Bax-induced apoptosis shortens the life span of DNA repair defect Ku70-knockout mice by inducing emphysema

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Abstract

Cells with DNA damage undergo apoptosis or cellular senescence if the damage cannot be repaired. Recent studies highlight that cellular senescence plays a major role in aging. However, age-associated diseases, including emphysema and neurodegenerative disorders, are caused by apoptosis of lung alveolar epithelial cells and neurons, respectively. Therefore, enhanced apoptosis also promotes aging and shortens the life span depending on the cell type. Recently, we reported that *ku70*^{-/-}*bax*^{-/-} and *ku70*^{-/-}*bax*^{+/-} mice showed significantly extended life span in comparison with *ku70*^{-/-}*bax*^{+/+} mice. Ku70 is essential for non-homologous end joining pathway for DNA double strand break repair, and Bax plays an important role in apoptosis. Our study suggests that Bax-induced apoptosis has a significant impact on shortening the life span of *ku70*^{-/-} mice, which are defective in one of DNA repair pathways. The lung alveolar space gradually enlarges during aging, both in mouse and human, and this age-dependent change results in the decrease of respiration capacity during aging that can lead to emphysema in more severe cases. We found that emphysema occurred in *ku70*^{-/-} mice at the age of three-months old, and that Bax deficiency was able to suppress it. These results suggest that Bax-mediated apoptosis induces emphysema in *ku70*^{-/-} mice. We also found that the number of cells, including bronchiolar epithelial cells and type 2 alveolar epithelial cells, shows a higher DNA double strand break damage response in *ku70* KO mouse lung than in wild type. Recent studies suggest that non-homologous end joining activity decreases with increased age in mouse and rat model. Together, we hypothesize that the decline of Ku70-dependent DNA repair activity in lung alveolar epithelial cells is one of the causes of age-dependent decline of lung function resulting from excess Bax-mediated apoptosis of lung alveolar epithelial cells (and their progenitor cells).

Keywords: Bax, Ku70, apoptosis, aging, life span, DNA damage, DNA repair, non-homologous end joining, emphysema, lung alveolar epithelial cells

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Introduction

There are many causes of death of aged animals including cancer, diabetes, neuronal disorders, infectious diseases, and other life-threatening diseases. We speculate that there are common age-dependent progressive changes in animal body that makes ageing as a risk factor for these different types of age-associated life-threatening diseases. As explained below, lung alveolar space is known to enlarge gradually according to age, probably due to the imbalance of cell death and cell division of lung alveolar epithelial cells. In this mini review, we will discuss the role of apoptosis in age-associated diseases, by focusing on Bax-induced apoptosis in emphysema. Our discussion is based on our recent study using mouse genetics as explained in detail below.

Ku70 knockout (KO) mouse and Ku70-Bax double KO mouse

Bax is a 21 kDa protein that is known to play an essential role in programmed cell death (PCD) or apoptosis.^{1,2} Bax belongs to the Bcl-2 family of proteins, which are an evolutionarily conserved family of proteins controlling cell death.^{1,2} Bax resides in the cytosol in healthy cells, but Bax translocates from the cytosol to mitochondria in response to apoptotic stresses (including DNA damage, protein misfolding, ER stresses, etc.)³ and Bax triggers cytochrome c release from the mitochondria, which in turn activates the caspase cascade, inducing cell death.² Ku70 is a 70 kDa protein that plays an essential role in the non-homologous end joining (NHEJ) pathway of DNA damage repair.⁴

Previously we found that Ku70 has anti-apoptotic activity through the suppression of mitochondrial translocation of Bax.⁵⁻⁷ Numerous studies also confirmed Ku70's anti-apoptotic activity as a Bax inhibitor in various types of cells.⁶⁻²⁰ In addition, it has been shown that Ku70-deficient cells are sensitive to apoptotic stresses that are not limited to DNA damaging stresses,^{5,7,21} supporting the hypothesis that Ku70 has anti-apoptotic activity independent from its previously known DNA damage repair activity. Furthermore, the Bax inhibiting peptide (BIP) designed from the Bax-binding domain of Ku70^{5,22} has been used to rescue damaged cells from Bax-mediated cell death in cell culture^{6,23-26} as well as in animal models.²⁷⁻³⁰ Taken together, these studies suggest that Ku70 has a biological activity as an inhibitor of Bax in addition to its well-known role in DNA repair.

