Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Deficiency of Atg6 impairs beneficial effect of metformin on intestinal stem cell aging in *Drosophila*

Hyun-Jin Na [a, 1], Jung-Hoon Pyo [a, 1], Ho-Jun Jeon [a], Joung-Sun Park [a], Hae-Young Chung [b, **], Mi-Ae Yoo [a, *]

[a] Department of Molecular Biology, Pusan National University, Busan, Republic of Korea
[b] Molecular Inflammation Research Center for Aging Intervention (MRCA), College of Pharmacy, Pusan National University, Busan, Republic of Korea

ARTICLE INFO

Article history:
Received 14 February 2018
Accepted 26 February 2018
Available online xxx

Keywords:
Drosophila
Intestinal stem cell
Atg6
Metformin
Aging
Centrosome amplification

ABSTRACT

Age-related changes of adult stem cell are crucial for tissue aging and age-related diseases. Thus, clarifying mechanisms to prevent adult stem cell aging is indispensable for healthy aging. Metformin, a drug for type 2 diabetes, has been highlighted for its anti-aging and anti-cancer effect. In *Drosophila* intestinal stem cell (ISC), we previously reported the inhibitory effect of metformin on age-related phenotypes of ISC. Here, we showed that knockdown of Atg6, a crucial autophagy-related factor, in ISC induces age-related phenotypes of ISC such as hyperproliferation, centrosome amplification, and DNA damage accumulation. Then, we revealed that metformin inhibits ISC aging phenotypes in Atg6-dependent manner. Taken together, our study suggests that Atg6 is required for the inhibitory effect of metformin on ISC aging, providing an intervention mechanism of metformin on adult stem cell aging.

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1. Introduction

Age-related changes of adult stem cell is associated with tissue aging and age-related diseases such as cancer [1]. Therefore, modulating stem cell aging could potentially mitigate such diseases and promote healthy aging. *Drosophila* midgut is now being increasingly recognized as a powerful model system to study the mechanisms of anti-cancer drugs [2]. *Drosophila* midgut epithelium consists of intestinal stem cells (ISCs), precursor enteroblasts (EBs), differentiated enterocytes (ECs, absorptive cells) and enteroendocrine cells (EEs, secretory cells) [3,4]. During aging, loss of proliferative homeostasis and hyperplasia occur in *Drosophila* midgut [5,6]. This tumor-promoting condition is attributed to ISC abnormalities, and chemotherapeutic screening of anti-cancer drugs successfully mitigate condition [7]. Moreover, the plausible age-related changes of ISC: hyperproliferation [5,6], centrosome amplification [8], and DNA damage accumulation [9] enable to find the mechanisms of anti-aging and anti-cancer effect of such drugs on adult stem cell aging.

Metformin, a widely used drug for type 2 diabetes, has gained interests owing to its anti-aging effect [10]. Studies have reported that metformin feeding extends lifespan of various model organisms [10–13]. Moreover, metformin treatment inhibits tumor incidence and progression through anti-proliferative effect in various types of cancer cells [14]. It was revealed that metformin targets multiple pathways, but the mechanism of action depends on cellular characteristics [15]. Previously, we documented that metformin inhibits age- and oxidative stress-induced ISC abnormalities via suppressing AKT/TOR pathway [16,17]. Based on this study, further exploring the mechanisms of action of metformin on ISC aging is needed for understanding metformin-mediated anti-aging effect of adult stem cell.

Autophagy-related genes are required to maintain stem cell integrity [18,19]. Atg6 (Autophagy-related gene 6, Beclin1 in mammals) is a core component of endosome formation, and has a crucial role in autophagy and endocytosis [20,21]. Loss of Atg6/Beclin1 is found with high frequency in human breast, ovarian, and prostate cancer [22–24]. Allelic loss of beclin1 is associated with activation of the DNA damage response *in vitro* [25]. Meanwhile, Atg6 and other autophagy related genes are known to be negatively regulated by AKT/TOR pathway [26]. Thus, revealing the role of

* Corresponding author. Department of Molecular Biology, Pusan National University, Busan, 46241, Republic of Korea.
** Corresponding author. Molecular Inflammation Research Center for Aging Intervention, College of Pharmacy, Pusan National University, 46241, Busan, Republic of Korea.
E-mail addresses: hyjung@pusan.ac.kr (H.-Y. Chung), mayoo@pusan.ac.kr (M.-A. Yoo).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.bbrc.2018.02.191
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Please cite this article in press as: H.-J. Na, et al., Deficiency of Atg6 impairs beneficial effect of metformin on intestinal stem cell aging in *Drosophila*, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.02.191

autophagy-related factor within the beneficial effect of metformin on ISC is needed to comprehensive understanding of the inhibitory mechanism of metformin on stem cell aging and cancer initiation.

