博士学位論文

miR-155 は BCL2 associated athanogene 5(BAG5) の
抑制を介したマイトファジー機構の制御に関与する

近 畿 大 学 大 学 院
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miR-155 inhibits mitophagy through suppression of

BAG5, a partner protein of PINK1

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論文題目

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miR-155 inhibits mitophagy through suppression of BAG5, a partner protein of PINK1

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ABSTRACT

Removal of dysfunctional mitochondria is essential step to maintain normal cell physiology, and selective autophagy in mitochondria, called mitophagy, plays a critical role in quality control of mitochondria. While in several diseases and aging, disturbed mitophagy has been observed. In stem cells, accumulation of damaged mitochondria can lead to deterioration of stem cell properties. Here, we focused on *miR-155-5p* (*miR-155*), one of the most prominent miRNAs in inflammatory and aged tissues, and found that *miR-155* disturbed mitophagy in mesenchymal stem cells (MSCs). As a molecular mechanism of *miR-155*-mediated mitophagy suppression, we found that BCL2 associated athanogene 5 (BAG5) is a direct target of miR-155. Reduction of BAG5 resulted in destabilization of PTEN-induced kinase (PINK1) and consequently disrupted mitophagy. Our study suggests a novel mechanism connecting aging and aging-associated inflammation with mitochondrial dysfunction in stem cells through a miRNA-mediated mechanism.

1. Introduction

Mitochondria have been recognized to involve in the process of aging. Accumulation of defective mitochondria is linked to impaired energy production and generation of ROS at toxic levels, resulting in cellular aging and age-related disorders [1]. When accumulation of dysfunctional mitochondria arises in stem cells, it directly affects stem cell activity and function, and can leads to deterioration of tissue turnover and regeneration [2—6].

In normal cell physiology, these mitochondria are tagged for clearance and are digested by a selective autophagy process called mitophagy. Mechanistically, degenerated mitochondria are identified by PINK1, then an E3 ligase Parkin is recruited and the channel protein VDAC1 is localized to the outer mitochondrial membrane; finally, the autophagic adaptor p62/SQSTM1 recognizes and triggers mitophagy [7]. Failed mitophagy has been observed in numerous diseases. Further, defective mitophagy was reported in

aging and/or aging-related diseases, with a suggested correlation between tissue degenerative and pathological changes in tissues or cells [8]. However, the detailed mechanisms and molecules responsible for the defects in mitophagy are largely unknown. Through recent studies, microRNAs (miRNAs) have been found to be involved in autophagy regulation. MiRNAs are a class of small non-cording RNAs that play important roles in the post-transcriptional regulation of various genes. Disrupted miRNA expression has been found in diseases such as cancer, and a connection between miRNA function and pathological changes is also reported; thus, miRNAs are considered important molecules to understand the cellular changes in diseases. In this study, we focused on miR-155, which is one of the major miRNA induced by inflammation and aging [9], and revealed that miR-155 could be a key molecule to explain aging-associated deterioration of mitochondrial quality.

2. Materials and methods

2.1. Ethics statement

All procedures involving animals were approved by the

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Institutional Animal Care and Use Committee at Kindai University and were performed in accordance with the institutional guidelines and regulations.

2.2. Isolation of bone marrow (BM) tissues

Four-week-old (young) and 1.5-year-old (aged) C57BL/6 N male mice were used for the experiment. Bone marrow tissues were prepared as reported previously [10].

2.3. Culture of human MSC-line UE6E7T-2

Immortalized human BMMSC line UE6E7T-2 [11] was purchased from the cell bank of the National Institutes of Biomedical Innovation, Health and Nutrition (Cell code JCRB1133, Osaka, Japan). UE6E7T-2 cells were cultured in 10% FCS-DMEM under 5% O_2 and 5% CO_2 at 37 °C.

