BAG2 Promotes Tumorigenesis and Metastasis by Regulating the Cathepsin B Cleavage in Triple-Negative Breast Cancer Cells

Kyung-Min Yang[1,4], Kyungwha Pang[1,3], Akira Ooshima[1], Jihee Lee[1,2,3], and Seong-Jin Kim[1,3,4]

[1]Precision Medicine Research Center, Advanced Institute of Convergence Technology, Seoul National University, Suwon, Kyunggi-do 16229, Republic of Korea. [2]Department of Biomedical Science, College of Life Science, CHA University, Seongnam-si, Republic of Korea, [3]Gilo Foundation and [4]Medpacto Inc., 92, Myeongdal-ro, Seocho-gu, Seoul, 06668, Republic of Korea

ABSTRACT

Breast cancer is classified into clinical subtypes based upon receptor expression (ER, PR, Her2). Among the breast cancer subtypes, triple-negative breast cancer (TNBC) refers to a form of breast cancer which lacks expression of ER, PR and HER2. Most of breast cancer treatments can be targeted for cancers that express hormonal receptors or HER2, whereas traditional chemotherapy does not work in TNBCs. Therefore, TNBC remains a clinical challenge for discovery novel specific target biomarker because of no targeted therapies for TNBC. In this study, we show that BAG2 promotes cancer progression by regulating the dual function of Cathepsin B in TNBC cells. We found that BAG2 is highly expressed in basal-like breast cancer cells, where it was associated with recurrence or distant metastasis-free survival in patients. In loss-of-function experiment, BAG2 knockdown strongly decreased the ability of tumor formation and lung metastasis *in vivo* in TNBC cells. Mechanistically, BAG2 induces metastasis through regulation of secretion of pro-form Cathepsin B and prevents lysosome-mediated cell death through decrease of intracellular single-chain form of Cathepsin B into cytoplasm. Thus, our results suggest that BAG2 promotes tumorigenesis and metastasis through regulation of Cathepsin B cleavage in TNBC cells and may be possible as a novel specific target for treatment of TNBC. [This work was supported by National R&D Program for Cancer Control, Ministry for Health and Welfare, Republic of Korea (HA17C0037)]

INTRODUCTION

- **Triple-negative breast cancer (TNBC) is an extremely aggressive subtype that is correlated with a poor prognosis and high mortality rates despite systemic therapy. Thus, there is an urgent need to discover new, effective therapeutic targets that may improve disease outcome and prognosis in patients with TNBC.**
- **Bcl-2-associated athanogene 2 (BAG2) as a co-chaperone binds to the Hsc70/Hsp70 ATPase domain and promote substrate release. The precise role of BAG2 in cancer progression and metastasis has not been extensively investigated,**
- **Cathepsin B (CTSB), a lysosomal cysteine protease possessing both endo- and exo-peptidase activities, is considered to participate in protein turnover. Aberrant expression/activity of CTSB has often been linked to progression and metastasis of malignant tumors, Although CTSB has been extensively studied in a wide variety of human cancers, the molecular basis regulating its dual functions as either a pro-oncogenic or pro-apoptotic enzyme is poorly understood. Furthermore, novel regulators that control the dual functions of CTSB in cancer progression have not been identified yet.**
- **Here, we show that BAG2 promotes cancer progression by regulating the dual function of CTSB in TNBC cells, implicating the potential for BAG2 to serve as a novel specific target for the treatment of TNBC.**

RESULTS (I)

