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ZFP36L1 and AUF1 Induction Contribute to the Suppression of Inflammatory Mediators Expression by Globular Adiponectin via Autophagy Induction in Macrophages

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Abstract

Adiponectin, a hormone predominantly originated from adipose tissue, has exhibited potent anti-inflammatory properties. Accumulating evidence suggests that autophagy induction plays a crucial role in anti-inflammatory responses by adiponectin. However, underlying molecular mechanisms are still largely unknown. Association of Bcl-2 with Beclin-1, an autophagy activating protein, prevents autophagy induction. We have previously shown that adiponectin-induced autophagy activation is mediated through inhibition of interaction between Bcl-2 and Beclin-1. In the present study, we examined the molecular mechanisms by which adiponectin modulates association of Bcl-2 and Beclin-1 in macrophages. Herein, we demonstrated that globular adiponectin (gAcrp) induced increase in the expression of AUF1 and ZFP36L1, which act as mRNA destabilizing proteins, both in RAW 264.7 macrophages and primary peritoneal macrophages. In addition, gene silencing of AUF1 and ZFP36L1 caused restoration of decrease in Bcl-2 expression and Bcl-2 mRNA half-life by gAcrp, indicating crucial roles of AUF1 and ZFP36L1 induction in Bcl-2 mRNA destabilization by gAcrp. Moreover, knock-down of AUF1 and ZFP36L1 enhanced interaction of Bcl-2 with Beclin-1, and subsequently prevented gAcrp-induced autophagy activation, suggesting that AUF1 and ZFP36L1 induction mediates gAcrp-induced autophagy activation via Bcl-2 mRNA destabilization. Furthermore, suppressive effects of gAcrp on LPS-stimulated inflammatory mediators expression were prevented by gene silencing of AUF1 and ZFP36L1 in macrophages. Taken together, these results suggest that AUF1 and ZFP36L1 induction critically contributes to autophagy induction by gAcrp and are promising targets for anti-inflammatory responses by gAcrp.

Key Words: Adiponectin, AUF1, Autophagy, Bcl-2, Inflammation, TTP

INTRODUCTION

Adipose tissue secretes a group of cytokines, collectively called as adipokines. It has been well documented that adipokines play important roles in diverse physiological responses, including metabolic homeostasis, energy metabolism, and immune functions (Ouchi *et al*., 2011). Therefore, adipose tissue behaves as a dynamic endocrine organ, rather than served as a simple storage organ for excessive energy. Among the various adipokines, adiponectin is the most abundantly present in the circulation, approximately accounting for 0.01% of the total plasma protein (Liu and Liu, 2009). Adiponectin exists as different forms in the plasma, such as low molecular weight (LMW, trimer), medium molecular weight (MMW, hexamer), and high-molecular oligomer (HMW, oligomer). In addition, only globular domain of adiponectin generated by proteolytic cleavage of the full-length adiponectin also exists in the circulation. Globular adiponectin (gAcrp) is more effective than full-length adiponectin on the suppression of TNF-α expression in macrophages (Park *et al*., 2007) and on fatty acid oxidation in skeletal muscle (Tomas *et al*., 2002), indicating that existence of only globular domain of adiponectin is functionally active.

Adiponectin has received much attention for its role as an intermediary between adipose tissue and other metabolic related organs. Interestingly, on the contrary to leptin and other adipokines, the plasma level of adiponectin exhibits inverse relationship with the mass of adipose tissue. The biological actions of adiponectin are mediated via binding to its specific receptors, mainly adiponectin receptor type 1 (AdipoR1) and adiponectin receptor type 2 (AdipoR2) (Perri *et al*., 2016).

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There is a growing appreciation that adiponectin plays a crucial role in the regulation of lipid metabolism, insulin sensitization, and improving whole body energy homeostasis (Liu and Liu, 2009; Wang and Scherer, 2016). In addition to its beneficial roles in metabolism, emerging evidence has indicated that adiponectin also possesses potent anti-inflammatory properties in various experimental conditions. For example, gAcrp induced tolerance to LPS-stimulated inflammatory mediators expression in macrophages (Pun *et al*., 2015), inhibited ER stress-mediated inflammasomes activation in hepatocytes (Khakurel and Park, 2018), and prevented vascular inflammatory responses via recruitment of ceramidase (Wang *et al*., 2014). Based on previous reports, it is well established that adiponectin acts as a potent anti-inflammatory mediator. However, underlying molecular mechanisms are not well defined.

Autophagy, a highly conserved self-digestive process, is accountable for removal of defective and dysfunctional cellular constituents, including damaged cellular organelles and mis-folded proteins, via sequestration of the target molecules within double membrane formation vesicles called as autophagosome (Levine and Klionsky, 2004). While autophagy can occur in normal physiological conditions, it is more extensively induced by intra- and/or extracellular stress, including starvation, hypoxia, and ER stress (He and Klionsky, 2009). It is well documented that autophagy plays an important role in numerous physiological responses and dysregulation of autophagy is accompanied with various pathologies, including neurodegeneration (Komatsu *et al*., 2006), autoimmune diseases (Levine *et al*., 2011), and cancer (Lee *et al*., 2018). Moreover, there has been increasing evidence demonstrating that autophagy plays a critical role in the modulation of inflammatory responses. Interestingly, recent studies have demonstrated that autophagy induction contributes to the anti-inflammatory responses by adiponectin (Pun *et al*., 2015; Kim *et al*., 2017). However, detailed molecular mechanisms for autophagy induction by adiponectin are not clearly understood.

Autophagic process is regulated via coordinated actions of the various autophagy-related genes (ATGs), such as Beclin-1, autophagy protein 5 (ATG5), and microtubule-associated proteins light chain 3B (LC3) (He and Klionsky, 2009; Huang and Brumell, 2014). Of the various ATGs, Beclin-1 plays a critical role in nucleation of phagophore and autophagosome formation. Autophagy induction by Beclin-1 is mediated by interaction with the class III phosphoinositide 3-kinase, also called as Vps34. Beclin-1 and Vps34 complex catalyzes the phosphorylation of phosphoinositides to produce phosphatidylinositol 3-phosphate (PI(3)P), thereby facilitating recruitment of the cargos and maturation of the autophagosome (Funderburk *et al*., 2010). The biological activity of Beclin-1 during the process of autophagy induction is regulated by various mechanisms. In particular, accumulating evidence suggests that binding of Bcl-2 family proteins with Beclin-1 through Bcl-2-homology-3 (BH3) results in dissociation between Beclin-1 and Vps34, which subsequently negatively regulates autophagosome formation (Deretic *et al*., 2013; McKnight and Zhenyu, 2013). In contrast, dissociation of Beclin-1 from Bcl-2 enhances interaction between Beclin-1 and Vps34, where kinase activity of Vps34 and autophagosome formation levels are enhanced (Levine *et al*., 2008). Therefore, association of Beclin-1 with Bcl-2 is a critical factor for the modulation of autophagy induction. Recently, it has been reported that gAcrp induces autophagy in macrophages by Bcl-2 mRNA destabilization and modulating association of Bcl-2 and Beclin-1 (Tilija Pun and Park, 2018). Given that association of Beclin-1 and Bcl-2 is a critical event modulating autophagy induction, suppression of Bcl-2 expression would be a critical event leading to autophagy induction and further anti-inflammatory responses by adiponectin. However, the molecular mechanisms underlying Bcl-2 mRNA destabilization are not clearly understood.

Stability of the mRNA is regulated by a group of AU-rich mRNA binding proteins (AUBPs), which binds with adenine uridine-rich elements (AREs) within 3'-untranslated region (3'-UTR) and modulates decay of the mRNA with a distinct effect on mRNA stability by target-dependent manner (Garneau *et al*., 2007). For example, binding of HuR and nucleolin induces Bcl-2 mRNA stabilization (Ishimaru *et al*., 2009, 2010), while binding of triestetraproline, (TTP), ARE/poly (U)-binding factor 1 (AUF1), and zinc finger RNA binding protein like 1 (ZFP36L1) results in mRNA destabilization of the target mRNA (Dodson and Shapiro, 2002). We have previously shown that induction of tristetraproln (TTP), acting as a mRNA destabilizer, contributes to adiponectin-induced decrease in Bcl-2 expression in macrophages (Tilija Pun and Park, 2018). To date, in addition to TTP, a number of different types of mRNA binding proteins have been identified and are implicated in modulating decay of mRNA in a gene-selective manner. While adiponectin has been shown to induce decrease in Bcl-2 mRNA destabilization, the molecular mechanisms involving various AUBPs are not well defined.