2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA was collected from MSCs using TRI Reagent® (Molecular Research Center Inc., Cincinnati, OH, USA) and reverse-transcribed with the PrimeScript® RT Master Mix Kit (TAKARA Bio Inc., Shiga, Japan). Quantitative real-time PCR of total cDNA was performed using Perfect real-time SYBR green II (TAKARA). Data are expressed as mean values ± SD of 3 replicates. Statistical significance was evaluated using Student's t-test with JMP software version 10.0.0 (SAS Institute, Cary, NC, USA). Primer sequences are listed in Table S1.

2.5. Western blot (WB) analysis

MSCs from each experiment were homogenized in SDS buffer and centrifuged at 9,000×g for 10 min at 4 °C to remove debris. The blotted membranes were blocked and probed overnight at 4 °C with primary antibodies (Table S2). Detection was performed with horseradish peroxidase (HRP)-conjugated secondary antibodies and Immunostar ® LD (Wako) detection reagents

2.6. Transfection of miRNA mimic or siRNA into UE6E7T-2 cells

Scrambled control RNA, the mimic sequence of miR-155 (UUAAUGCUAAUCGUGAUAGGGGUU, GeneDesign, Inc., Osaka, Japan) or siRNA against BAG5 (sense, GAU AUC AAC AAA UUA UUG AdTdT and antisense, UCA AUA AUU UGU UGA UAU CdTdT, Japan Bio Services Co., Ltd., Saitama, Japan) were transfected into cells using Lipofectamine® RNAiMAX (Thermo Fisher Scientific) following the manufacturer's instructions. The cells were analyzed after 48 h of transfection.

2.7. Transfection of miR-155 or MT-KeimaRed expression plasmids into UE6E7T-2 cells

To observe only the cells expressing scrambled RNA or the mimic sequence of miR-155, miExpress™ EGFP-hsa-miR-155 plasmid (GeneCopoeia Inc. Rockville, MD, USA) or the scrambled control sequence expression plasmid CmiR0001-MR04 (GeneCopoeia, Inc.) were co-transfected with a MT-KeimaRed expression plasmid (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) using a CUY21 electroporator (NEPA Gene, Tokyo, Japan). After 24 h of electroporation, the cells were treated with CCCP at 1 μM for 24 h followed by fluorescence observation using a Keyence BZX-710 microscope (Keyence, Osaka, Japan).

2.8. Immunoprecipitation

Plasmids coding Ty1-tagged PINK1 were transfected into HEK293 cells using Lipofectamine 3000 (Thermo Fisher Scientific). Whole cell lysates or fractionated samples were incubated with 2 μg of Ty1 antibody (Diagenode Co. Ltd., Toyama, Japan) for 8 h at 4 °C in immunoprecipitation buffer (50 mM Tris [pH 7.3], 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Triton X-100) with 1 mM PMSF and protease inhibitors (Sigma-Aldrich). Immunocomplexes were then isolated with protein G-Sepharose beads (Thermo Fisher Scientific). Bound proteins were retrieved from Sepharose beads by boiling in Laemmli buffer containing β-mercaptoethanol. To detect ubiquitinated PINK1, cells were treated with the proteasome inhibitor MG132 at 1 nM for 6 h before sampling.

2.9. Statistical analysis

Significant differences were determined by Tukey-Kramer HSD test or Student's t-test, as appropriate. P values less than 0.05 were considered significant.

3. Results

3.1. Upregulation of miR-155 and downregulation of mitophagy related genes is observed in aged bone marrow tissues

To investigate the function of miR-155 in mitophagy, we observed the expression level of miR-155 and mitophagy-related genes in young and aged BM. Consistent with our previous results [9], miR-155 expression was upregulated in aged BM (Fig. 1A). In contrast, the expression of Atg5, Atg5-12 conjugate, Atg16L1, and Pink1 proteins was downregulated in aged BM (Fig. 1B), whereas free Atg12 and Beclin1 proteins were upregulated.