To further examine the physiological significance of Ku70-dependent inhibition of Bax-induced cell death, we generated *ku70*^{-/-}*bax*^{-/-} and *ku70*^{-/-}*bax*^{+/-} mice and compared their phenotype with Ku70 single KO (*ku70*^{-/-}) mice, which are known to have an accelerated aging phenotype.³¹ We speculated that accelerated aging of *ku70*^{-/-} mice might be, at least in part, due to the increased Bax-induced apoptosis because of the absence of Ku70's inhibition against Bax. After 10 years of effort to develop mouse colonies and analyze the life span of these mutant mice, we found that Bax deficiency was able to extend the life span of Ku70 KO mice (median life span of *ku70*^{-/-}, *ku70*^{-/-}*bax*^{-/-}, and *ku70*^{-/-}*bax*^{+/-} were 26 (n=55), 37.5 (n=46, *P* < 0.001), and 38 (n=23, *P* < 0.01) weeks, respectively), suggesting that Bax-mediated apoptosis plays a role in inducing premature death in *ku70*^{-/-} mice.³² This result supports the hypothesis that the absence of Ku70 and the lack of its Bax inhibitory function may lead to Bax hyperactivation, which accelerates the development of age-associated diseases that shorten the life span of *ku70*^{-/-} mice. In addition, the increased accumulation of DNA damage due to the absence of Ku70 can trigger the DNA damage response to indirectly initiate apoptosis through p53-dependent Bax activation. We suspect that both of these mechanisms of Bax activation are contributing to the premature death observed in *ku70*^{-/-} mice. Although the restoration of the abnormal aging phenotype in *ku70*^{-/-} mice by Bax deficiency does not fully prove the role of Ku70 as a Bax inhibitor (since other mechanisms can explain this phenotype), the fact that Bax deficiency was able to extend the life span of Ku70 KO mice implies very important roles for Ku70 and Bax in the development of age-associated life-threatening diseases. In this article, we will discuss the previously unrecognized role of Ku70 and Bax to regulate the progression of age-dependent enlargement of lung alveolar space that causes the decrease of respiration activity of aged animals.³³⁻³⁶

Cell death or cellular senescence?

Cell death and cellular senescence are two major responses to irreparable DNA damage, and these responses prevent the proliferation of mutated cells. Since apoptosis removes unwanted damaged cells (including cells with potentially cancerous mutations), apoptosis is considered to be

Figure 1 Roles of apoptosis and cellular senescence in aging

beneficial for longevity.³⁷ On the other hand, the presence of senescent cells is deleterious to surrounding cells since senescent cells secrete inflammatory cytokines that induce chronic inflammation and lead to other deleterious local tissue changes such as fibrosis.³⁸ Therefore, cellular senescence, rather than apoptosis, is considered to be the causative cellular event that induces organismal aging. In fact, a recent study showed that removal of senescent cells by genetic engineering was able to extend the life span of mice.³⁹ However, our evidence demonstrates that age-dependent degenerative diseases occur in part due to apoptosis of essential cells. Thus, apoptosis can have both positive and negative impacts on longevity (Figure 1).

A previous study showed that the deletion of the DNA damage response gene *cdkn1a* was able to extend the life-span of mice with dysfunctional telomeres,⁴⁰ which suggests that DNA damage responses, including apoptosis and cellular senescence, have significant influences on the life span determination of mutant mice with genomic instability. Importantly, Maslov *et al.*⁴¹ reported that remaining permanent DNA damages or significantly increased mutation rates were not detected in mutant mice with defects in DNA repair that show abnormal aging phenotype. This study further suggests that the DNA damage response, rather than DNA damage (or mutations) itself, may play an important role in aging-associated diseases that are accelerated in DNA repair defective mice.⁴¹ Cellular senescence seems to have a certain role in the induction of age-associated diseases in defective DNA repair mutant mice.⁴² However, the suppression of cellular senescence by *p21* knockdown in *ku80* null mice⁴³ or the removal of senescent cells in mice with dysfunctional telomeres⁴⁴ did not result in a significant life span extension in mice, although there was a delay in the development of age-associated disorders.⁴⁴ Very recently, approximately 20% life span extension of wild type mouse (i.e. DNA repair pathways and telomere lengths are normal) was achieved by the removal of senescent cells in mice using genetic engineering which induces apoptosis in senescent cells,³⁹ supporting the hypothesis that cellular senescence plays an important role in the progression of aging and age-associated life-threatening diseases.