Thus, to better understand the molecular mechanisms underlying autophagy induction by adiponectin, we investigated the role of AUBPs in the modulation of Bcl-2 mRNA stability by gAcrp. Herein, we demonstrated for the first time that gAcrp induced increase in AUF1 and ZFP36L1 in macrophages. Moreover, AUF1 and ZFP36L1 induction play a critical role in Bcl-2 mRNA destabilization by gAcrp and causes dissociation of Beclin-1 and Bcl-2, which in turn leads to autophagy induction in macrophages. Furthermore, we also observed that AUF1 and ZFP36L1 also play crucial roles in the suppression of inflammatory mediators expression by gAcrp.

MATERIALS AND METHODS

Materials

All the cell culture reagents were purchased from Hyclone Laboratories (South Logan, UT, USA) unless indicated elsewhere. Recombinant human globular adiponectin was obtained from Peprotech Inc. (Rocky Hill NJ, USA). Primary antibodies against ZFP36L1 (Catalog. No. 2119), Bcl-2 (Catalog. No. 3498), Beclin-1 (Catalog. No.3738), and β-actin (Catalog. No. MA5-15739) were procured from Cell Signaling Technology Inc. (Beverly, MA, USA); AUF1 (Catalog. No. 07-260) was procured from Merck Millipore. The secondary antibodies conjugated with horse radish peroxidase (HRP) were obtained from Pierce Biotechnology (Rockford, IL, USA). The TNF-α ELISA kit was procured from BioLegend (San Diego, CA, USA).

Cell culture of RAW 264.7 macrophages

Raw 264.7 murine macrophage cell line was purchased from Korean Cell Line Bank (Seoul, Korea) and was routinely cultured in Dulbecco's modified Eagle medium (DMEM), appended with 10% (v/v) Fetal Bovine Serum and 1% (v/v) pen-

Table 1. Primer sequences used for quantitative RT-PCR

Target gene	Primer	Nucleotide sequence
AUF1	F	5'-GGATGTCTGGTTGTGGCTTT-3'
	R	5'-AGCUAUUUUGAUGUCCACC-3'
ZFP36L1	F	5'-GGAGAGCAAAAAUGUUGCU-3'
	R	5'-AGCAACAUUUUUGCUCUCC-3'
Bcl-2	F	5'-AGGAGCAGGTGCCTACAAGA-3'
	R	5'-GCATTTTCCCACCACTGTCT-3'
TNF-α	F	5'-GAGGGAAATTTGAGCACCAG-3'
	R	5'GCTACGACGTGGGCTACAG-3'
IL-1β	F	5'-GCCTCGTGCTGTCGGACCCATAT-3'
	R	5'-GCTACGACGTGGGCTACAG-3'
IL-6	F	5'-ACAACCACGGCCTTCCCTACTT-3'
	R	5'-CACGATTTCCCAGAGAACATGTG-3'
IL-18	F	5'-GACTCTTGCGTRAACTTCAAGG-3'
	R	5'-CAGGCTGTCTTTTGTCAACGA-3'
IFN-β	F	5'-AACTCCACCAGCAGACAGTG-3'
	R	5'-TGAGGACATCTCCCACGTCA-3'
AdipoR1	F	5'-ACGTTGGAGAGTCATCCCGTAT-3'
	R	5'-TCTTGAAGCAAGCCCGAAAG-3'
AdipoR2	F	5'-AGCCTCTATATCACGGGAGCTG-3'
	R	5'-GCTGATGAGAGTGAAACCAGATGT-3'
GAPDH	F	5'-ACCACAGTCCATGCCATCAC-3'
	R	5'-TCCACCACCCTGTTGCTGTA-3'

icillin-streptomycin at 37°C in an incubator supplemented with 5% CO_2.

Isolation and culture of murine peritoneal macrophages

All the animal experiments were conducted under the guidelines issued by the Yeungnam University Research Committee for proper handling of laboratory animals. The experimental protocols were reviewed and approved by YU institutional animal care and use committee (YU-2017-005). Peritoneal macrophages were isolated as described previously (Pun *et al*., 2015). Briefly, 7- to 8-week old male C57BL/6N mice were intraperitoneally injected with 4% Brewer thioglycollate medium (1 ml) to induce accumulation of macrophages in the peritoneum region. On the third day of injection, the cells in the peritoneum were collected and washed with ice-cold Hank's balanced salt solution (HBSS, calcium and magnesium free). The cells were collected by centrifugation at 12,000 rpm for 5 min and resuspended in RBC lysis buffer to remove red blood cells. Cells were then mixed in RPMI 1640 media supplemented with 10% Fetal Calf Serum (FCS) and 1% penicillin-streptomycin and routinely cultured in an incubator at 37°C.

ELISA for TNF-α detection

The amount of secreted TNF-α was measured essentially described as previously (Oh *et al*., 2018). Briefly, cells were seeded at a density of 5×10^4 cells/well in 96-well plates. After treatments as indicated, the cell culture media were collected and used to determine the amount of secreted TNF-α using TNF-α ELISA kits (Biolegend) according to the manufacturer's instructions.

Table 2. Sequences of small interfering RNA used in transfection

Target gene	Primer	Nucleotide sequence
AUF1	F	5'-GGUGGACAUCAAAAUAGCU-3'
	R	5'-AGCUAUUUUGAUGUCCACC-3'
ZFP36L1	F	5'-GGAGAGCAAAAAUGUUGCU-3'
	R	5'-AGCAACAUUUUUGCUCUCC-3'
AdipoR1	F	5'-GACUUGGCUUGAGUGGUGU-3'
	R	5'-ACACCACUCAAGCCAAGUC-3'
AdipoR2	F	5'-AGAGUGAAGCCACCUGGUU-3'
	R	5'-AACCAGGUGGCUUCACUCU-3'

RNA isolation, reverse transcription (RT) and quantitative PCR (qPCR)

To measure mRNA expression levels of AUF1 and ZFP36L1, total RNA was extracted by Qiagen (Hilden, Germany) lysis solution in accordance with the manufacturer's instructions and reverse transcribed using Go Script reverse transcription system (Promega, Maison, WI, USA). Quantitative real-time PCR (qPCR) was then performed to amplify cDNA using Roche Light Cycler 2.0 (Mannheim, Germany) and an absolute QPCR SYBR green capillary mix system (Thermo Scientific, Waltham, MA, USA) at 95°C for 15 min, with 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 45 s. The mRNA levels of target genes were determined using the comparative threshold (C_t) method after the target mRNA is normalized to the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of primers used for the PCR amplification are listed in Table 1.

Transient gene silencing by transfection with small interfering RNAs (siRNAs)

RAW 264.7 macrophages were seeded at a density of 7×10^5 cells/dish in 35-mm dishes. Cells were transfected with either siRNA targeting specific genes or scrambled control siRNA using Hiperfect transfection reagent (Qiagen) according to the manufacturer's instructions. The efficiency of gene silencing of the target genes was monitored by Western blot analysis 24 h after transfection. The siRNA duplexes were synthesized by Bioneer (Daejeon, South Korea) and sequences for the siRNAs used are listed in Table 2.