3.2. miR-155 suppresses mitophagy

To observe the effect of miR-155 on mitophagy regulation, we transfected the mimic RNA of mmu-miR-155-5p into mouse primary BMMSCs or hsa-miR-155-5p into the human MSC-line UE6E7T-2, and examined the expression of ATG5, ATG5-12 conjugate, ATG16L1, and PINK1, which showed reduced expression in aged BM tissues by WB. In the mouse BMMSCs, transfection with the miR-155 mimic decreased the expression of ATG5, ATG5-12 conjugate, ATG16L1, and PINK1 protein (Fig. 2A). Further, in the
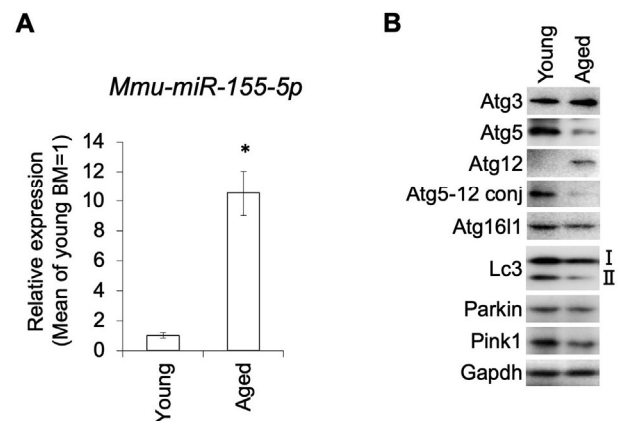

