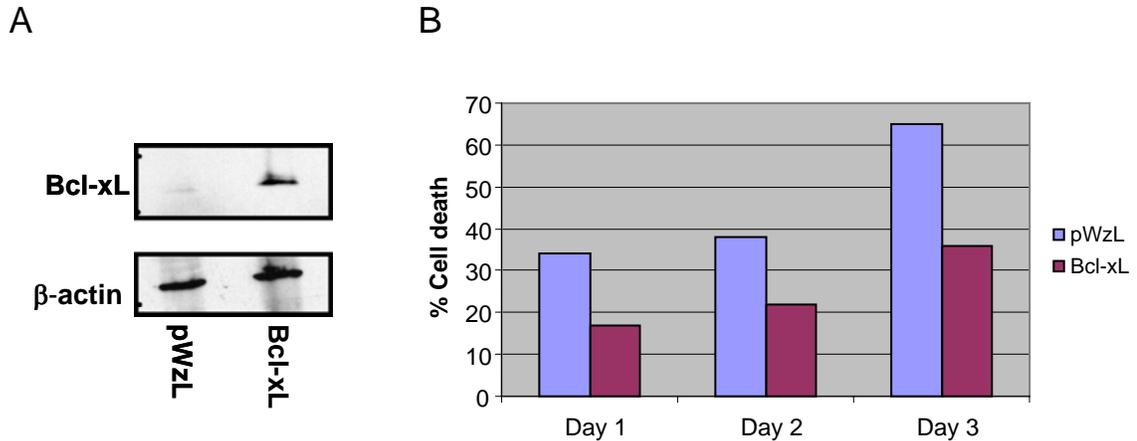


Figure 13. Bcl-xL overexpression in m5-7 cells. Cells were retrovirally infected with either Bcl-xL or vector only (pWzL). **(A)** Overexpression of Bcl-xL by Western blot analysis. **(B)** m5-7 cells were grown in the absence of serum and cell death assayed by PI exclusion at the indicated time points.

We next expressed Bcl-xL in the m5-7 cells as shown in figure 13A. Expression of Bcl-xL partially protected these cells from cell death. As shown in figure 13B, after complete serum withdrawal, the percent of cell death was approximately 2-fold greater in vector only than in Bcl-xL cells expressing. Then we examined whether Bcl-xL overexpression resulted in the expected cell cycle entry delay and enhanced arrest in G0. Vector only and Bcl-xL expressing m5-7 cells were arrested by either serum starvation or contact inhibition and then stimulated to re-enter the cell cycle. Time points were taken after release. Bcl-xL expressing cells entered the cell cycle after vector only (Fig. 14A and B). Although the cell cycle entry delay observed was not as strong as we have seen previously in 3T3s, it was consistent throughout our experiments. We did not see any difference in cell size between Bcl-xL and vector only during arrest (Fig. 14C).