Preparation of cellular extracts and western blot analysis

The expression levels of the genes of interest were determined as described previously (Kim *et al*., 2015). Briefly, RAW 264.7 macrophages or murine peritoneal macrophages were seeded at a density of 1×10^6 cells per well in 35-mm dish. After treatments as indicated, total proteins were extracted using RIPA lysis buffer containing phosphatase and protease inhibitors cocktail (Thermo Scientific). For immunoblot analysis, 20-30 μg of proteins were loaded, resolved by 10% SDS-PAGE, and transferred to Polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS)/Tween 20 for 1 h to block non-specific binding and incubated with the primary antibody in 3% bovine serum albumin (BSA) for overnight at 4°C. After washing with PBS/T, the membranes were incubated with the secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. The images of the blots were

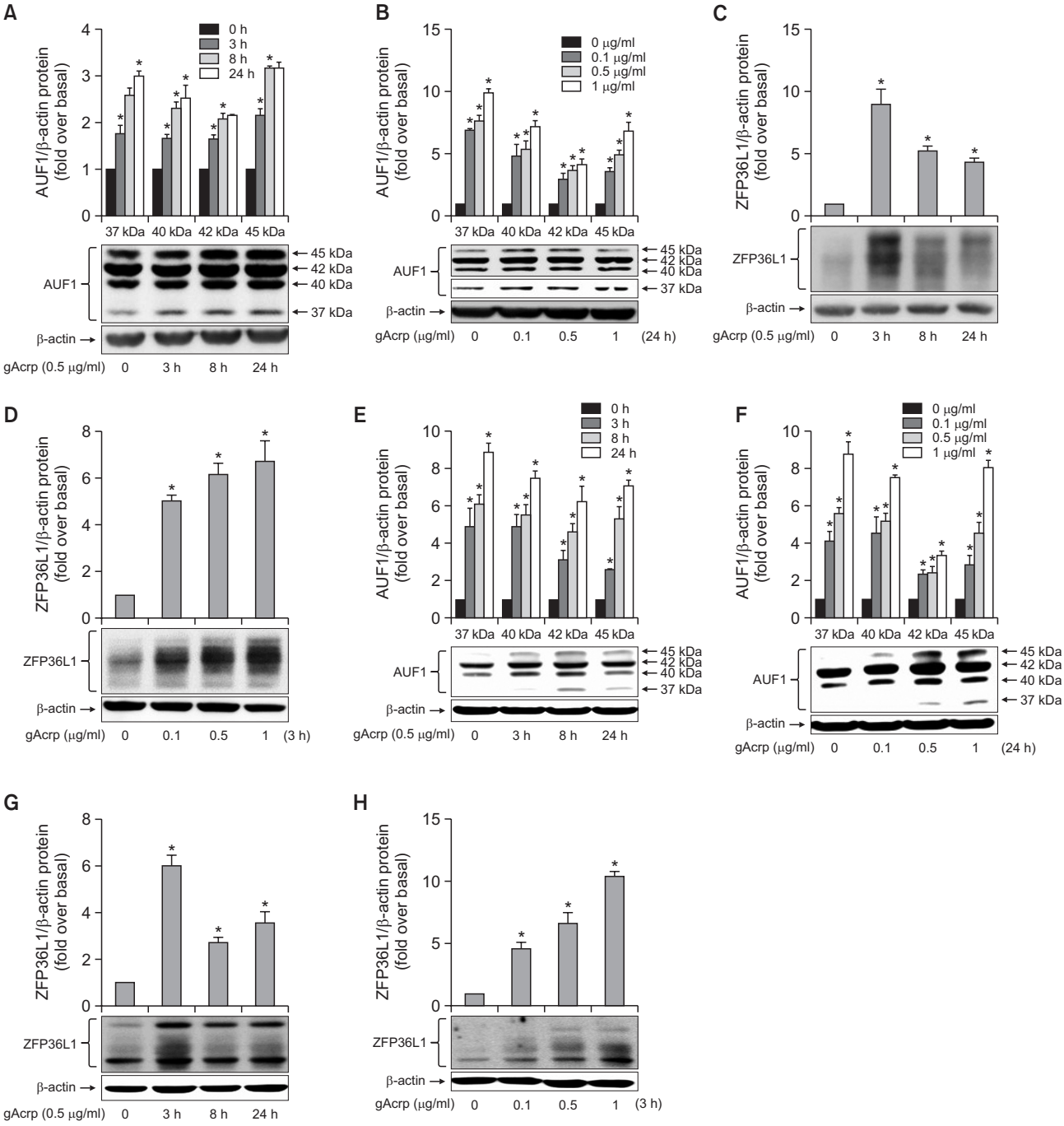

Fig. 1. Effects of globular adiponectin on AUF1 and ZFP36L1 protein expression in macrophages. (A, B) RAW 264.7 macrophages were incubated with gAcrp (0.5 μg/ml) for the indicated time periods (A) or concentrations of gAcrp for 24 h (B). Protein expression of AUF1 was measured by Western blot analysis. (C, D) RAW 264.7 macrophages were stimulated with gAcrp (0.5 μg/ml) for different time periods (C) or concentrations of gAcrp for 3 h (D). Protein expression of ZFP36L1 was measured by Western blot analysis. (E, F) Macrophages were isolated from murine peritoneum and the cells were stimulated with gAcrp (0.5 μg/ml) for the indicated time periods (E) or concentrations of gAcrp for 24 h (F). AUF1 protein expression level was measured by Western blot analysis. (G, H) Primary murine peritoneal macrophages were isolated and stimulated with gAcrp (0.5 μg/ml) for the indicated time periods (G) or indicated concentrations of gAcrp for 3 h. ZFP36L1 protein expression level was determined by Western blot analysis. In all the Western blot analysis, representative images from three independent experiments are shown and β-actin was used as internal loading control. Expression levels of AUF1 and ZFP36L1 were quantified by densitometric analysis after normalization to the level of β-actin and are shown above each Western blot image. Values are represented as the fold changes relative to the control cells (fold over basal) and are presented as mean ± SEM (n=3), *p<0.05; compared to the control cells.

finally captured using a chemiluminescent substrate solution and Fujifilm LAS-4000 mini (Fujifilm, Tokyo, Japan). The membranes were stripped and reprobed with β- actin antibody as internal loading control.

Confocal microscopic analysis

Cells were plated in 8-well chamber slides at a density of 5×10^4/well. After overnight incubation, cells were transfected with siRNA targeting AUF1, ZFP36L1, or scrambled siRNA and further co-transfected with the plasmid expressing LC3 tagged with enhanced green fluorescent protein (eGFP) using Fugene HD transfection reagent (Promega) according to the manufacturer's instruction. After treatment with gAcrp, cells were fixed with 4% paraformaldehyde solution. The formation of LC3 puncta, indicating autophagosome formation, was captured using an A1 Confocal Laser Microscope System (Nikon, Tokyo, Japan).

Immunoprecipitation (IP) assay

RAW 264.7 macrophages were plated in 100-mm dish at a density of 5.5×10^6 cells/dish. Cells were transfected with siRNA targeting AUF1, ZFP36L1, or scrambled siRNA, and further incubated with gAcrp for 24 h. Total proteins were extracted with IP lysis buffer that contains 150 mM NaCl, 50 mM HEPES, 1% NP-40, 5 mM EDTA, 1 mM PMSF and 0.5 mM DTT. Protein G Agarose (Thermo Scientific) was added to the cellular lysates and incubated for overnight at 4°C with gentle rocking. The protein in the supernatant (500 μg) was obtained and incubated with an anti-Beclin1 antibody (diluted 1:200) overnight at 4°C. The immune complexes were captured by incubation with protein G agarose for 4 h followed by centrifugation at 5,000 g for 5 min. The bead pellets containing immune complexes were washed IP lysis buffer, suspended in denaturation buffer, and heated at 95°C for 10 min. Finally, the samples were used for determination of Bcl-2 levels interacting with Beclin-1 by Western blot analysis using antibody against Bcl-2.

Statistical analysis

Values from at least three independent experiments are expressed as mean ± SEM. The data were analyzed by one-way ANOVA combined with Tukey's post hoc multiple comparison tests in Graph Pad Prism software version 5.01 (La Jolla, CA, USA). Differences between groups were considered to be significant at $p<0.05$.

RESULTS

Globular adiponectin induces increase in AUF1 and ZFP36L1 expression in macrophages

To investigate whether mRNA binding proteins are implicated in autophagy induction by gAcrp, we first examined the effects of gAcrp on the expression of AUF1 and ZFP36L1, which act as mRNA destabilizing proteins, in RAW 264.7 macrophages. As shown in Fig. 1, treatment with gAcrp induced significant increase in the expression of all four isoforms of AUF1 in a time- and dose-dependent manner (Fig. 1A, 1B). In addition, gAcrp also significantly increased ZFP36L1 protein expression in a dose- and time-dependent manner (Fig. 1C, 1D). Maximum effect on ZFP36L1 protein expression was observed at 3 h treatment and it was gradually decreased by prolonged exposure. Therefore, we selected 3 h incubation for the following experiments. The effects of gAcrp on the expression of AUF1 and ZFL36L1 in macrophages were further confirmed in primary macrophages isolated from peritoneum of mice. As anticipated, gAcrp treatment generated similar effects on the expression of AUF1 (Fig. 1E, 1F) and ZFP36L1 (Fig. 1G, 1H) in primary peritoneal macrophages. Taken together, these data clearly indicate that gAcrp induced significant increase in the expression of AUF1 and ZFP36L1 in macrophages.

