Autophagy positively regulates Wnt signaling in mice with diabetic retinopathy

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Abstract. Diabetic retinopathy (DR) is a microvascular complication of diabetes. Aberrant Wnt signaling activation plays a pathological role in DR. However, the underlying mechanisms of aberrant Wnt signaling in DR remain unknown. Autophagy has been reported to be involved in the pathophysiology of DR. The present study aimed therefore to investigate the regulatory effects of autophagy on Wnt signaling in DR. Wnt signaling was activated in the retina of db/db mice combined with an increase in the expression of the autophagic proteins microtubule-associated protein 1A/1B-light chain 3 and beclin-1 and a decrease in the expression of the autophagic protein P62. Inhibition of autophagy by 3-methyladenin decreased Wnt signaling in diabetic retinas, indicating a potential association between Wnt signaling and autophagy. Rapamycin, an autophagy inducer, upregulated Wnt signaling in the retina of normal C57BL/6J mice. In cultured Müller cells, rapamycin induced autophagy and activated Wnt signaling, while chloroquine, an autophagy inhibitor, inhibited autophagy and downregulated Wnt signaling, suggesting that autophagy could regulate Wnt signaling in mice retina and retinal cells. In summary, this study demonstrated that autophagy may positively regulate Wnt signaling in diabetic retinas, indicating a potential mechanism of Wnt signaling upregulation in DR and a possible novel therapeutic target of DR.

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Introduction

Diabetic retinopathy (DR) is a microvascular complication of diabetes and a major cause of vision loss in Western countries. The incidence of diabetes worldwide will reach >366 million by the end of 2030 (1). It is reported that nearly all patients with type 1 diabetes will develop some manifestation of DR, and that ~50-80% of patients with type 2 diabetes will have DR within 20-25 years of developing the condition (2). With the growing population of individuals with diabetes, the incidence of DR will increase gradually, which poses a major threat to the global population and a costly burden to health care systems. The exact mechanism of DR is unclear, and the current treatments for diabetic retinopathy, such as anti-vascular endothelial growth factors and laser coagulation, have limited efficacies for patients with DR (3). Wnt signaling is an evolutionally conserved signaling pathway that serves essential roles in tissue development and adult homeostasis (4). Dysregulated Wnt signaling plays pathogenic roles in a variety of human diseases, including DR (5,6). Upregulated Wnt signaling increases retinal inflammation and secretion of vascular endothelial growth factor (VEGF) and results in retinal neovascularization in diabetic animal models (5,7). Blockage of the Wnt signaling pathway has anti-angiogenic and anti-inflammatory effects in DR (8,9). However, the underlying mechanism of aberrant Wnt signaling activation in DR remains to be determined.

Autophagy is a catabolic process in which cellular components are degraded by lysosomes (10). Autophagy is essential for retinal development and vision formation (11). Previous studies have demonstrated that autophagy is involved in the pathogenesis of DR (12-14). For example, retinal autophagy is upregulated in diabetic human and mice, where it has been demonstrated to play dual roles in DR, a protective role in mild stress [induced by 50 mg/l in *vitro*-modified heavily-oxidized glycated LDL (HOG-LDL)] in cultured human retinal capillary cells and a detrimental role in severe stress (200 mg/l HOG-LDL) (13). High glucose increases endoplasmic reticulum stress and induces autophagy in Müller cells (14).

The interplay between autophagy and Wnt signaling has been previously reported. For example, Kallistatin, which is a

Wnt signaling inhibitor, can induce apoptosis and autophagy in breast cancer cells, thereby reducing the rates of tumor angiogenesis and vascular growth (15). A previous study on lung cancer demonstrated that the tumor suppressor candidate 3 protein could induce expression of autophagy related proteins, which activates Wnt/β-catenin signaling in human non-small lung cancer cells (16). The association between autophagy and Wnt signaling in cancer field is well established (17). However, whether autophagy might regulate Wnt signaling in DR has not yet been investigated.

The present study aimed to investigate the possible mechanism of abnormal Wnt signaling in DR and hypothesized that autophagy may have a regulatory effect on Wnt signaling in DR. Meanwhile, the investigation of autophagy in DR may shed a light on how autophagy was changed in DR and the possible function of autophagy in the retina.