Based on our recent findings showing that Bax deficiency extended the life span of *ku70*^{-/-} mice, we propose

that Bax-mediated apoptosis has a significant impact on survival and life span determination in mice with defective DNA repair. Previous studies have shown that *ku70*^{-/-}, *ku80*^{-/-} and *ku70*^{-/-}, *ku80*^{-/-} mice exhibit similar abnormal aging phenotypes, including shortened life spans. Since Ku70 protein levels become very low in *ku80*^{-/-} cells,^{31,45} *ku80*^{-/-} mice are expected to be similar to *ku70*^{-/-} mice and have an increased DNA damage response as well as a lower threshold to initiate Bax-mediated apoptosis. Therefore, we speculate that Bax-induced apoptosis has a significant role to shorten the life span of *ku80* KO mice as well. Cellular senescence is known to be induced by two major pathways, p53- or retinoblastoma (RB) protein-dependent cell cycle arrest pathways.⁴⁶ In general, DNA damage triggers p53-dependent cellular response, i.e. apoptosis or cellular senescence, if DNA damage cannot be repaired.⁴⁶ The main mediators of p53-dependent DNA damage response are Bax (apoptosis) and p21 (cell cycle arrest and cellular senescence), respectively.⁴⁶ Interestingly, the deletion of p21 was not able to extend the survival of *ku80*^{-/-} mice, despite the significant decrease of cellular senescence was confirmed by cultured fibroblast experiments.⁴³ This previous study suggests that p21-dependent cellular senescence may not cause life span shortening, though p21-independent cellular senescence may still induce aging-associated dysfunction in *ku80*^{-/-} and *p21*^{-/-} mice. Importantly, our recent study shows that Bax deficiency is able to extend the survival and life span of *ku70*^{-/-} mice. Previous studies showed that cell death inhibition of DNA-damaged cells increases the emergence of senescent cells.³⁸ If this is the case, it is expected that *ku70*-*bax* double KO mice would have more cellular senescence than *ku70* single KO mice because cell death is suppressed by *bax* deletion. If cellular senescence was the cause of a shortened life span in mice rather than cell death, *bax* deletion would be predicted to shorten the life span of *ku70*^{-/-} mouse by increasing cellular senescence. The result, however, was the opposite. Overall, our results suggest that promoting cell survival by the inhibition of Bax may have a greater beneficial impact on extending the survival period of prematurely aging animals despite the potential risk of increased cellular senescence by the inhibition apoptosis.

Ku70 KO mice showed age-associated changes much earlier than wild type mice, such as kyphosis and alopecia.³¹ Interestingly, Bax deficiency slowed down these phenotypes in *ku70*^{-/-} mice.³² This result suggests that the absence of Bax-induced apoptosis can slow down the progression of organismal aging in *ku70*^{-/-} mice. *Ku70* KO mice lost subcutaneous fat at the age of six-months old, whereas Bax-deficient *ku70* KO mice retained this fat layer.³² The maintenance of this fat tissue in Bax-deficient *ku70* KO mice suggests that Bax deficiency improved overall health condition of *ku70*^{-/-} mice as discussed in detail in our recent report.³²

How does Bax deficiency extend the life span in *ku70* KO mice?

If Bax-induced apoptosis enhances aging in *Ku70* KO mice, the next important issue is to understand the cell types that

are the targets of excess Bax-induced apoptosis. *Ku70* KO mice have immune deficiency and this defect is likely to be one of the causes of their shortened life span.³¹ However, *Bax* deletion does not improve this immune deficiency because *Bax* deletion cannot overcome the decreased lymphocyte development due to the absence of NHEJ DNA repair pathway that is required for T-cell receptor maturation and IgG gene arrangement in B-cells.³² Therefore, an improvement of immune function is not likely the reason for life span extension in this case.