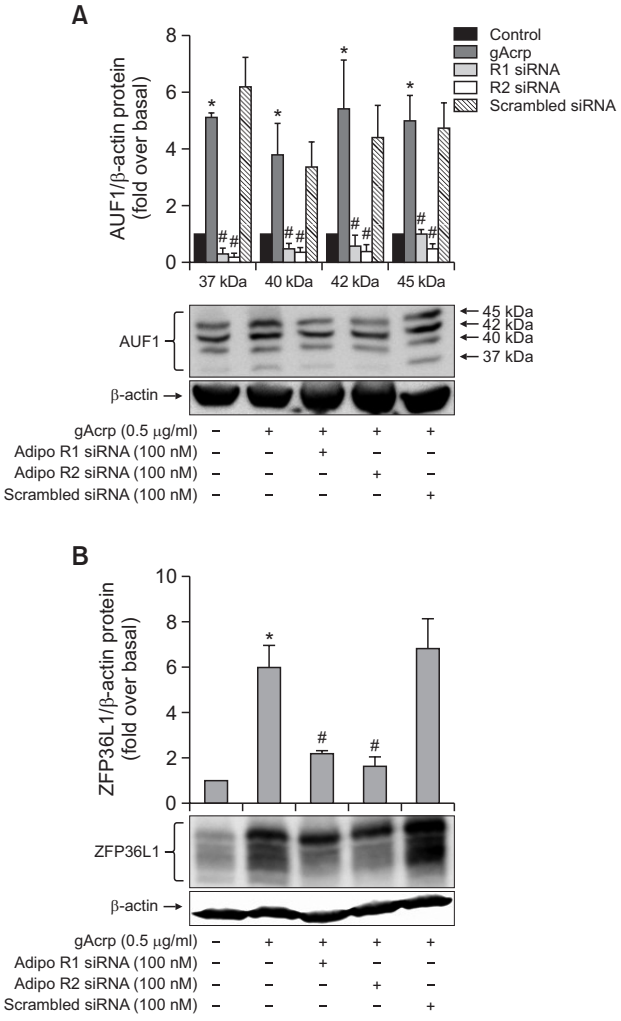

Fig. 2. Identification of the adiponectin receptor type involved in adiponectin-induced increase in AUF1 and ZFP36L1 expression in RAW 264.7 macrophages. RAW 264.7 macrophages were transfected with siRNA targeting AdipoR1, AdipoR2, or scrambled control siRNA. After 24 h incubation, cells were stimulated with gAcrp (0.5 μg/ml) for 24 h. Protein expression levels of AUF1 (A) and ZFP36L1 (B) were determined by Western blot analysis. Representative images from three independent experiments are shown along with the β-actin as an internal loading control. Expression levels of AUF1 and ZFP36L1 were quantified by densitometric analysis (normalized to the level of β-actin) and are shown above Western blot image. Values represent the fold change relative to control (fold over basal) and are expressed as mean ± SEM (n=3). *$p<0.05$ compared to the control cells; #$p<0.05$ compared to the cells treated with globular adiponectin, but not transfected with adiponectin receptor siRNA.

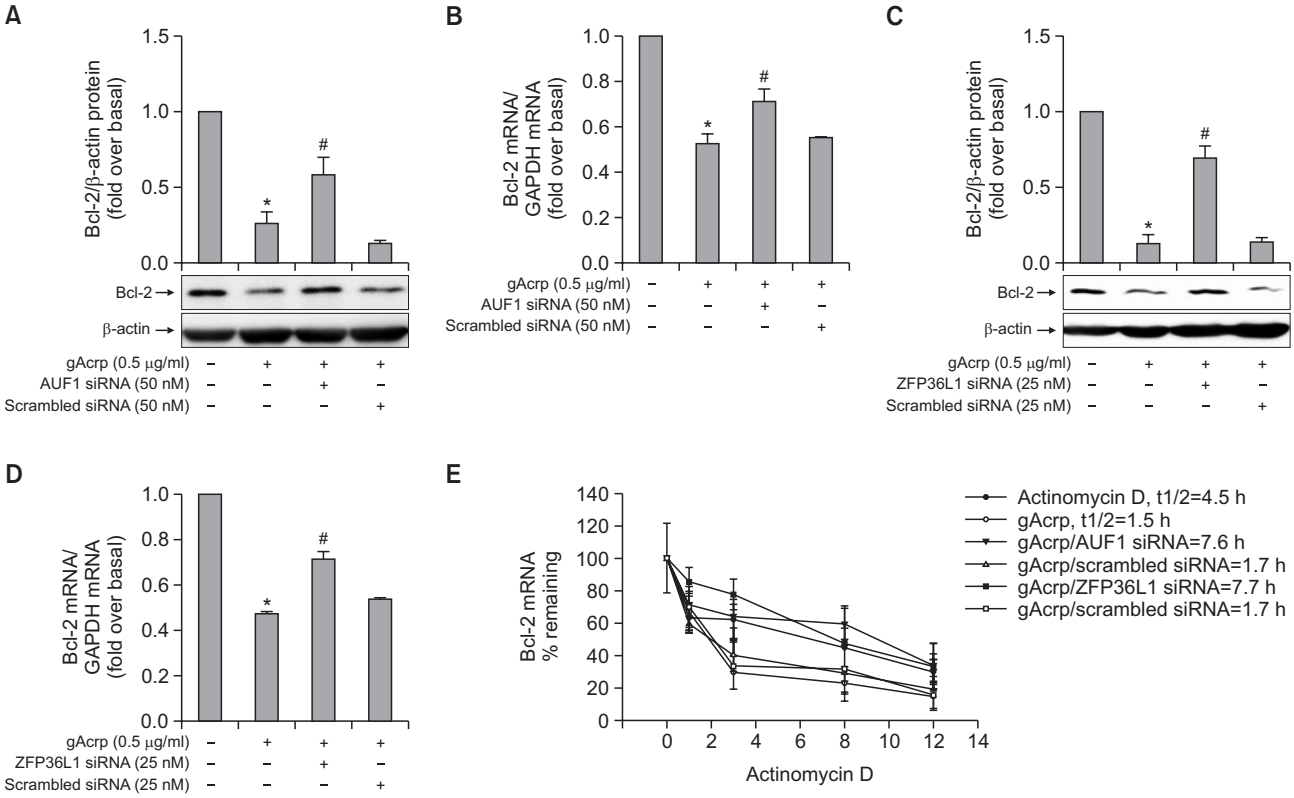

Fig. 3. Role of AUF1 and ZFP36L1 induction in Bcl-2 mRNA destabilization by globular adiponectin in RAW 264.7 macrophages. (A, B) RAW 264.7 macrophages were transfected with AUF1 siRNA or scrambled control siRNA followed by treatment with gAcrp (0.5 μg/ml) for 24 h. (A) Bcl-2 protein expression level was determined by Western blotting. Representative images from three separate experiments are shown along with β-actin as an internal loading control. (B) Bcl-2 mRNA expression level was determined by qPCR analysis. Values represent the fold changes relative to the control cells and are presented as mean ± SEM (n=3). *p<0.05 compared to the control cells; $^{\#}p$<0.05 compared to the cells treated with gAcrp, but not transfected with AUF1 siRNA. (C, D) RAW 264.7 macrophages were transfected with siRNA targeting ZFP or scrambled control siRNA followed by treatment with gAcrp (0.5 μg/ml) for 24 h. (C) Bcl-2 protein expression level was measured by Western blotting. Representative images from three separate experiments are shown along with β-actin as an internal loading control. (D) Bcl-2 mRNA expression level was determined by qPCR analysis, as indicated in methods section. Values represent the fold changes relative to the control cells and are presented as mean ± SEM (n=3). *p<0.05 compared to the control cells; $^{\#}p$<0.05 compared to the cells treated with gAcrp, but not transfected with ZFP36L1 siRNA. In the Western blot analysis, expression level of Bcl-2 was quantified by densitometric analysis and are shown above each Western blot image. Values are represented as the fold changes relative to the control cells and are presented as mean ± SEM (n=3), *p<0.05 compared to the control cells; $^{\#}p$<0.05 compared to the cells treated with globular adiponectin, but not transfected with AUF1 or ZFP36L1 siRNA. (E) After transfection with AUF1 siRNA or ZFP36L1 siRNA, the cells were pretreated with gAcrp for 24 h followed by further treatment with actinomycin D (2.5 mg/ml) for up to 12 h. Accumulation of Bcl-2 mRNA was determined by qPCR analysis and the half-life was calculated as percentage of the remaining Bcl-2 mRNA.