Materials and methods

Animals. Heterozygous BKS.Cg-Dock7m$^{+/+}$ Leprdb/J mice (stock no. 000642) were purchased from Jackson Laboratory. Homozygous db/db mice and control mice were obtained by crossing heterozygous db/+ mice. C57BL/6J [wild-type (WT)] mice were purchased from the Laboratory Animal Center of Xiamen University. All animals were housed in a specific-pathogen-free facility and maintained in 12-h light/dark cycle. All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology 'Statement for the Use of Animals in Ophthalmic and Vision Research'. The animal protocols were approved by the Xiamen University Experimental Animal Ethics Committee (approval no. XMULA20190022).

Cell culture. The rat Müller (rMC-1) cell line was a gift from Dr Vijay Sarthy of Northwestern University (Evanston, IL, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin streptomycin solution (all from Gibco; Thermo Fisher Scientific, Inc.) and placed at 37˚C in a humidified incubator containing 5% CO_2.

Induction/inhibition of autophagy in vitro. rMC-1 cells were plated in a 6-well plate at a density of 5x10^5/well. After 24 h, rMC-1 cells were treated with rapamycin (4 μM) which was dissolved in DMSO (both from Sigma-Aldrich; Merck KGaA) or with the same amount of DMSO as a vehicle for 24 h. To suppress the autophagy in rMC-1 cells, chloroquine (1 μM; Sigma-Aldrich) was used to treat rMC-1 cells for 6 h and DMSO was used as vehicle control.

Induction/inhibition of autophagy in vivo. An autophagy inhibitor 3-methyladenin (3-MA; Sigma-Aldrich; Merck KGaA; 25 mg/kg) was dissolved in DMSO and intraperitoneally injected into db/db mice (5 months old; average weight, 54 g) every other day for 30 days (18), while the same amount of DMSO was used as vehicle control in 5-month-old db/db mice with the similar manner. Similarly, rapamycin (Sigma-Aldrich; Merck KGaA; 25 mg/kg) was dissolved in DMSO and intraperitoneally injected into C57 mice (3 months old; average weight, 28 g) once a day for 30 days to induce autophagy, while DMSO was used as vehicle control (19). At least six mice were used in each group. Mice were sacrificed using carbon dioxide (30% cage volume/min) and the retinas were collected for subsequent experiments and stored in a -80˚C freezer for further use.

Western blotting. The treated rMC-1 cells and retinal tissues were lysed by radioimmunoprecipitation assay (RPAP) buffer (Sigma-Aldrich; Merck KGaA) and the total protein concentrations were measured by bicinchoninic acid assay. Each amount of protein (25 μg per lane) was resolved by electrophoresis through 12% Tris-glycine SDS polyacrylamide gel and electrotransferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween (TBST)-20 for 2 h at room temperature. The membrane was incubated with the primary antibodies (dilution 1:1,000) at 4˚C overnight. The primary antibodies against microtubule-associated protein 1A/1B-light chain 3B (LC3B; cat. no. ab48394), P62 (cat. no. ab56416) and VEGF (cat. no. ab46154) were obtained from Abcam. The primary antibodies against Beclin-1 (cat. no. sc-48341) and β-catenin (cat. no. sc-7199) were purchased from Santa Cruz Biotechnology, Inc. Primary antibodies against non-phosphorylated-β-catenin (n-p-β-catenin; cat. no. 4270S) and β-actin (cat. no. 4970) were purchased from Cell Signaling Technology, Inc. After three washes with TBST, the membranes were incubated with 1:5,000 dilution of a HRP-conjugated goat anti-mouse antibody (Sigma-Aldrich, cat. no. A4416) or an HRP-conjugated anti-rabbit antibody (Sigma-Aldrich; Merck KGaA; cat. no. A6154) in TBST containing 1% dry milk for 1 h. After four washes with TBST, the bands were detected using super ECL detection Reagent (Shanghai Yeasen Biotechnology Co., Ltd.) and the band intensities were semi-quantified by densitometry using Quantity One-1D analysis software (Bio-Rad Laboratories, Inc.).