Fig. 1. Expression levels of miR-155 and mitophagy-related proteins in aged bone marrow (BM) tissue. A, Expression level of miR-155 in aged BMMSCs. Asterisk indicates a significant difference compared with the BM of young mice at P < 0.05 (N = 3). B, Expression of mitophagy related proteins in aged BMMSCs.
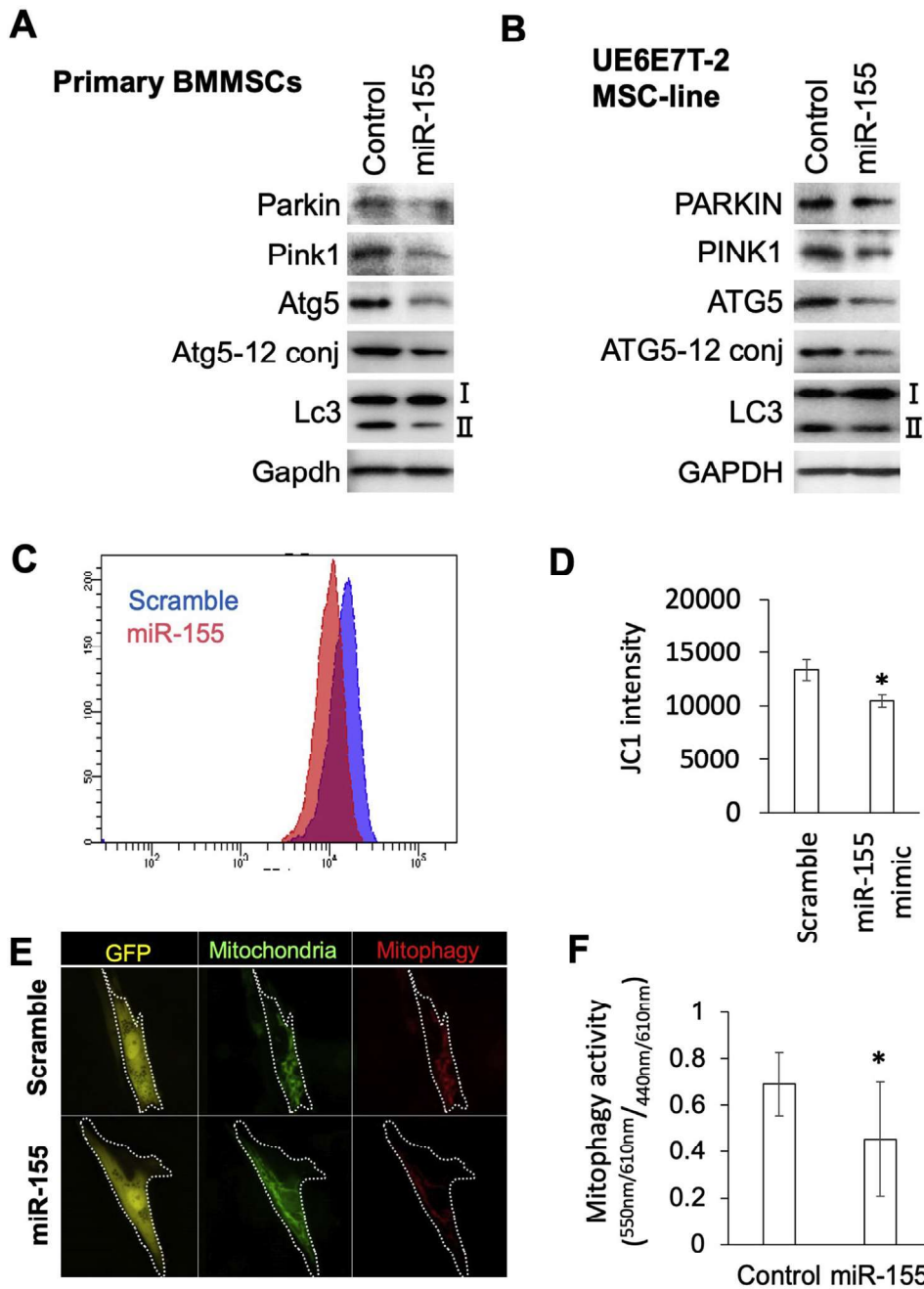
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Fig. 2. Mitophagy-related protein expression and mitophagy activity were suppressed by miR-155. A, Western blot (WB) analysis for mitophagy-related proteins using miR-155 transfected mouse primary BMMSCs. Atg5-12 conj. is the conjugated form of Atg12 with Atg5 that indicates activated autophagy. B, WB analysis for mitophagy-related proteins using miR-155 transfected human MSC-line, UE6E7T-2. C, FACS analysis of JC-1 stained UE6E7T-2 cells transfected with miR-155 mimic. Overlay of scrambled RNA-transfected control (blue) onto miR155 mimic-transfected cells (red). D, Quantification of JC-1 fluorescence intensity by FACS. Asterisk indicates a significant difference between the two groups at P < 0.05 (N = 3). E, Fluorescence observation of mitophagy activity with MT-KeimaRed in UE6E7T-2 cells transfected with scrambled RNA or miR-155 mimic. F, Quantification of mitophagy activity by comparing the differential fluorescence of MT-KeimaRed. Asterisk indicates a significant difference between the two groups at P < 0.05 (N = 10).

human MSC-line, *miR-155* transfection reduced the expression of PINK1, ATG5, and ATG5-12 conjugate proteins (Fig. 2B). We then investigated whether suppression of PINK1, ATG5, and ATG5-12 weakens mitophagy activity and the deterioration of mitochondrial quality. We thus assessed mitochondrial membrane potential ($\Delta\psi_M$) using the JC-1 fluorescent dye. In the presence of 1 μM CCCP, a decrease in the 600-nm fluorescence was observed indicating that mitochondrial membrane potential was reduced by the miR-155 mimic (Fig. 2C–D). To confirm that the reduced $\Delta\psi_M$ was

caused by mitophagy disruption, we co-transfected a plasmid encoding MT-mKeima-Red fluorescent protein, which enables visualization of mitophagy by two different excitation wavelengths 440 nm/610 nm for total mitochondria and 550 nm/610 nm for mitochondria under mitophagy processing, along with EGFP-*hsa-miR-155* in UE6E7T-2 cells. As a control we co-transfected MT-mKeima-Red and a plasmid encoding EGFP with a control sequence. In EGFP-*miR-155* transfected cells, CCCP-induced mitophagy signal was clearly suppressed and showed

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approximately 40% reduction compared to the control (Fig. 2E–F).