Ku70 KO mice have a defect in brain development due to increased neuronal cell death during embryogenesis,⁴⁷ and we confirmed that the brain weight of *ku70*^{-/-} mice was approximately 50% of wild type mice at three months of age.³² We also found that complete Bax deletion (i.e. *ku70*^{-/-}*bax*^{-/-} mice) was able to suppress excessive neuronal apoptosis in *ku70*^{-/-} mice, and brain weight was significantly restored similar to wild type.³² Therefore, restoration of brain function may be one of the reasons why *ku70*^{-/-}*bax*^{-/-} mice have longer life span than *ku70*^{-/-}. However, there seems to be another potentially more important reason why Bax deficiency was able to extend the life span of *ku70* KO mice. To be noted, *ku70*^{-/-}*bax*^{+/-} mice as well as *ku70*^{-/-}*bax*^{-/-} mice showed the similar extension of survival compared to *ku70*^{-/-} mice.³² Interestingly, brain weights of *ku70*^{-/-}*bax*^{+/-} mice were similar to those of *ku70*^{-/-} mice and the number of survived neurons in the brain were also similar to that of *ku70*^{-/-} mice.³² Probably, the remaining Bax protein (*bax*^{+/-} cells express approximately 50% Bax protein in comparison with wild type (*bax*^{+/+}) cells³²) induced excess neuronal cell death in *ku70*^{-/-} mouse. These results imply that there are reasons other than suppression of neuronal apoptosis that help *ku70*^{-/-}*bax*^{+/-} mice live longer than *ku70*^{-/-} mice. As explained in the next paragraph, we found that the lung structure was damaged progressively during aging in *ku70*^{-/-} mice, and this abnormality was commonly restored in *ku70*^{-/-}*bax*^{-/-} and *ku70*^{-/-}*bax*^{+/-} mice. This observation suggests that the lung is another important target organ (maybe more important than the brain) affected by Bax-induced cell death to shorten the life span of *ku70*^{-/-} mice.

As reported in our recent study,³² we found that *ku70*^{-/-} mice showed accelerated age-dependent enlargement of lung alveolar spaces, known as emphysema (example histology are shown in Figure 2). As shown in Figure 2, the lung alveolar sizes and structures of wild type and *ku70*^{-/-} mice are similar to each other at an early age. However, the lung alveolar size of *ku70*^{-/-} mice became significantly larger than wild type mice with age. These histological results suggest that the expansion of alveolar space of *ku70*^{-/-} mice is an age-dependent process of progressive alveolar degeneration, rather than defects in the lung development. Emphysema and the consequential chronic obstructive pulmonary disease (COPD) lead to declines in respiratory activity⁴⁸ and triggers secondary life-threatening diseases, such as pulmonary artery hypertension and heart failure.⁴⁹ Importantly, Bax deficiency (both *bax*^{-/-} and *bax*^{+/-}) was able to suppress the incidence of emphysema in *ku70*^{-/-} mice (Figure 2).³² These observations suggest that Bax-mediated cell death induces emphysema in *ku70*^{-/-} mice.

Bax deficiency suppresses emphysema in Ku70 KO mice

Figure 2 Ku70-deficient mice show accelerated age-dependent emphysema, and Bax deficiency suppressed emphysema in Ku70-deficient mice. Lung histology of wild type, *ku70*^{-/-}, *bax*^{-/-}, and *ku70*^{-/-}*bax*^{-/-} mice are shown. Lung structures of newborn wt (a) and *ku70*^{-/-} (b) mice are similar, indicating that lung development during embryogenesis was not affected by Ku70 deficiency. However, at the age of three-months old, alveolar space of *ku70*^{-/-} mice (d) became much larger than wt mice (c) showing that Ku70 deficiency causes emphysema. Bax deficiency was able to suppress enlargement of alveolar space (e) suggesting that Bax-induced apoptosis causes emphysema in *ku70*^{-/-} mice. Bax KO (*bax*^{-/-}) (f) and wild type mice (c) showed the similar lung alveolar size at the age of three months old. In panel A and B, sections were stained by phospho-H2AX antibody detecting DNA double strand break (DSB) response (Red Arrows, brown staining). Number of cells with DNA DSB damage is higher in *ku70*^{-/-} lung (b) than wt lung (a). Experiments were performed as previously reported in Ngo *et al.*³² (A color version of this figure is available in the online journal.)