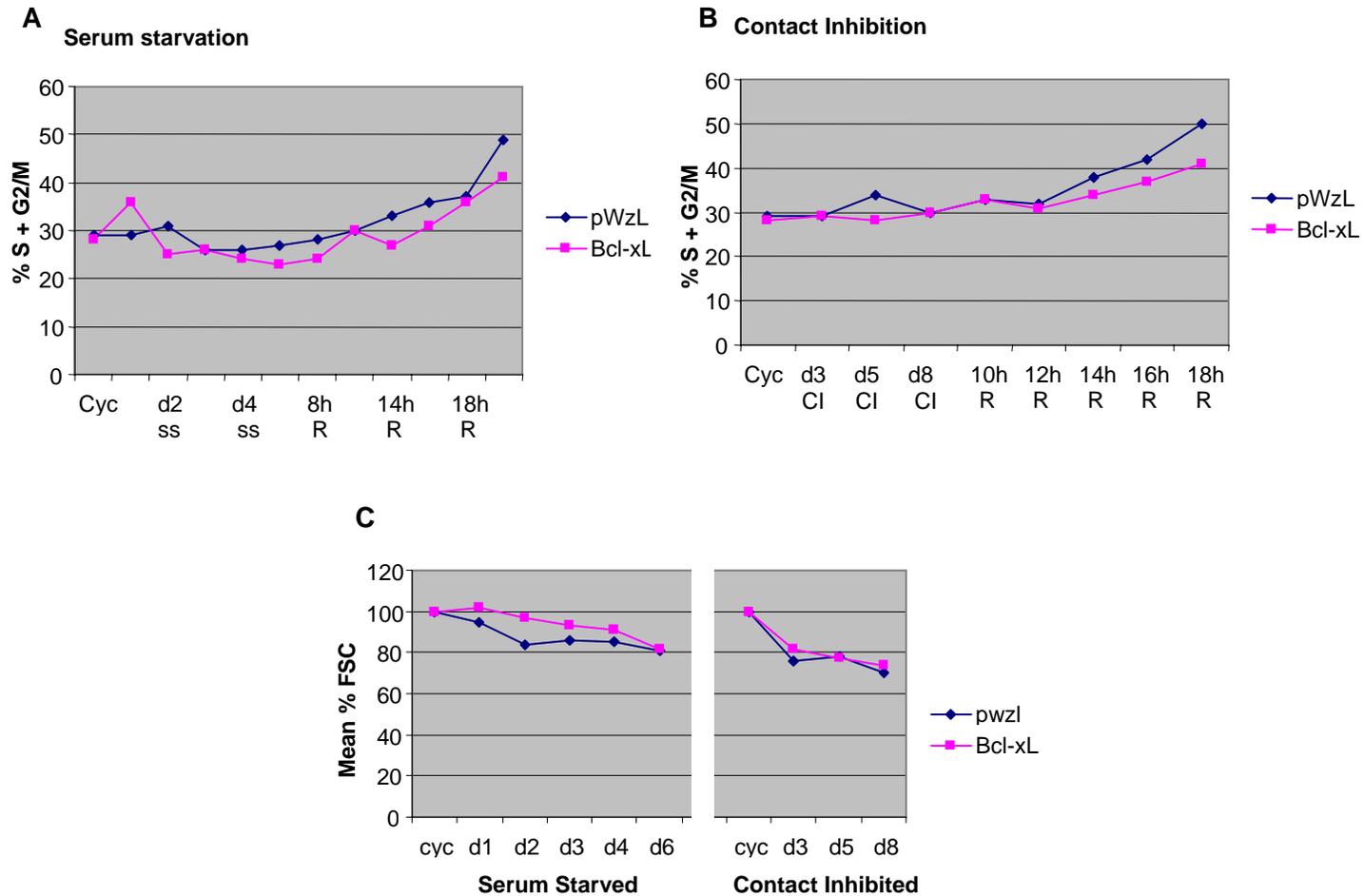


Figure 14. Arrest phenotype in Bcl-xL expressing m5-7 cells. Cells were arrested by either serum starvation (ss) or by contact inhibition (CI). Cells were collected before arrest (Cycling= cyc), during arrest (d=days of arrest) and after release (R) at the indicated time points (h=hours after release). Percentage of cells in S and G2/M phase were obtained from cell cycle profiles by PI/FACS. (A) Cell cycle arrest was induced by serum starvation. Cells were stimulated into the cell cycle by re-addition of serum to the media after day 4 (d4) of arrest. (B) Contact Inhibition induced arrest. Cells were re-plated after day 8 (d8) of arrest to induce cell cycle entry. (C) Cell size for A and B. Cell size was assayed by measuring the forward scatter (FSC) using flow cytometry. 100% = Mean FSC for cycling cells.

Because of the weak phenotype observed and because no difference in cell size was observed it may be possible that the expression of Bcl-xL in these cells is not sufficient. Also protection from cell death by Bcl-xL expression was not as efficient as observed in other cells overexpressing Bcl-xL. We still decided to examine whether inhibition of autophagy by Atg5 suppression (Dox treated) in Bcl-xL m5-7 cells inhibited the ability of Bcl-xL to lower RNA content and cell size. Again we arrested the cells by either serum starvation or contact inhibition. Figure 15 compares arrested Bcl-xL or vector expressing cells in the presence or absence of Dox. In this experiment we found that inhibition of autophagy in serum starved Bcl-xL expressing cells resulted in higher (20% more) RNA content than in Bcl-xL cells where autophagy was not inhibited (Fig. 15A). No difference in cell size was observed (Fig. 15A). On the other hand, when comparing Bcl-xL cells arrested by contact inhibition we did not observe any difference in RNA content nor in cell size between Dox treated and untreated controls (Fig 15B). In addition Dox treatment had no effect on p27 upregulation in Bcl-xL m5-7 cells (Fig. 15C).