3.3. miR-155 disturbs mitophagy through PINK1 suppression

To identify the molecules targeted by *miR-155* in human MSCs, we performed qRT-PCR for mitophagy-related genes and found that the expression level of *BECN1*, *ATG5*, *12*, *16L1*, *LC3B*, *PARKIN*, and *PINK1* did not change (Supplementary Fig. 1), though the protein expression of ATG5, ATG5-12 conjugate, and PINK1 was affected by miR-155. We thus hypothesized that miR-155 is involved in the post-transcriptional regulation of ATG5, ATG5-12 conjugate, or PINK1 by targeting their accessory proteins. Based on prediction analysis using miRDB [12], DIANA-Tarbase v7.0 software [13] and previous reports, we hypothesized that Bcl-2-associated athano-gene 5 (BAG5), a stabilizer of PINK1 [14], could be a direct target of miR-155.

Consistent with the hypothesis, BAG5 expression was repressed in the miR-155 mimic-transfected UE6E7T-2 cells (Fig. 3A–B). Luciferase assay using the Luc-BAG5 3′UTR showed that miR-155 targeted the 3′UTR of BAG5 (Fig. 3C).

3.4. Suppression of BAG5 by miR-155 induced PINK1 ubiquitination

We then confirmed that BAG5 interacts with PINK1 and that its reduction results in PINK1 destabilization through Co-IP and WB analysis. Co-IP showed that PINK1 binds BAG5 (Fig. 4A). Treatment with siRNA against BAG5 (siBAG5) resulted in approximately 80% reduction of BAG5 mRNA with decreased expression of PINK1 (Fig. 4B and C). To determine whether the interaction of BAG5 and PINK1 involves PINK1 stability, we assessed the ubiquitination of
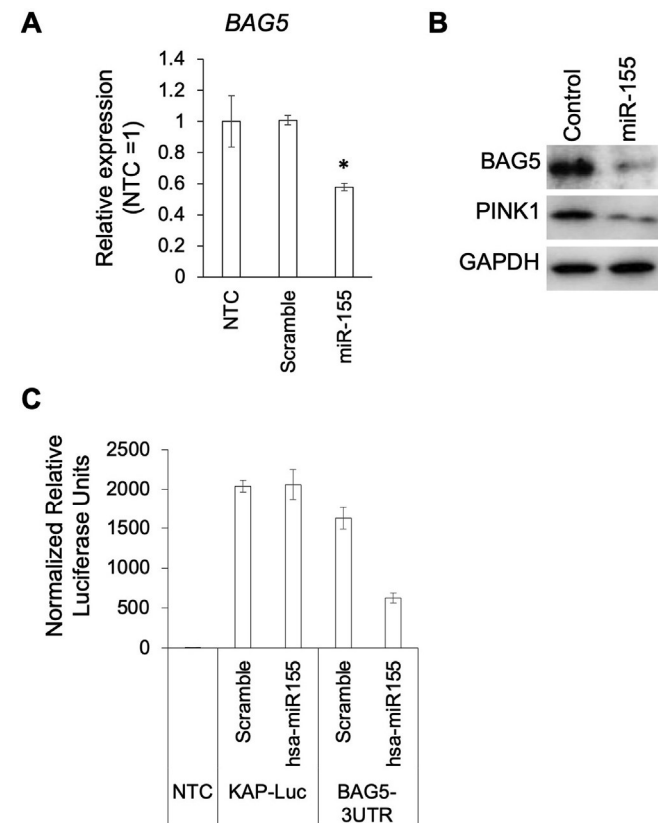