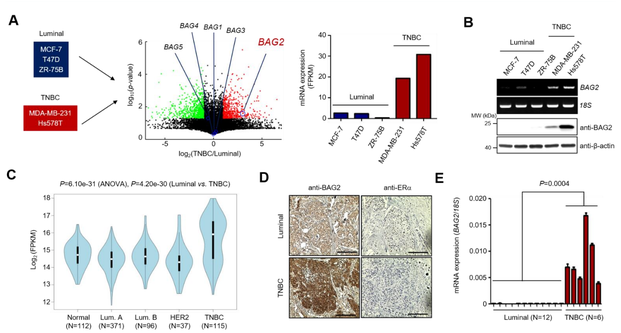


Figure 1. BAG2 Is Specifically Overexpressed in TNBC Cells and Primary TNBC Tissues. (A) Volcano plot of differential expression between luminal (MCF-7, T47D, ZR-75B) and TNBC (MDA-MB-231, Hs578T) cell lines using RNA sequencing. (B) RT-PCR and immunoblot analysis of BAG2 mRNA and protein expression in luminal and TNBC cell lines. (C) Box-plots of *BAG2* expression in different subtypes of breast cancer and normal tissues, obtained from RNA sequencing datasets of Genomic Data Commons (GDC). (E) Histological analysis of BAG2 and ERα in luminal (upper) and TNBC (lower) tissues of breast cancer. (F) Quantitative RT-PCR of BAG2 gene expression in 12 luminal tissues and 6 TNBC tissues.

RESULTS (II)

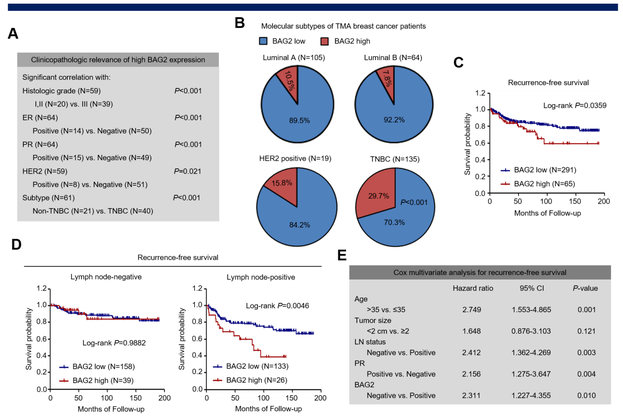


Figure 2. Overexpression of BAG2 Has Clinical Relevance and Prognostic Value. (A) Associations of high BAG2 expression with clinical parameters. (B) Pie charts on the percentages of low and high BAG2 in patients with different breast cancer subtypes. (C) Kaplan-Meier analysis showing and recurrence-free survival depending on BAG2 expression level. (D) Kaplan-Meier analysis showing the association between BAG2 expression and recurrence-free survival according to the lymph node status. (E) Cox proportional hazards regression model.

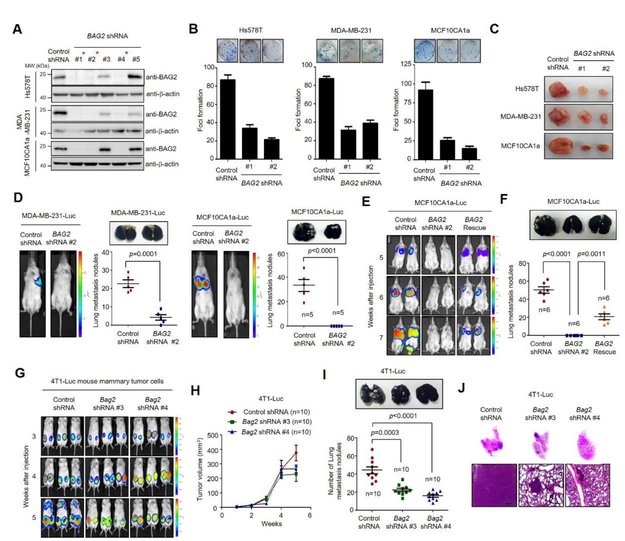


Figure 3. Depletion of BAG2 Significantly Suppresses the Tumorigenicity and Lung Metastasis of TNBC Cells. (A) Immunoblot analysis showing the knockdown efficiency of five different *BAG2*-specific shRNAs (#1-#5) in TNBC cell lines. * indicates the most effective *BAG2*-specific shRNA. (B) Focus forming assay of BAG2-depleted TNBC cells. (C) Tumor formation of control and BAG2-deficient TNBC cells. (D) Representative bioluminescent (BLI) imaging and lung metastasis nodules of NOD/SCID mice. (E and F) Representative BLI imaging of NOD/SCID mice showing lung metastasis from control and BAG2-depleted MCF10CA1a-Luc cells along with BAG2 Rescue. (G) Representative BLI imaging of Balb/c mice showing primary tumors and spontaneous lung metastases generated by control or BAG2-depleted 4T1-Luc cells. (H) Tumor volume curves showing primary tumor growth. (I) Representative whole lung image stained with India ink showing metastatic nodules (top) and scatter plot on the number of spontaneous lung metastatic nodules (bottom). (J) Representative image of H&E staining of lungs showing spontaneous lung metastatic nodules.