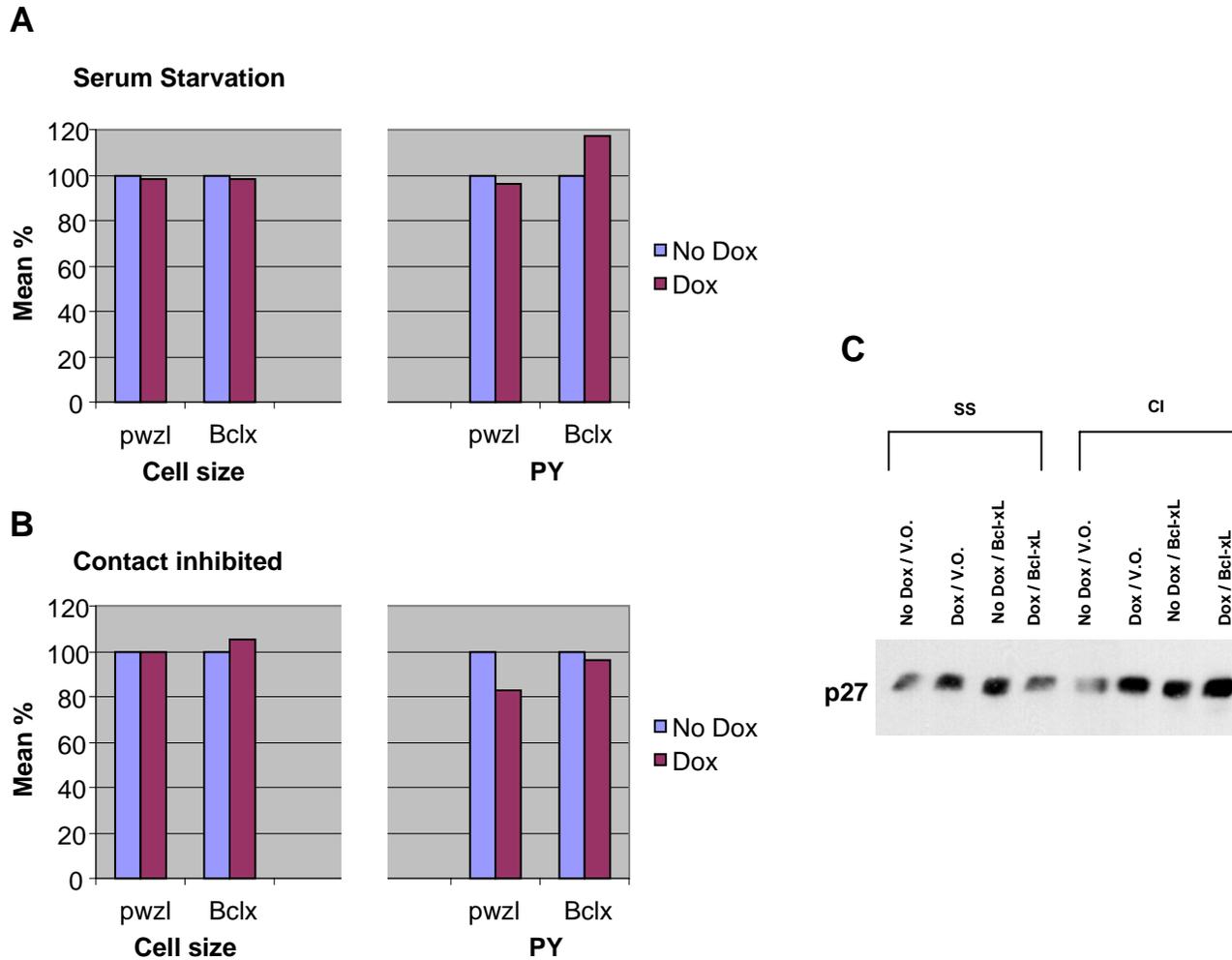


Figure 15. Effect of autophagic inhibition during arrest in Bcl-xL m5-7. Shows only arrested cells. Cells were stained with both 7-AAD and Pyronin Y (PY) and analyzed by FACS. Cell size was assayed by measuring the forward scatter (FSC). 100% = Mean PY or FSC for cycling cells. (A) m5-7 cells expressing either Bcl-xL or vector only (pWzL) were arrested by serum starvation (0.2% FBS containing media) for 5 days. (B) Arrested by contact inhibition for 5 days. (C) Western blot analysis for A and B. Serum starved (ss) and contact inhibition (CI).