The age-dependent alveolar space enlargement is known to occur in mice (including wild type mice) and humans.^{33–36} The imbalance of apoptosis and the division of alveolar cells (e.g. epithelial cells, endothelial cells, and fibroblasts) is likely the mechanism of this degenerative disease,^{50,51} but the exact details of this imbalance are not well understood. Among several apoptosis-inducing proteins, our recent finding suggests that Bax plays a major role in age-dependent emphysema. If this is the case, then *bax*^{-/-} mice is expected to have a decreased incidence or delayed age-dependent emphysema compared to *wild type* mice. However, age-dependent changes of lung function of *bax*^{-/-} mice have not been well characterized, and thus future study investigating this issue in detail is clearly

necessary to understand the mechanism of age-dependent degeneration of the lung. The *bax*^{-/-} mice do not show a significantly extended life span in comparison with wild type mice.⁵² Since global *bax* gene knockout results in the survival of both essential cells as well as unwanted cells, the beneficial effects of Bax deficiency such as suppression of emphysema may be cancelled by the negative effects caused by the absence of clearance of unwanted cells, including senescent and/or mutated cells. Further study using conditional *bax* gene knockout in specific cell types in the lung (such as lung alveolar epithelial cells) will be necessary to determine the role of Bax-induced cell death in the age-dependent enlargement of lung alveolar space.

Alveolar epithelial cells may depend on Ku70-dependent DNA repair pathway

The NHEJ and homologous recombination (HR) pathways are major mechanisms of DNA double strand break (DSB) repair.⁵³ An important distinction among these two pathways is that non-dividing cells such as stem (or progenitor) cells at a quiescent state can only use the NHEJ pathway, and not the HR pathway, since the HR pathway requires cell cycle progression.⁵³ Interestingly, DNA DSB damage responses, such as H2AX phosphorylation, were detected in bronchiolar and alveolar cells in *ku70*^{-/-} mice at higher frequency than in wild type mice (Figure 2).³² Ku70 is known to be essential for NHEJ, and thus *ku70*^{-/-} has an apparent defect in NHEJ. Our observations suggest that the NHEJ DNA repair pathway has a significant role in repairing DNA damage in certain cell types in the bronchia and alveoli. As shown in Figure 3, some (but not all) of the phosphorylated H2AX-positive cells (i.e. DNA DSB damage response positive cells) were double stained by pro-SPC, which is the cell surface marker for type 2 alveolar epithelial (AT2) cells, suggesting that at least AT2 cells have a higher dependency on NHEJ to repair DNA DSB. Interestingly, clusters of multiple AT2 cells were often observed in *ku70*^{-/-}*bax*^{-/-} mice that were not seen in wild type and *ku70*^{-/-} mice (Figure 4). These results imply that (1) AT2 cells require Ku70 (and Ku70-dependent DNA DSB repair pathway, i.e. NHEJ pathway) to repair DNA DSB damage and that (2) the absence of Bax-mediated apoptosis allows AT2 cells to survive after DNA damage. Probably, these cells enter cell division to utilize the HR pathway, and that is why clusters of multiple AT2 cells were formed in the lung of *ku70*^{-/-}*bax*^{-/-} mice. AT2 cells as well as bronchiolar

cells are known to function as progenitor cells, producing type 1 lung alveolar (AT1) cells that maintain the structure of lung alveoli.⁵⁴⁻⁵⁶ Therefore, the increased death of AT2 and bronchiolar cells due to the DNA damage in *ku70*^{-/-} may cause a shortage of progenitor cells producing AT1 that results in the acceleration of age-dependent enlargement of lung alveoli.

Why these lung cells do not use cell cycle-dependent HR pathway to repair DNA DSB instead of choosing to die by Bax-mediated apoptosis is unknown. The HR pathway is more accurate than the NHEJ pathway to repair DNA damage⁵³; therefore, it is surprising that these progenitor cells depend on the error prone NHEJ pathway.