Here, we showed that knockdown of Atg6 in ISC induces age-related phenotypes of ISC and intestinal hyperplasia. We also revealed that Atg6 is required for the inhibitory effect of metformin on the age-related phenotypes in *Drosophila* midgut ISCs.

2. Materials and methods

2.1. Fly stocks

Fly stocks were maintained at 25 °C on standard food under a ~12 h/12 h light/dark cycle. Food consisted of 79.2% water, 1% agar, 7% cornmeal, 2% yeast, 10% sucrose, 0.3% bokinin, and 0.5% propionic acid. To avoid larval overpopulation, 50-60 adult flies per vial were transferred to new food vials every 2-3 days. *Oregon-R* (*OR*) was used as wild type. *UAS-Atg6^{RNAi}* (#22122) was obtained from the Vienna Drosophila RNAi Center (Vienna, Austria). Temperature-inducible *Su(H)GBE-lacZ; esg-Gal4,tub-Gal80^{ts},UAS-GFP/CyO* (*esg^{ts} > GFP*) was kindly provided by Benjamin Ohlstein [4]. *Dl-Gal4/TM6* was acquired from Steven X. Hou [27]. Temperature-inducible *Dl^{ts}-Gal4* was obtained from a cross of *Dl-Gal4* and *tub-Gal80^{ts}* (#7108, Bloomington Drosophila Stock Center, Bloomington, IN, USA). *Su(H)-Gal4;tub-Gal80^{ts},UAS-GFP/SM1 (Su(H)^{ts} > GFP)* was kindly provided by Bruno Lemaitre [28]. The *esg^{ts} > GFP/+ and esg^{ts} > GFP + Atg6^{RNAi}* flies were obtained from a cross of *esg^{ts} > GFP* females and *OR or UAS-Atg6^{RNAi}* males. *Dl^{ts}>+ and Dl^{ts} > Atg6^{RNAi}* flies were obtained from a cross of *Dl^{ts}* females and *OR or UAS-Atg6^{RNAi}* males. *Su(H)^{ts} > GFP/+, and Su(H)^{ts} > GFP + Atg6^{RNAi}* flies were obtained from a cross of *Su(H)^{ts} > GFP* females and *OR or UAS-Atg6^{RNAi}* males.

2.2. Temperature-controlled expression

For transgene expression at specific developmental stages, the Gal80^{ts} technique was used [29]. The flies were set up and maintained at 22 °C until adulthood. After maintaining the flies at 29 °C for 7 days, the midguts were dissected and analyzed.

2.3. Metformin feeding assay

Two-day-old or 5-day-old *esg^{ts} > GFP* flies were fed 5 mM metformin (Sigma Aldrich, St. Louis, MO, USA) mixed in standard food for 7 days at 29 °C. Flies were transferred to new metformin-containing food vials every 2-3 days.

2.4. Paraquat feeding assay

Flies with control or metformin-fed for 6 days from 5-day-old were fed 10 mM paraquat (methyl viologen, PQ, Sigma-Aldrich) in standard media for 18-20 h at 25 °C. After feeding, the midguts were dissected and analyzed.