Both adiponectin receptor type 1 and type 2 mediate expression of AUF1 and ZFP36L1 by globular adiponectin in RAW 264.7 macrophages

Physiological responses by adiponectin are initiated by binding and activation of the specific receptors, mainly adiponectin receptor type 1 (adipoR1) and type 2 (adipoR2). We next identified the specific type of receptor involved in increase of AUF1 and ZFP36L1 expression by gAcrp and found that transfection of siRNA targeting adipoR1 and adipoR2 abrogated gAcrp-induced increase in AUF1 and ZFP36L1 expression (Fig. 2A, 2B). These findings indicate that both adipoR1 and adipoR2 signaling mediate increase in AUF1 and ZFP36L1 expression by gAcrp in macrophages.

AUF1 and ZFP36L1 induction plays a critical role in Bcl-2 mRNA destabilization by globular adiponectin in RAW 264.7 macrophages

We next examined if AUF1 and ZFP36L1 induction plays a functional role in the suppression of Bcl-2 expression by gAcrp. As shown in Fig. 3A, gAcrp treatment caused significant decrease in Bcl-2 protein expression in RAW 264.7 macrophages consistent with the previous reports. The inhibitory effect of gAcrp on Bcl-2 protein expression was markedly restored by transfection of siRNA targeting AUF1. Essentially similar effect on Bcl-2 mRNA level was observed by knock down of AUF1 siRNA (Fig. 3B), indicating that AUF1 induction contributes to the suppression of Bcl-2 expression by gAcrp. We further observed that gene silencing of ZFP36L1 also abrogated suppression of Bcl-2 expression by gAcrp both at mRNA and protein levels (Fig. 3C, 3D), suggesting that ZFP36L1 induction also plays a role in the modulation of Bcl-2

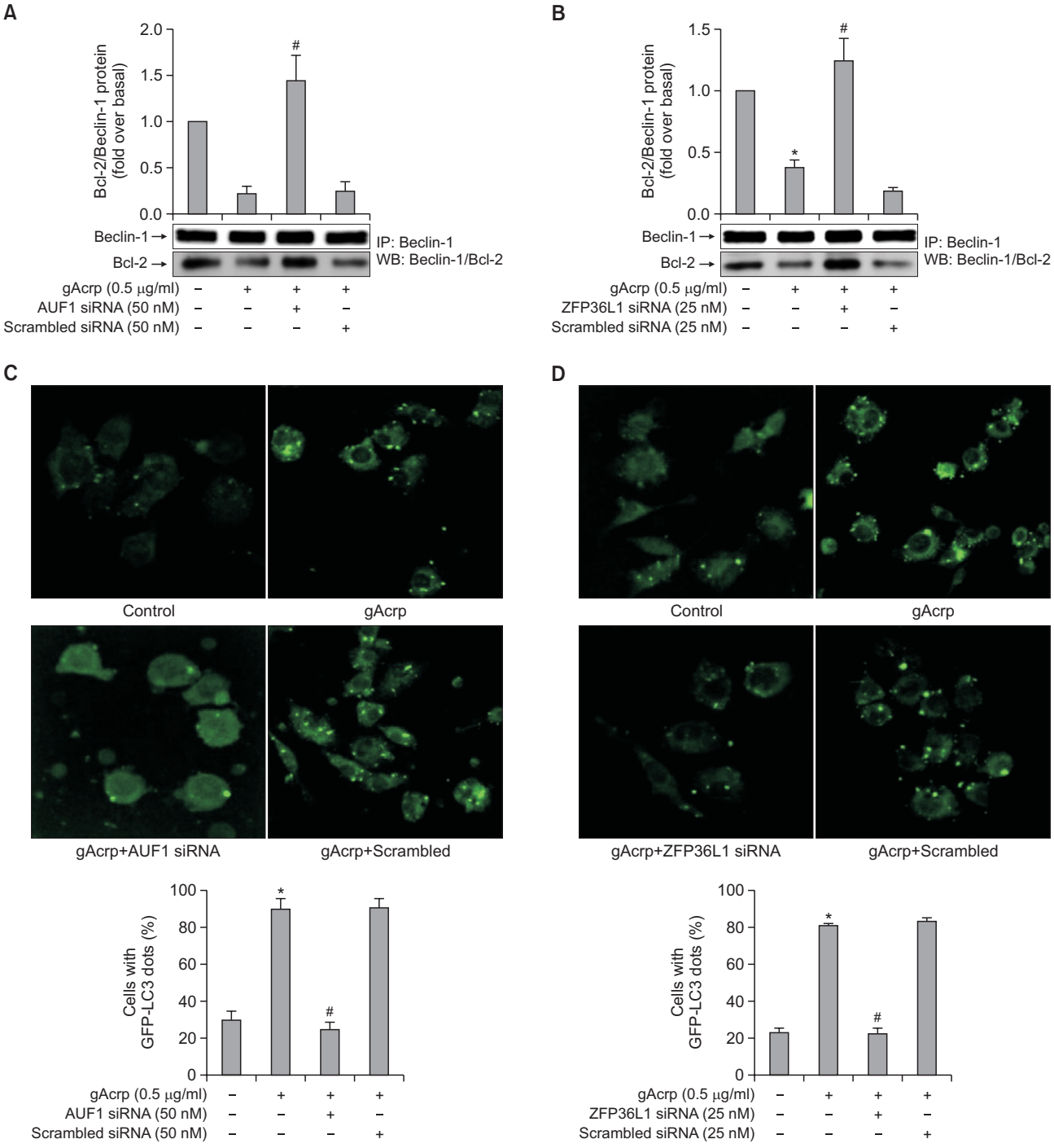

Fig. 4. Role of AUF1 and ZFP36L1 induction in the modulation of Bcl-2/Beclin-1 interaction and autophagosome formation by globular adiponectin in RAW 264.7 macrophages. (A, B) RAW 264.7 macrophages were transfected with AUF1 siRNA (A) or ZFP36L1 siRNA (B). After 28 h of transfection, cells were stimulated with gAcrp (0.5 μg/ml) for an additional 24 h. Association between Bcl-2 and Beclin-1 was analyzed by immunoprecipitation using anti-Beclin-1 antibody and further Western blot analysis with anti-Bcl-2 antibody. The representative images from three independent experiments are shown. Data from densitometric analysis are presented above each Western blot image. Values are shown as the fold increases relative to the control and are indicated as mean ± SEM (n=3). *p<0.05 compared to the control cells; #p<0.05 compared to the cells treated with gAcrp, but not transfected with AUF1 or ZFP36L1 siRNA. (C, D) RAW 264.7 macrophages were transfected with siRNA targeting AUF1, ZFP36L1, or scrambled siRNA. Cells were further co-transfected with eGFP-tagged LC3 plasmids (eGFP-LC3) followed by treatment with gAcrp for an additional 24 h. The autophagosome formation was analyzed by confocal microscopic analysis, indicated by LC3 puncta (green dots). The representative images from three independent experiments are shown along with the quantitation of LC3 dots in the lower panel.