Statistical analysis. GraphPad Prism version 7 (GraphPad Software, Inc.) was used for statistical analyses. Data were presented as the means ± standard error of the mean. Comparison between two groups was performed using Student's t-test. At least three independent measurements were conducted for each assay. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Wnt signaling is activated and autophagy is stimulated in retinas from db/db mice. Previous studies reported that Wnt signaling is abnormally activated in diabetic retinas and serves a pathogenic role in DR (7,20). In the present study, db/db mouse, a genetic type 2 diabetic animal model, was selected as animal model of diabetic retinopathy (21). The protein expression of non-p-β catenin and β-catenin was evaluated by western blotting (Fig. 1A) and non-p-β catenin expression was found to be significantly increased in db/db retinas (Fig. 1B), while β-catenin expression was unchanged (Fig. 1C). Protein expression of the target gene of Wnt signaling VEGF was also significantly upregulated in db/db retinas compared with those in control retinas (Fig. 1D and E). Furthermore, the expression of the autophagic proteins LC3BI, LC3BII (Fig. 1F), Beclin-1 (Fig. 1H)

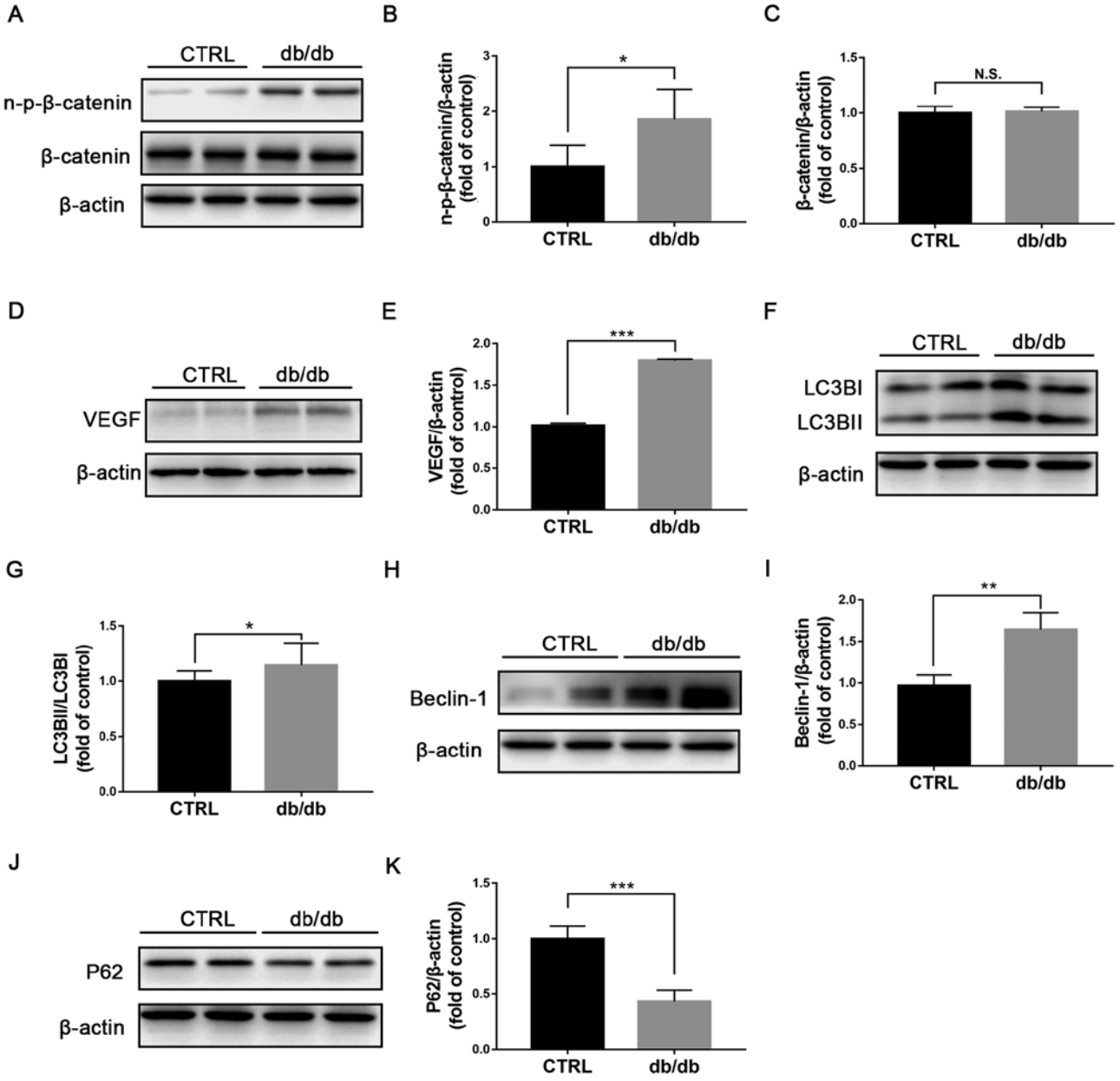

Figure 1. Positive relationship between Wnt signaling pathway and autophagy in db/db retinas. (A) Expression of n-p-β-catenin and β-catenin was evaluated by western blotting in the retinas of WT and db/db mice. (B) n-p-β-catenin and (C) β-catenin were quantified by densitometry and normalized to β-actin levels (n=6). (D and E) Expression of the downstream target gene of Wnt signaling VEGF was evaluated and quantified in WT or db/db retinas (n=6). Expression of (F) LC3BI and LC3BII (LC3BI/II), (H) Beclin-1 and (J) P62 was evaluated by western