Fig. 4. Interaction between BAG5 and PINK1 is essential for PINK1 stability. A. IP showing interaction between PINK1 and BAG5. The IP sample was prepared with anti-HA antibody and HA-BAG5 overexpressing HEK293 cells. Subsequent immunoblot (IB) analysis was performed with anti-PINK1 and anti-BAG5 antibodies. B. Suppression of BAG5 using siRNA against BAG5 (siBAG5). C, Detection of ubiquitination in the immunoprecipitated (IP) fraction with Ty1-tagged PINK1 (Ty1-PINK1) and anti-Ty1 antibody in the siBAG5-treated cells. D, Detection of ubiquitination in the IP fraction with Ty1-PINK1 and anti-Ty1 antibody in the miR-155 mimic-treated cells. E, Detection of ubiquitination in the IP fraction with Ty1-PINK1 and anti-Ty1 antibody in cells co-transfected with miR-155 mimic and BAG5 plasmid.

PINK1 by overexpressing Ty1-tagged PINK1 followed by immuno-precipitation with an anti-Ty1 antibody and immunoblotting with an anti-Ubiquitin (Ub) antibody. Treatment with siBAG5 signifi-cantly increased the ubiquitinated proteins immunoprecipitated with anti-Ty1 (PINK1) antibody (Fig. 4C). Consistent with our hy-pothesis that miR-155 suppresses BAG5 and that reduced BAG5 sequentially induces PINK1 ubiquitination and destabilization, transfection with the miR-155 mimic clearly induced an increase in the ubiquitinated signal (Fig. 4D). In contrast, BAG5 overexpression blocked the miR-155-induced ubiquitination of PINK1 (Fig. 4E).

4. Discussion

In this study we found that Atg5, Atg5-12 conjugate, Atg16, and Pink1 were downregulated in aged BM tissues and miR-155

Fig. 3. MiR-155 targets BAG5 in MSCs. A, qRT-PCR analysis for BAG5 in UE6E7T-2 cells treated with miR-155 mimic. B, WB analysis for BAG5 in UE6E7T-2 cells treated with miR-155 mimic. C, Luc assay using a Luc-BAG5 3′UTR construct and miR-155 mimic.
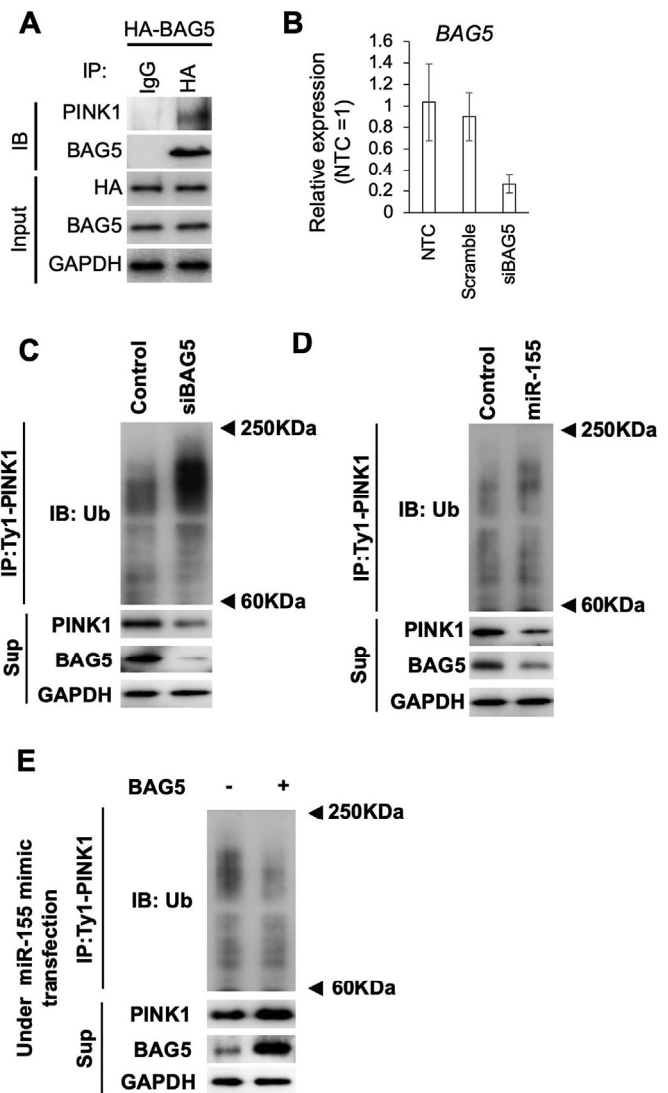
overexpressing cells. To determine whether the mitophagy mediated mitochondrial quality control was disturbed by miR-155, we performed FACS analysis using the JC-1 fluorescent dye that reflects the mitochondrial inner membrane potential with CCCP, an inducer of mitophagy. In this experiment, cells maintaining normal mitochondrial clearance showed higher JC-1 intensity compared to that in cells with affected mitophagy activity. As expected, transfection with the miR-155 mimic decreased JC-1 intensity, indicating that the quality or quantity of mitochondria was affected by miR-155. We then co-transfected cells with the MT-KeimaRed plasmid and EGFP-*hsa-miR-155* plasmid, and found that miR-155 expression induced approximately 40% reduction of mitophagy in the human MSC model. Sun et al. reported that the MT-Keima signal in the dentate gyrus region of aged mice was downregulated by 75% compared with that in young mice [15]. This evidence supports the idea that mitophagy is suppressed in aged cells/tissues and indicates that KeimaRed is useful to detect mitophagy.