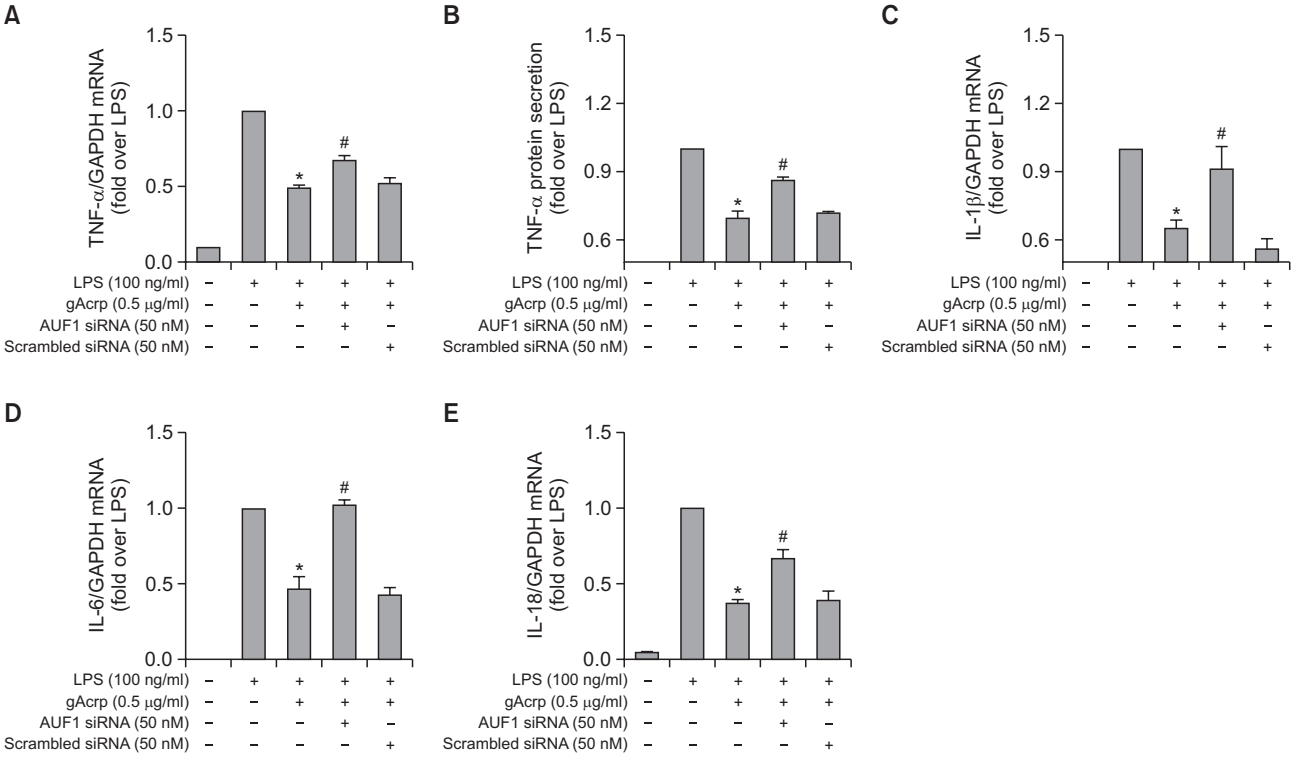

Fig. 5. Role of AUF1 in the suppression of inflammatory mediators expression by globular adiponectin in RAW 264.7 macrophages. (A, B) After transfection with AUF1 siRNA or scrambled siRNA, cells were treated with gAcrp (0.5 μg/ml) for 24 h followed by LPS treatment for an additional 2 h (A) or 4 h (B). (A) TNF-α mRNA levels were determined by qPCR analysis. (B) Cell culture media were collected and the amount of secreted TNF-α was measured by enzyme-linked immunosorbent assay (ELISA). (C-E) After transfection with AUF1 siRNA, cells were treated with gAcrp for 24 h followed by further stimulation with LPS for an additional 6 h. Messenger RNA levels of IL-1β (C), IL-6 (D) and IL-18 (E) were determined by qPCR. Values are expressed as mean ± SEM (n=3). *p<0.05 compared with the cells treated with LPS; #p<0.05 compared with the cells treated with LPS and gAcrp together.

expression by gAcrp.

We next confirmed whether AUF1 and ZFP36L1 induction is implicated in gAcrp-induced Bcl-2 mRNA destabilization. To examine the stability of Bcl-2 mRNA, cells were treated with actinomycin D, an inhibitor of *de novo* mRNA synthesis, followed by further treatment with gAcrp. The half-life of Bcl2 mRNA was then determined by qRT-PCR. As indicated in Fig. 3E, gAcrp treatment significantly reduced the half-life of Bcl-2 mRNA (half-life was 4.5 h in control cells vs 1.5 h by treatment with gAcrp), whereas gene silencing of AUF1 and ZFP36L1 caused restoration of the decreased Bcl-2 mRNA half-life (half-life was 7.6 h and 7.7 h, respectively). Collectively, these results indicate that AUF1 and ZFP36L1 induction play key roles in Bcl-2 mRNA destabilization by gAcrp in macrophages.

AUF1 and ZFP36L1 modulates interaction of Bcl-2 and Beclin-1 and autophagosome formation in RAW 264.7 macrophages

Decreased Bcl-2 levels may lead to reduction in Bcl-2/Beclin-1 association and suppression of Bcl-2 and Beclin-1 interaction enhances autophagosome formation. We next examined whether AUF1 and ZFP36L1 induction regulates association of Bcl-2 and Beclin-1 by immunoprecipitation followed by Western blot analysis. As shown in Fig. 4A, gAcrp treatment caused significant decrease in interaction of Bcl-2 with Beclin-1, but it was restored by gene silencing of AUF1. Knock down of ZFP36L1 also resulted in the similar effects on the interaction between AUF1 and ZFP36L1 modulated by gAcrp (Fig. 4B). These results indicate that AUF1 and ZFP36L1 induction plays a role in the modulation of Bcl-2/Beclin-1 interaction by gAcrp in macrophages. To further examine the role of AUF1 and ZFP36L1 in autophagy induction by gAcrp, we assessed if AUF1 and ZFP36L1 induction are implicated in gAcrp-induced autophagosome formation. For this, cells were transfected with the plasmid expressing LC3II tagged with GFP and autophagosome formation was measured by confocal microscopic analysis. Treatment with gAcrp resulted in significant increase in LC3 puncta formation. However, it was prominently abrogated by transfection with siRNA targeting AUF1 and ZFP36L1 without significant effects by transfection with scrambled control siRNA (Fig. 4C, 4D). These results collectively indicate that AUF1 and ZFP36L1 induction contributes to gAcrp-induced autophagy activation by modulating interaction of Bcl-2 and Beclin-1.

AUF1 induction mediates suppression of inflammatory mediators expression by globular adiponectin in RAW 264.7 macrophages

It has been shown that adiponectin inhibits expression of inflammatory mediators expression in macrophages via autophagy induction. Since AUF1 induction is implicated in gAcrp-induced autophagy induction, we next explored whether AUF1 plays a role in the anti-inflammatory responses by gAcrp. As shown in Fig. 5, gAcrp treatment significantly decreased LPS-