blotting. (G) Level of LC3BI/II was quantified by densitometry and the band intensity ratio of LC3BII to LC3BI (LC3BII/LC3BI) was calculated and compared (n=6). Levels of (I) Beclin-1 and (K) P62 were quantified by densitometry and normalized to β-actin levels (n=6). *P<0.05, **P<0.01 and ***P<0.001. n-p-β-catenin, non-phosphorylated-β-catenin; N.S., non-significant; db, diabetic; LC3B, microtubule-associated protein 1A/1B-light chain 3; WT, wild-type; VEGF, vascular endothelial growth factor.

and P62 (Fig. 1J) was determined by western blotting. The ratio LC3BII/LC3BI (Fig. 1G) and expression of Beclin-1 (Fig.1I) were significantly elevated, whereas P62 expression (Fig. 1K) was significantly reduced in db/db retinas compared with the control group, indicating an induction of autophagy in the retina of db/db mice. Taken together, these findings indicated that Wnt signaling and autophagy in db/db retinas may be related.

Autophagy inhibition downregulates Wnt signaling in db/db retinas. To further clarify the relationship between autophagy and Wnt signaling, db/db mice were treated with 3-MA, a selective PI3K inhibitor that inhibits autophagy (22). After one-month treatment, the ratio LC3BII/LC3BI (Fig. 2A and B) and Beclin-1 expression (Fig. 2C and D) were significantly decreased, whereas P62 expression was significantly increased in the retinas of db/db mice treated with 3-MA compared with those treated with vehicle (Fig. 2C and E), suggesting a successful inhibition of autophagy in db/db retinas. Furthermore, the protein expression of n-p-β-catenin was significantly decreased in the retinas of 3-MA-treated db/db mice compared with the retinas of vehicle-treated db/db mice (Fig. 2F and G), and the protein expression of β-catenin (Fig.2G and H) was unchanged. Protein expression of VEGF