RESULTS (III)

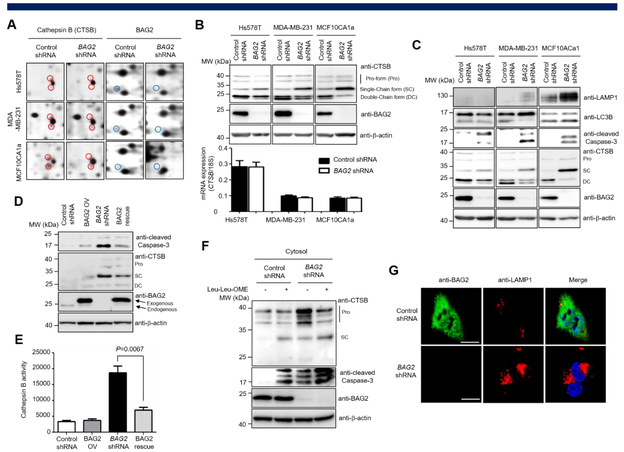


Figure 4. Depletion of BAG2 Induces Lysosomal-Mediated Cell Death by Releasing the Single Chain Form of CTSB into the Cytosol and Impairing Lysosome Biogenesis. (A) Magnified 2-DE gel image displaying the spots of CTSB (double chain form; red) and BAG2 (blue) proteins. (B) Immunoblot analysis (top) and qRT-PCR (bottom) of CTSB and BAG2 in control and BAG2-depleted TNBC cells. (C) Immunoblot analysis of control and BAG2-depleted TNBC cells. (D) Immunoblot analysis of MCF10CA1a-Luc cells stably expressing control shRNA, BAG2 protein, *BAG2*-specific shRNA, or BAG2 Rescue. (E) CTSB activity of each fraction eluted from (D). (F) Immunoblot analysis of control and BAG2-deficient cells treated with or without Leu-Leu-OME. (G) Immunofluorescence analysis of endogenous BAG2 (green) and LAMP-1 (red) in control and BAG2-depleted MDA-MB-231 cells.

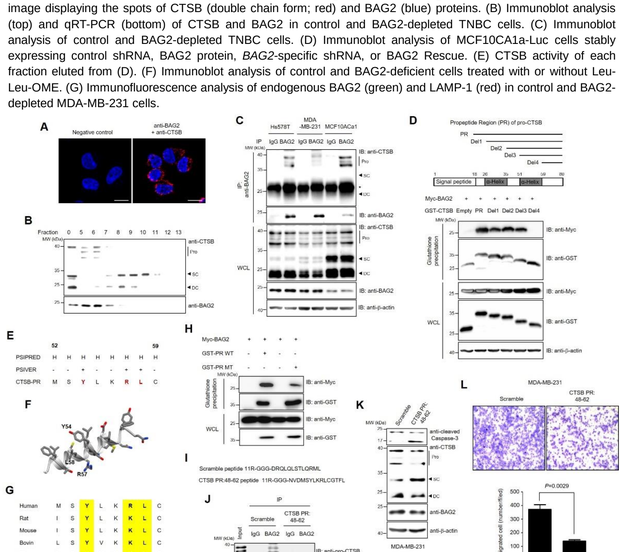


Figure 5. BAG2 Regulates Auto-Cleavage of Pro-CTSB by Binding to the Propeptide Region. (A) Confocal image of in situ PLA showing the interaction (red) between BAG2 and CTSB. (B) Size distribution of CTSB following size exclusion chromatography. (C) Immunoprecipitation assay showing endogenous interaction between BAG2 and CTSB in TNBC cells. (D) GST pull-down assay of Myc-tagged BAG2 bound to GST-fused deletion mutants of the propeptide region of pro-CTSB. (E) The predicted interaction sites determined by PSIVER software. (F) The putative BAG2-binding sites (Y54, R57, and L58) on a predicted three-dimensional structure of CTSB PPR. (G) The conserved sequence in CTSB PPR among different mammalian species. (H) Immunoprecipitation assay showing BAG2-binding region in CTSB PPR. (I) Peptide sequences of scramble or CTSB PPR (48-62). (J) Immunoprecipitation assay, immunoblot analysis, trans-well migration showing the inhibitory effect of CTSB PPR (48-62) peptide in MDA-MB-231 cells.