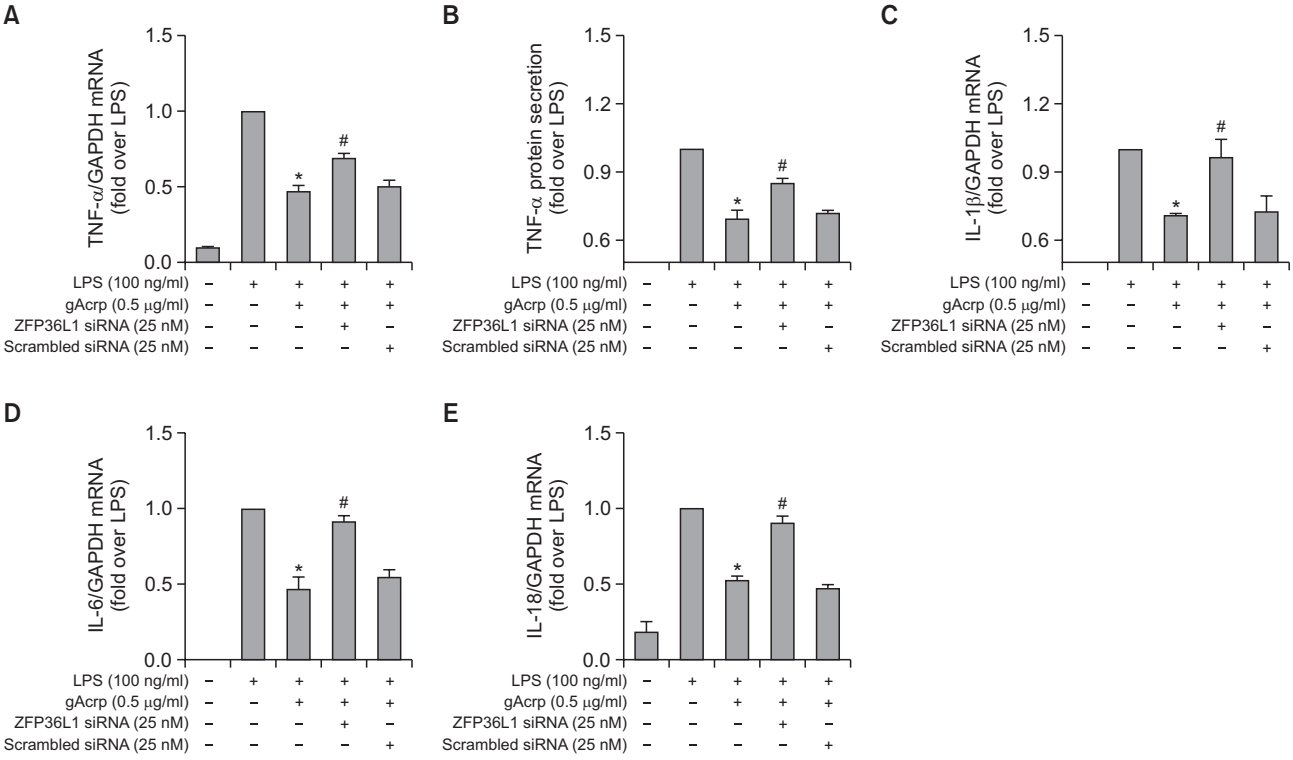

Fig. 6. Role ZFP36L1 induction in the suppression of inflammatory mediators expression by globular adiponectin in RAW 264.7 macrophages. (A, B) After transfection with ZFP36L1 siRNA or scrambled siRNA, cells were further treated with gAcrp for 24 h, followed by LPS treatment for an additional 2 h (A) and 4 h (B). TNF-α mRNA levels were determined by qPCR. (B) The amount of secreted TNF-α was determined by ELISA. (C-E) After transfection with ZFP36L1 siRNA, cells were treated with gAcrp for 24 h followed by treatment with LPS for an additional 6 h. The mRNA levels of IL-1β (C), IL-6 (D), and IL-18 (E) were determined by qPCR. Values were expressed as mean ± SEM (n=3). **p*<0.05 compared with the cells treated with LPS. #*p*<0.05 compared with the cells treated with LPS and gAcrp together.

stimulated TNF-α expression. However, it was returned to almost normal level by gene silencing of AUF1 both at mRNA and secretion levels (Fig. 5A, 5B). Moreover, transfection with AUF1 siRNA had similar effects on other inflammatory mediators, including IL-1β (Fig. 5C), IL-6 (Fig. 5D), and IL-18 (Fig. 5E) mRNA expression, clearly indicating that AUF1 induction plays a critical role in the suppression of anti-inflammatory mediators expression by gAcrp in macrophages.

ZFP36L1 induction is implicated in the suppression of inflammatory mediators expression by globular adiponectin in RAW 264.7 macrophages

We further examined the role of ZFP36L1 in the inhibition of inflammatory mediators expression by gAcrp and found that gene silencing of ZFP36L1 abrogated the suppressive effect of gAcrp on LPS-stimulated inflammatory mediators expression, including TNF-α both at mRNA (Fig. 6A) and protein secretion (Fig. 6B) level, IL-1β (Fig. 6C), IL-6 (Fig. 6D) and IL-18 (Fig. 6E) mRNA expressions, essentially similar effects by transfection with AUF1 siRNA, suggesting that ZFP36L1 induction also contributes to the anti-inflammatory responses by gAcrp in macrophages.

DISCUSSION

There has been increasing evidence that adiponectin possesses potent anti-inflammatory properties. However, its underlying mechanisms are still largely unknown. Autophagy, a highly conserved self-digestive process, is implicated in a number of physiological responses and accumulating evidence suggests that autophagy modulates inflammatory responses in a complicated manner (Deretic *et al*., 2013). We and others have demonstrated that autophagy induction mediates anti-inflammatory responses by adiponectin in various experimental conditions (Qi *et al*., 2014; Zhao *et al*., 2014; Kim *et al*., 2017). However, the molecular mechanisms by which adiponectin induces autophagy are not clearly understood. In the current study, we have demonstrated for the first time that AUF1 and ZFP36L1, acting as Bcl-2 mRNA destabilizing protein, play a key role in globular adiponectin-induced autophagy activation by modulating interaction of Bcl-2 with Beclin-1.

We have recently demonstrated that globular adiponectin (gAcrp) induces destabilization of Bcl-2 mRNA through TTP induction (Tilija Pun and Park, 2018). Stability of the mRNA is modulated by a number of AU-rich element mRNA binding proteins (AUBPs). In this study, we further investigated the roles of other mRNA binding proteins in Bcl-2 mRNA destabilization by gAcrp and found that gAcrp treatment induced significant increase in AUF1 and ZFP36L1 expression in macrophages (Fig. 1). In addition, gene silencing of AUF1 and ZFP36L1 restored suppression of Bcl-2 expression and decreased half-life of Bcl-2 mRNA by gAcrp (Fig. 3). These results collectively indicate the pivotal roles of AUF1 and ZFP36L1 in

the modulation of Bcl-2 expression by gAcrp. Furthermore, we showed that AUF1 and ZFP36L1 play functional roles in autophagy induction (Fig. 4) and suppression of inflammatory mediators expression (Fig. 5, 6) by gAcrp. In the modulation of inflammatory cytokines expression, knock-down of AUF1 and ZFP36L1 restored suppression of various inflammatory mediators, including TNF-α, IL-1β, IL-6, and IL-18. However, it did not significantly affect expression of IFN-β (Supplementary Fig. 2), indicating that AUF1 and ZFP36L1 induction is implicated in the modulation of inflammatory mediators expression by gAcrp in a gene selective manner. While previous studies have revealed that AUF1 and ZFP36L1 regulates stability of Bcl-2 mRNA (Ishimaru *et al*., 2010; Zekavati *et al*., 2014), biological roles of these mRNA binding proteins in physiological responses by adiponectin have not been explored. To the best of our knowledge, this is the first report to demonstrate the effects of gAcrp on the expression of AUF1 and ZFP36L1, and their roles in the modulation of autophagy induction and inflammatory responses by gAcrp.

In this study, gAcrp increased protein expression of AUF1 and ZFP36L1 both in murine macrophage cell line and primary peritoneal macrophages. However, we observed that gAcrp did not significantly affect mRNA levels of AUF1 and ZFP36L1 (Supplementary Fig. 1), implying that gAcrp-induced increase in AUF1 and ZFP36L1 expression would be determined at translational or post-translational level. With regards to the regulation of AUF1 expression, JNK signaling (Glaser *et al*., 2006) and NADPH oxidase (Zhou *et al*., 2008) have been shown to induce AUF1 expression. Interestingly, chaperone phosphorylation of Hsp27 promotes proteolysis of AUF1 in a proteasome-dependent fashion (Knapinska *et al*., 2011), suggesting that proteasomal degradation might be a possible mechanism for the modulation of AUF1 expression by gAcrp. In contrast to AUF1, to our knowledge, little is known about the mechanisms underlying ZFP36L1 expression, while the functional role of ZFP36L1 has been an intense area of research for the modulation of various target mRNA stability. Although we did not investigate the mechanisms underlying the increases of AUF1 and ZFP36L1 expression by gAcrp in this study, further studies seeking to the mechanisms by which gAcrp induces AUF1 and ZFP36L1 expression would provide further insights for anti-inflammatory responses by adiponectin.

Autophagic process is coordinately regulated by various genes-related with autophagy (ATGs). Among various ATGs, Beclin-1 plays an important role in the initiation of autophagy induction through making a complex formation with class III PI3K, also known as Vps34, which contributes to nucleation of the phagophore and maturation of the autophagosome (Funderburk *et al*., 2010). Interestingly, interaction of Bcl-2 with Beclin-1 through BH-3 domain impedes autophagy inducing activity of Beclin-1 by interfering with the formation of the Beclin-1/Vps34 complex. Therefore, interaction of Bcl-2 with Beclin-1 is considered a critical step for the modulation of autophagy induction (Levine *et al*., 2008). Expression and activity of Bcl-2 are modulated by different mechanisms. For examples, estrogen receptor-α regulated transcription of Bcl-2 via either protein-protein interaction with c-Jun or by direct binding to estrogen responsive element in c-Jun (Shibata *et al*., 2005). In addition, oxidative stress induced ubiquitination of Bcl-2 (Li *et al*., 2017). Moreover, post-transcriptional regulation of Bcl-2 mRNA plays an important role in the determination of Bcl-2 expression level (Otake *et al*., 2004). Stability of the mRNA is regulated by binding of group of proteins to ARE elements. Bcl-2 mRNA contains several motifs for ARE binding protein (AUBP) and each AUBP has a distinct effect on mRNA stability (Garneau *et al*., 2007; Nakayama *et al*., 2016). For example, binding of HuR and nucleolin induces Bcl-2 mRNA stabilization (Ishimaru *et al*., 2009, 2010), while binding of AUF1 and ZFP36L1 results in Bcl-2 mRNA destabilization (Ishimaru *et al*., 2010), indicating that modulation of mRNA stability may be a critical factor for determining the expression level of Bcl-2.