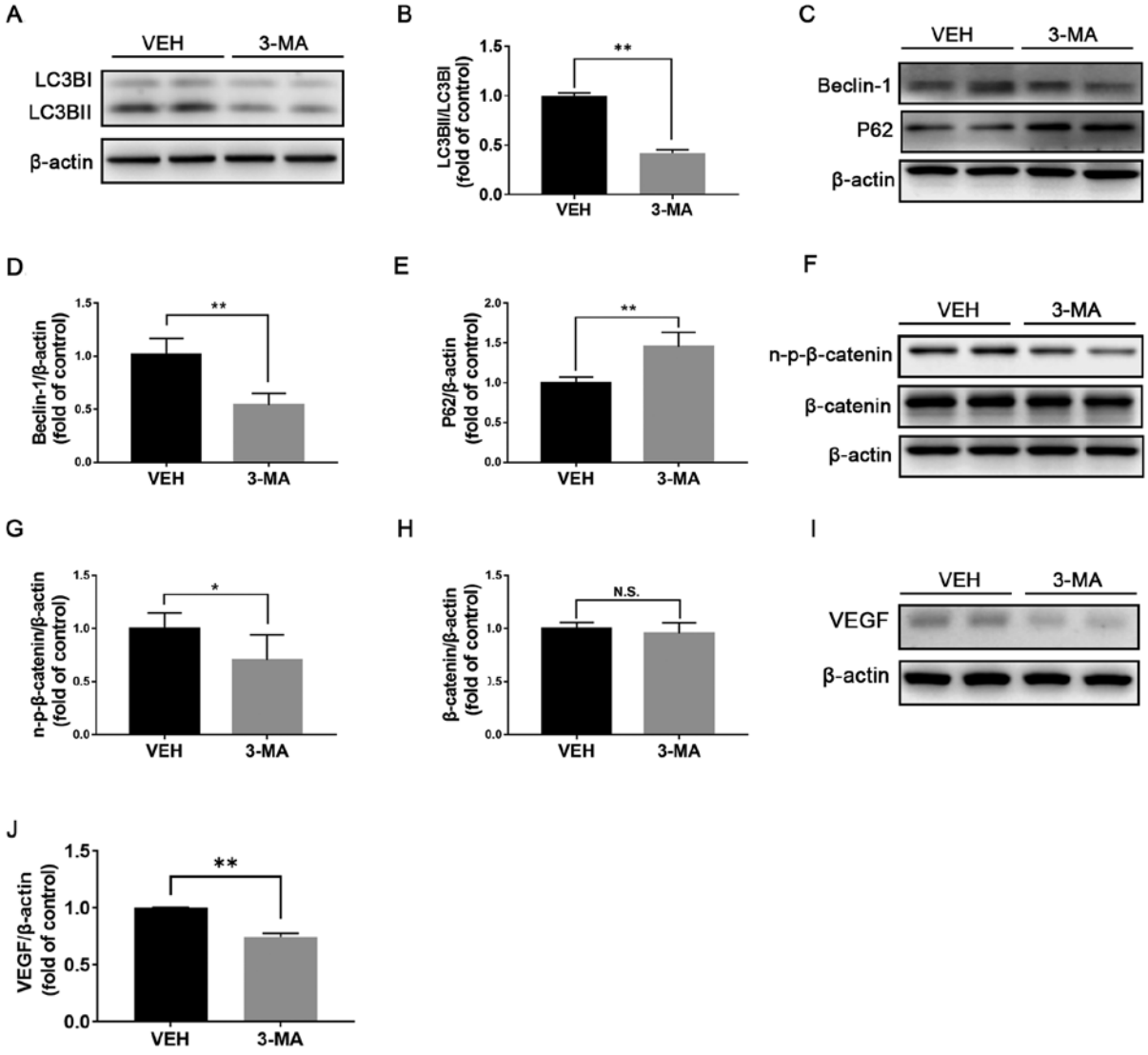

Figure 2. Inhibition of autophagy downregulates Wnt signaling in db/db retinas. Representative images of western blotting for (A) LC3BI/II, (C) Beclin-1, P62, (F) n-p-β-catenin and β-catenin in db/db mice treated with VEH or 3-MA. (B) Protein levels of LC3BI/II were quantified by densitometry and LC3BII/LC3BI was calculated and compared (n=6). Protein levels of (D) Beclin-1, (E) P62, (G) n-p-β-catenin and (H) β-catenin were quantified by densitometry and normalized to β-actin levels (n=6). (I and J) Protein expression of the downstream target gene of Wnt signaling VEGF was evaluated and quantified in db/db mice treated with VEH or 3-MA (n=6). *P<0.05 and **P<0.01. n-p-β-catenin, non-phosphorylated-β-catenin; N.S., non-significant; LC3B, microtubule-associated protein 1A/1B-light chain 3; VEGF, vascular endothelial growth factor; VEH, vehicle; 3-MA, 3-methyladenin; db, diabetic.

was significantly decreased in the retinas of 3-MA-treated db/db mice (Fig. 2I and J). In addition, the weight and blood glucose levels of db/db mice were not significantly changed before or after treatment of 3-MA (Table SI). Taken together, these results demonstrated that inhibition of autophagy may be accompanied with inactivation of Wnt signaling, indicating a regulatory effect of autophagy on Wnt signaling.