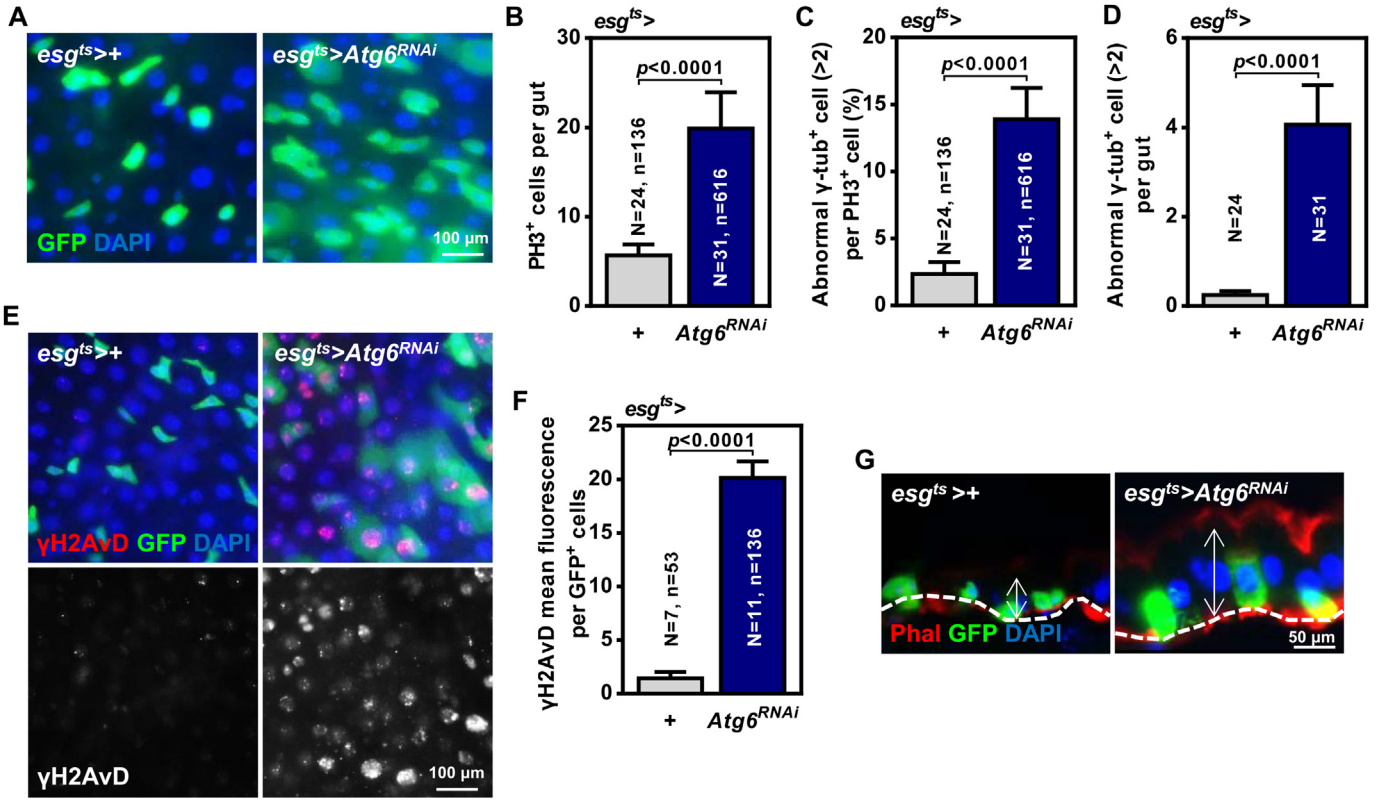

Fig. 1. Age-related phenotypes of ISC induced by ISC/EB-specific knockdown of Atg6. (A) After 7 days induction, the number of esg-GFP-positive cells (ISC/EB, green) increased in midgut of *esg^{ts} > Atg6^{RNAi}* flies. (B) The number of PH3-positive cells (mitotic ISCs), (C) The number of mitotic ISCs with supernumerary centrosomes, and (D) the number of mitotic ISCs with abnormal centrosomes per gut increased in *esg^{ts} > Atg6^{RNAi}* flies. (D) Immunofluorescence staining of γH2AvD (red) indicates the increase of γH2AvD level in esg-GFP-positive cells (green) in midgut of *esg^{ts} > Atg6^{RNAi}* flies. (E and F) Images and measured data of γH2AvD fluorescence in esg-GFP-positive cells. (G) Longitudinal-section of midgut. Phalloidin (Phal, red) marks visceral muscle. Throughout the figures, nucleus is visualized by DAPI staining (blue). Error bars represent standard error, and *p*-values were calculated using two-tailed unpaired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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