Adiponectin has been shown to decrease Bcl-2 expression in macrophages and cancer cells (Mistry *et al*., 2008; Tilija Pun and Park, 2018). We have previously shown that TTP induction contributes to adiponectin-induced Bcl-2 mRNA destabilization in macrophages (Tilija Pun and Park, 2018). To date, in addition to TTP, a number of different types of AUBPs have been identified and modulates mRNA decay in a gene-selective manner. For example, AUF1 binds with ARE at 3'-UTR region of the target mRNA and induces degradation of mRNA by rapid shortening of poly-A tail followed by exosomal degradation (Raineri *et al*., 2004; Funakoshi *et al*., 2007; Garneau *et al*., 2007). Four isoforms of AUF1 ($p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$, and $p45^{AUF1}$) resulting from differential splicing process have been identified and each isoform exhibits varied ARE-binding affinities (Wagner *et al*., 1998). Therefore, relative expression levels of individual isoforms, rather than the total levels of AUF1, would determine the net stability of the target mRNA (Raineri *et al*., 2004). Herein, we observed that gAcrp significantly increased all four isoforms of AUF1. Although we didn't further address to the role of each AUF1 isoform in this study, further studies seeking to identify the specific AUF1 isoform involved would provide the further insights into the mechanisms for the modulation of Bcl-2 expression by gAcrp.

ZFP36 protein family members, consisting of three members, including ZFP36 (called as TTP), ZFP36L1, and ZFP36L2, also binds to ARE motif in target mRNAs and promotes their degradation (Blackshear, 2002). We have previously shown that ZFP36L (TTP) plays a role in suppressing Bcl-2 expression by gAcrp. In this study, we further examined whether ZFP36L1 plays a role in the regulation of Bcl-2 expression by gAcrp and found that ZFP36L1 induction also contributes to Bcl-2 mRNA destabilization. This is the first report to demonstrate the effect of adiponectin on ZFP36L1 expression and its role in gAcrp-induced autophagy activation and regulation of inflammatory responses. ZFP36L1 has also been shown to play a critical role in apoptosis induction in malignant B cells (Zekavati *et al*., 2014). Given the previous reports that adiponectin potently suppresses tumor growth (Dalamaga *et al*., 2012), further studies to investigate the role of ZFP36L1 in the modulation of tumor growth by gAcrp would also be worth considering.

Adiponectin exerts physiological responses by binding to its receptors. To date, adiponectin receptor type 1 (adipoR1) and type 2 (adipoR2) have been identified as main adiponectin receptors. While T-cadherin was reported to play a role in adiponectin-mediated cardioprotection in mice and acts as an adiponectin receptor (Denzel *et al*., 2010), its detailed biological roles as adiponectin receptor remain elusive. AdipoR1 and adipoR2 share 96.8% of identity in human and 95.2% in mice and generate similar metabolic actions by adiponectin. However, interestingly, adipoR1 and adipoR2 signaling are dependent on the type of tissues and other experimental conditions. For instance, gAcrp modulated expression of p62, an adaptor

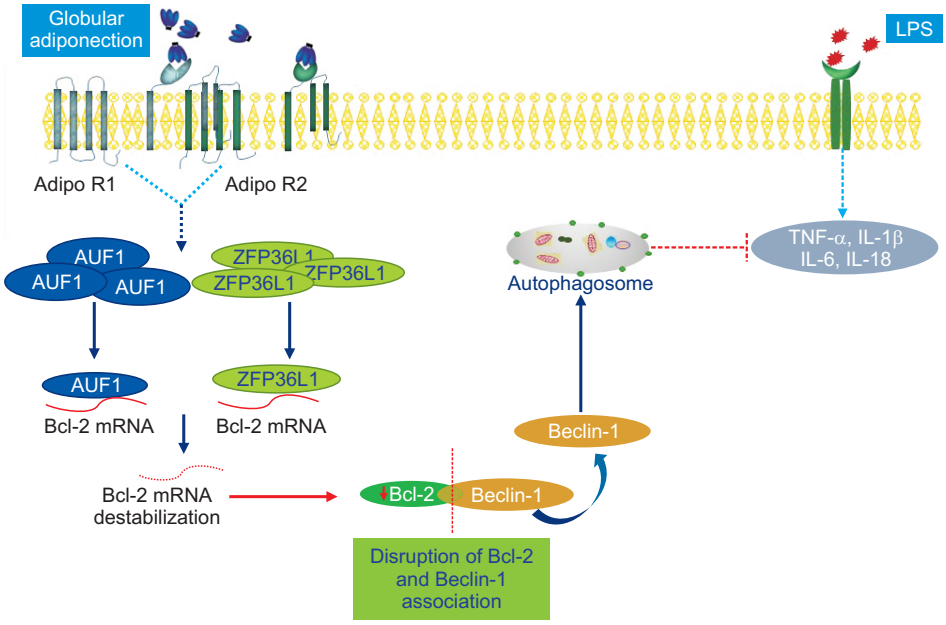

Fig. 7. Proposed model for the involvement of AUF1 and ZFP36L1 in autophagy induction by globular adiponectin leading to the suppression of inflammatory cytokines expression in macrophages. Globular adiponectin suppresses inflammatory mediators expression via autophagy induction in macrophages. In the present study, we examined the molecular mechanisms underlying autophagy induction by globular adiponectin. Globular adiponectin induces increase in AUF1 and ZFP36L1 expression involving both adipoR1 and adipoR2 signaling. AUF1 and ZFP36L1 bind with Bcl-2 mRNA and induces destabilization of Bcl-2 mRNA. As a consequence, Bcl-2 expression level is decreased and interaction between Bcl-2 and Beclin-1 is inhibited. Bcl-2 acts as an autophagy inhibitory protein and dissociation of Beclin-1 from Bcl-2 facilitates autophagosome formation. Moreover, AUF1 and ZFP36L1 induction play critical roles in the suppression of LPS-stimulated various inflammatory cytokines expression by gAcrp. Detailed signaling mechanisms underlying AUF1 and ZFP36L1 induction by gAcrp remain to be determined.

molecule involved in autophagy induction, via adipoR2 signaling (Tilija Pun and Park, 2017), whereas adipoR1 signaling play a predominant role in the phosphorylation of DAPK1 and TTP induction by gAcrp (Tilija Pun and Park, 2018). In this study, we found that transfection of siRNA targeting both adipoR1 and adipoR2 significantly attenuated gAcrp-induced increase in AUF1 and ZFP36L1 expression (Fig. 2), indicating that both adipoR1 and adipoR2 signaling contribute to AUF1 and ZFP36L1 induction by gAcrp. Collectively, these reports support the notion that the predominant role of adiponectin receptors (adipoR1 or adipoR2) would be determined by the cellular environments. We couldn't address the mechanisms for these contradictory results and detailed mechanisms underlying what determines the differential roles of adiponectin receptors remain to be further investigated.

In conclusion, the data presented in this study have demonstrated that AUF1 and ZFP36L1 induction plays a pivotal role in autophagy induction and suppression of inflammatory mediators expression by globular adiponectin by inducing destabilization of Bcl2 mRNA (Fig. 7). This is the first report to demonstrate the effect of globular adiponectin on AUF1 and ZFP36L1 expression in macrophages and further their roles in gAcrp-induced Bcl2 mRNA destabilization and autophagy induction. Given that autophagy induction plays a critical role in anti-inflammatory responses by adiponectin, AUF1 and ZFP36L1 would be promising targets for the modulation of inflammatory responses by adiponectin.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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