Induction of autophagy activates Wnt signaling in WT retinas. To further investigate the effect of autophagy on the modulation of Wnt signaling, rapamycin was used to induce autophagy in normal WT retinas and the expression of proteins associated with Wnt signaling was evaluated by western blotting. Following administration of rapamycin, the ratio LC3BII/LC3BI (Fig. 3A and B) and expression of Beclin-1 (Fig.3C and D) were significantly increased, while P62 expression was significantly decreased in the retinas (Fig. 3E and F), suggesting the successful induction of autophagy in the retinas of WT mice. In addition, protein expression of n-p-β-catenin (Fig.3G and H) was significantly increased in the retinas of rapamycin-treated WT retinas compared with those of vehicle-treated WT mice and the expression of β-catenin was unchanged (Fig. 3G and I). Furthermore, protein expression of VEGF was significantly increased in rapamycin-treated retinas (Fig. 3J and K). Taken together, these findings suggested that induction of autophagy may activate Wnt signaling in the retina.

Induction of autophagy upregulates Wnt signaling in rMC-1 cells. Müller cells are the primary glial cells that contribute to the pathological responses in DR (23). A previous study demonstrated that inactivation of Wnt signaling in Müller cells attenuates inflammatory responses in diabetic retinas (24). To determine on which cells autophagy may