CHAPTER IV

DISCUSSION

Our results show that 3-MA treatment in Bcl-xL expressing cells inhibits the ability of Bcl-xL to enhance arrest in G0 suggesting that Bcl-xL enhanced arrest might be mediated in part through autophagy. We have also shown that upon 3-MA treatment in Bcl-xL cells there appears to be an accumulation of cells in the S-phase of the cell cycle. Preliminary data by BrdU incorporation (data not shown) suggests that 3-MA treated Bcl-xL cells are arrested in S-phase. These results are in agreement with a recent study that shows that cell cycle arrest in G1 and/or S-phase may contribute to autophagy (Liang 2007).

On the other hand, no effect was seen in 3-MA treated Bcl-2 overexpressing cells. In the presence of 3-MA, Bcl-2 expressing cells displayed enhanced arrest and cell cycle entry delay. In agreement with our results the Craig Thompson group found that silencing of Bcl-xL by siRNA in *bak*, *bax* double knock out MEF resulted in a marked suppression in the formation of autophagosomes after etoposide treatment²⁹. They observed that silencing of Bcl-2 did not suppress the autophagic response. Although these results suggest functional differences between Bcl-xL and Bcl-2, it might be possible that Bcl-xL has a stronger phenotype that can be more readily observed. For example, when we measured PY staining in 3-MA treated Bcl-2 cells during serum starvation, we found that there was an increase in RNA content although this increase was not as significant as in 3-MA treated Bcl-xL cells.

Our finding that 3-MA treatment in Bcl-2 expressing cells had no effect could reflect results of other studies that have shown that Bcl-2 binds beclin 1 and inhibits autophagy^{38, 48-50}. This suggests that inhibition of autophagy by 3-MA in Bcl-2 expressing cells is redundant, because Bcl-2 carries this same function. Still other studies suggest that Bcl-xL binds beclin1 and that it is possible that Bcl-xL is required for the induction of Atg5-Atg12 through regulation of Beclin 1²⁹. In this respect, it is possible that Bcl-2 and Bcl-xL perform opposing regulating roles in autophagy.

Another possible explanation could be that upon induction of cell death a blockage in the apoptotic pathway might lead to the activation of autophagy. This has been shown to be true in *bax* *-/-* *bak* *-/-* MEF and IL-3 dependent bone marrow cells. In the case of Bcl-2 and Bcl-xL overexpressing cells there is also protection against cell death and upon a death stimulus autophagy might be activated. The activation of autophagy in Bcl-2 and Bcl-xL cell might not be as strong as in *bax*, *bak* deficient cells, because these cells still express Bax, and Bak and can still activate apoptosis although with greater resistance than vector only cells. There also might be differences in the degree of protection between Bcl-2 and Bcl-xL overexpressing cells.

As stated earlier a recent study shows that accumulation of p27 is sufficient to induce autophagy³¹. In our experiments, p27 upregulation was not affected when Bcl-xL FL5.12 cells were treated with 3-MA, suggesting that p27 upregulation might be upstream of the class III PI3 kinase/Beclin 1 complex. On the other hand, 3-MA treated Bcl-xL expressing 3T3 appear to have a reduction

in the amount of p27. This would indicate that p27 is downstream of the class III PI3 kinase/Beclin 1 complex. Further investigation is necessary to better understand this mechanism.

In conclusion, Bcl-xL may arrest in G0 in part through autophagy. This does not appear to be true for Bcl-2 although this may be due to a weaker phenotype. Further studies are necessary to confirm this function of Bcl-xL. Upon induction of autophagy LC3 localizes to the autophagosome. Taking advantage of fluorescent microscopy we will be transfecting Bcl-xL expressing cells with GFP-LC3. During normal conditions GFP-LC3 is expected to be seen diffused throughout the cytoplasm. In contrast, during starvation conditions a punctuate pattern should be observed suggesting localization of LC3 to the autophagosomal membrane. This system will allow us to determine whether Bcl-xL cells have a stronger autophagic response during starvation conditions.

Whether Bcl-xL induces autophagy through the upregulation of p27 remains to be investigated. It would be interesting to investigate whether the Bcl-xL cell cycle phenotype can be reproduced in Bcl-xL expressing cells by Beclin-1 or Atg5 upregulation in the absence of p27.

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