RESULTS (IV)

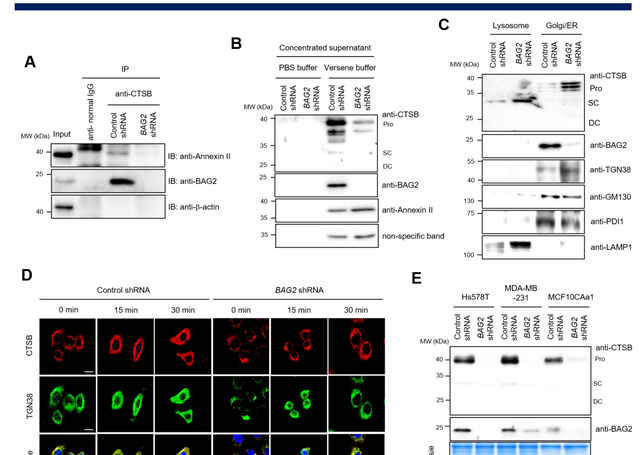


Figure 6. BAG2 Interacts with Pro-CTSB/Annexin II Complex on the Surface of TNBC Cells and Facilitates Pro-CTSB Secretion through Regulation of TGN Trafficking. (A) Immunoprecipitation assay for endogenous interaction between CTSB and annexin II in control and BAG2-depleted MDA-MB-231 cells. (B) Immunoblot analysis showing CTSB, BAG2, and annexin II bound to the cell surface in a Ca^{2+}-dependent manner. (C) Immunoblot analysis showing lysosome and Golgi/ER fractions of control and BAG2-depleted MDA-MB-231 cells. (D) Golgi exit assay of control and BAG2-depleted MDA-MB-231 cells. (E) Immunoblot analysis showing secreted pro-CTSB and BAG2 in conditioned media of control and BAG2-depleted TNBC cells.

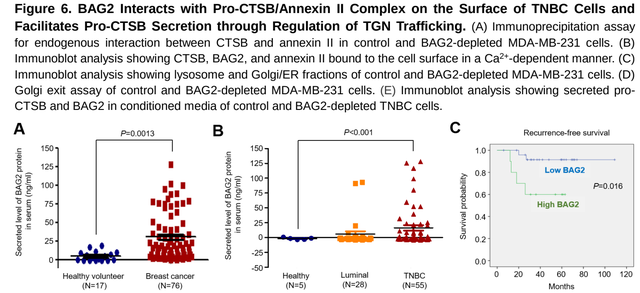
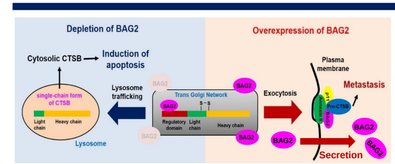


Figure 7. BAG2 Is Specifically Secreted into the Serums of Patients with Aggressive Breast Cancer. (A, B) ELISA analysis showing BAG2 protein levels in the serums of breast cancer patients. (C) Kaplan-Meier analysis showing recurrence-free survival depending on secreted BAG2 protein level in patients with breast caner.

SUMMARY



- **BAG2 overexpression in TNBC is strongly correlated with poor clinical outcomes.**
- **Silencing BAG2 induces apoptosis through increase of single-chain cathepsin B.**
- **BAG2 controls the secretion of pro-CTSB through TGN38-positive vesicles.**
- **BAG2 proteins are secreted into serums and may serve as a positive factor for the risk of mortality in patients with breast cancer.**

CONCLUSION

In conclusion, our results present a novel mechanism regarding BAG2-mediated regulation of the dual functions of CTSB via controlling the auto-cleavage processing of CTSB, thereby exerting a unique function in TNBC progression. In addition, given that the oncogenic function of CTSB is strongly linked to breast cancer progression, our findings highlight BAG2 as a promising and novel therapeutic target for TNBC.