As a mechanism for the *miR-155*-mediated mitophagy regulation, we discovered that BAG5 is a direct target of *miR-155*. Upon mitochondrial dysfunction, PINK1 is stabilized on the outer mitochondrial membrane and activates PARKIN to catalyze the ubiquitination of mitochondrial proteins for autophagic clearance of damaged mitochondria [16]. BAG5 directly interacts with and protects PINK1 from degradation by the ubiquitin proteasome system [14]. Consistent with this notion, we confirmed that BAG5 suppression using siRNA significantly increased the ubiquitination and degradation of PINK1 protein. To demonstrate that miR-155 alters the ubiquitination status of PINK1, we prepared HEK293 cells expressing Ty1-epitope tagged PINK1, and isolated Ty1-PINK1 by immunoprecipitation after transfecting the *miR-155* mimic. Consistent with our hypothesis, the immunoprecipitated fraction in which PINK1 protein was concentrated, was highly ubiquitinated by treatment with the miR-155 mimic, and over-ubiquitination was cancelled by co-transfection with a BAG5 encoding plasmid. These results indicate that ubiquitination status is regulated by the amount of BAG5, and that reduction of BAG5 by miR-155 induces excessive ubiquitination of PINK1.

Defective mitophagy has been recently discovered in neurodegenerative diseases such as Alzheimer's disease [17] and Parkinson's disease [18]. Importantly, upregulation of miR-155 expression [19,20] and defective PINK1 expression [21,22] are also observed in neuronal diseases. Bueno et al. reported that impaired mitochondria in IPF and aging lungs were associated with low PINK1 expression [23]. In these diseases, some extracellular stressors are considered to induce excessive miR-155 expression and suppression of mitophagy-related molecular activity; for example, both amyloid-β (Aβ) [20] and oxidative stress [24] have been demonstrated as strong inducers of miR-155. The molecular pathway demonstrated in this study could be a link connecting upregulated miR-155 and downregulated PINK1 in a pathological environment.

We report a novel molecular pathway that regulates mitophagy through aging-associated miRNA, wherein upregulated miR-155 targets BAG5 followed by PINK1 destabilization. Although further investigations on the role of miR-155 in mitophagy are clearly required, the present study proposes an attractive opportunity to develop a therapeutic strategy to improve stem cell qualities for regenerative medicine and to control various aging-associated dysfunctions.

Declaration of competing interest

All authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.01.022.

Transparency document

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