Figure 4 Cell clusters of multiple type 2 alveolar epithelial cells exist in Bax-deficient Ku70 KO mice. Lung sections of wt, *ku70*^{-/-} and *ku70*^{-/-}*bax*^{-/-} mice were stained by pro-SPC antibody detecting type 2 alveolar epithelial (AT2) cells. AT2 cell does not form multiple cell clusters in wt and *ku70*^{-/-} mice, but AT2 cell clusters were often detected in *ku70*^{-/-}*bax*^{-/-} mice. Experiments were performed as previously reported in Ngo et al.³² (A color version of this figure is available in the online journal.)

DNA double strand break damage response were detected in type 2 pneumocytes

Figure 3 DNA double strand break (DSB) responses were detected in type 2 alveolar epithelial cells (pneumocytes) and bronchiolar epithelial cells. Serial sections (5 μm sections) of the lung from three-months old *ku70*^{-/-} mice were stained by antibodies detecting phosphor-H2AX (DNA DSB response marker) and pro-SPC (type 2 alveolar epithelial cell marker). IgG from normal rabbit serum was used as a negative control. Red arrows show pro-SPC-positive lung cells that were also phosphor-H2AX-positive. Experiments were performed as previously reported in Ngo et al.³² (A color version of this figure is available in the online journal.)

One speculation is that these cells may have a strict restriction of cell division even when these cells receive DNA DSB damage, and that is why these cells depend on the NHEJ pathway to repair DNA damage. This restriction of cell division may be a safety mechanism to maintain efficient gas exchange area (lung alveolar space) by limiting cell doubling of alveolar epithelial cells, and to suppress expansion of cancerous mutated cells. In fact, abnormally increased numbers of AT2 cells were detected in some of Bax-deficient *ku70*^{-/-} mice.³² However, if this is the case, this safety mechanism may be one of the causes of age-dependent alveolar enlargement since NHEJ activity in the lung seems to decline with the progression of age, as we will discuss in the next paragraph, and thus lung progenitor cells may be forced to undergo apoptosis due to the accumulation of DNA damage, rather than enter the cell cycle to use the HR pathway.

Ku70-dependent NHEJ DNA repair activity decreases as the lungs age

Lee *et al.*⁵⁷ reported that the NHEJ activity was significantly decreased by aging in rat lung (whole lung homogenates were used to measure the NHEJ activity). Caloric restriction (CR), which is considered to be beneficial for healthy aging, significantly suppressed the decline of NHEJ activity in these lungs, and CR increased the levels of Ku70 in the lung.⁵⁷ Messenger RNA expression of genes involved in NHEJ (e.g. XRCC4 and XRCC5) has been also reported to be significantly decreased in the lungs of aged mice (24-months old) in comparison with young mice (three-months old),⁵⁸ suggesting that the decrease in NHEJ activity occurs indeed as a result of aging. Together with our recent findings, we hypothesize that the age-dependent decrease in Ku70-dependent NHEJ activity in the lung (especially in alveolar epithelial progenitor cells such as AT2) is among the major contributors in age-associated emphysema. Notably, a recent study showed that Ku70 protein levels (in total lung homogenate) were decreased by cigarette smoke in mouse lung with emphysema,⁵⁹ supporting our hypothesis that the decrease of Ku70 expression promotes emphysema.

Conclusion

Our recent study demonstrated that Bax deficiency was able to extend the life span of *ku70* null mice.³² To our knowledge, this is the first report showing a significant role of an apoptosis-inducing gene in the induction of premature death in mutant mice with defects in DNA repair. We propose that the pro-apoptotic protein Bax has a significant role in age-dependent diseases, especially emphysema. Our studies investigating the phenotype of *ku70*^{-/-} mice also revealed that Ku70 is essential in order to maintain normal lung alveoli structures during aging. It is not well understood how the DNA damage response influences the development of age-associated emphysema; therefore, further study is clearly needed to better understand the role of Ku70 in the maintenance of homeostasis in the pulmonary organs.

Authors' contribution: SM, JP, and JN contributed the writing sentences of this manuscript based on the experimental results produced by themselves as well as by AB, IP-F, KW, and MM. All authors contributed to the development of the proposed hypothesis about the biology of aging and the interpretation of the experimental results.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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