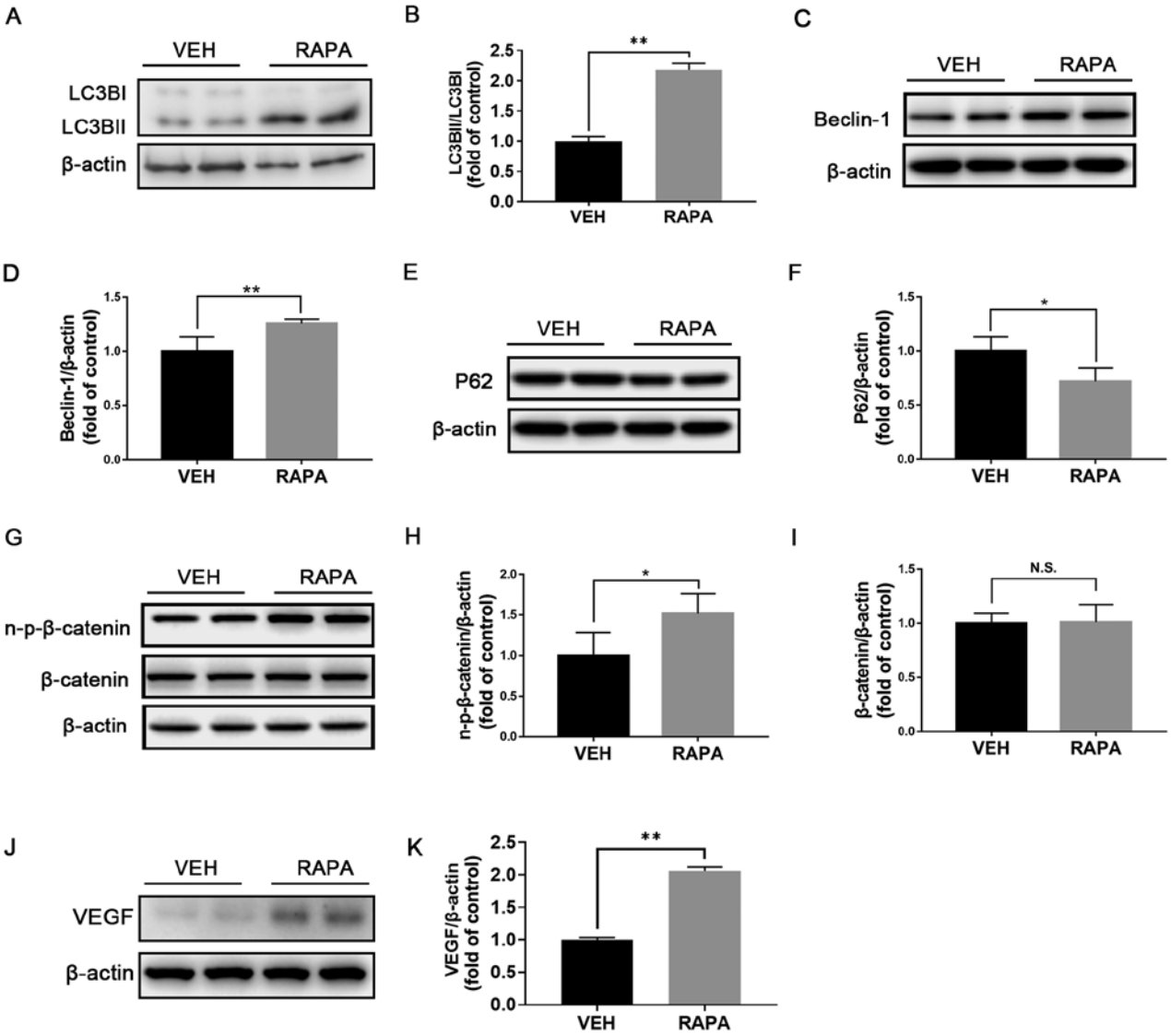

Figure 3. Induction of autophagy by RAPA activates Wnt signaling in C57BL/6J retinas. Expression of autophagy proteins (A) LC3BI/II, (C) Beclin-1 and (E) P62 was evaluated by western blotting in the retina of C57BL/6J mice treated with VEH or RAPA. (B) Protein levels of LC3BI/II were quantified by densitometry and LC3BII/LC3BI were calculated and compared (n=6). Protein levels of (D) Beclin-1 and (F) P62 were quantified by densitometry and normalized to β-actin levels (n=6). (G) Expression of n-p-β-catenin and β-catenin was evaluated by western blotting in the retina of C57BL/6J mice treated with VEH or RAPA. (H and I) Levels of (H) n-p-β-catenin and (I) β-catenin were quantified by densitometry and normalized to β-actin levels (n=6). (J and K) Expression of the downstream target gene of Wnt signaling VEGF was evaluated and quantified in the retina of C57BL/6J mice treated with VEH or RAPA (n=6). *P<0.05 and **P<0.01. n-p-β-catenin, non-phosphorylated-β-catenin; N.S., non-significant; LC3B, microtubule-associated protein 1A/1B-light chain 3; VEGF, vascular endothelial growth factor; VEH, vehicle; RAPA, rapamycin.

have an effect, rMC-1 cells were treated with rapamycin and the expression of autophagic proteins and key components of Wnt signaling was evaluated. The ratio LC3BII/LC3BI was significantly increased (Fig. 4A and B) and P62 expression was significantly decreased in rMC-1 cells treated with rapamycin compared with those treated with vehicle (Fig. 4A and C), suggesting that autophagy was induced in rMC-1 cells. Furthermore, protein expression of n-p-β-catenin was significantly increased in rapamycin-treated rMC-1 cells (Fig. 4A and D), whereas β-catenin expression was unchanged (Fig. 4A and E). Taken together, these results demonstrated that autophagy may activate Wnt signaling in rMC-1 cells.

Inhibition of autophagy downregulates Wnt signaling in rMC-1 cells. We treated rMC-1 cells with chloroquine, which is a commonly used autophagy inhibitor (25). Following chloroquine treatment, the ratio LC3BII/LC3BI was significantly decreased (Fig. 5A and B), whereas expression of P62 was significantly increased in rMC-1 cells (Fig. 5A and C). The expression of n-p-β-catenin was significantly decreased in rMC-1 cells treated with chloroquine compared with those treated with vehicle (Fig. 5A and D), whereas the protein expression of β-catenin was unchanged (Fig. 5A and E). Furthermore, the ratio LC3BII/LC3BI was significantly increased in high glucose-treated rMC-1 cells compared with control cells (Fig. 5F and G). In addition, the autophagy inhibitor 3-MA inhibited the upregulation of LC3BII/LC3BI in high glucose-treated rMC-1 cells (Fig. 5H and I), suggesting that autophagy may play a regulatory role through rMC-1 cells in diabetic retinas.

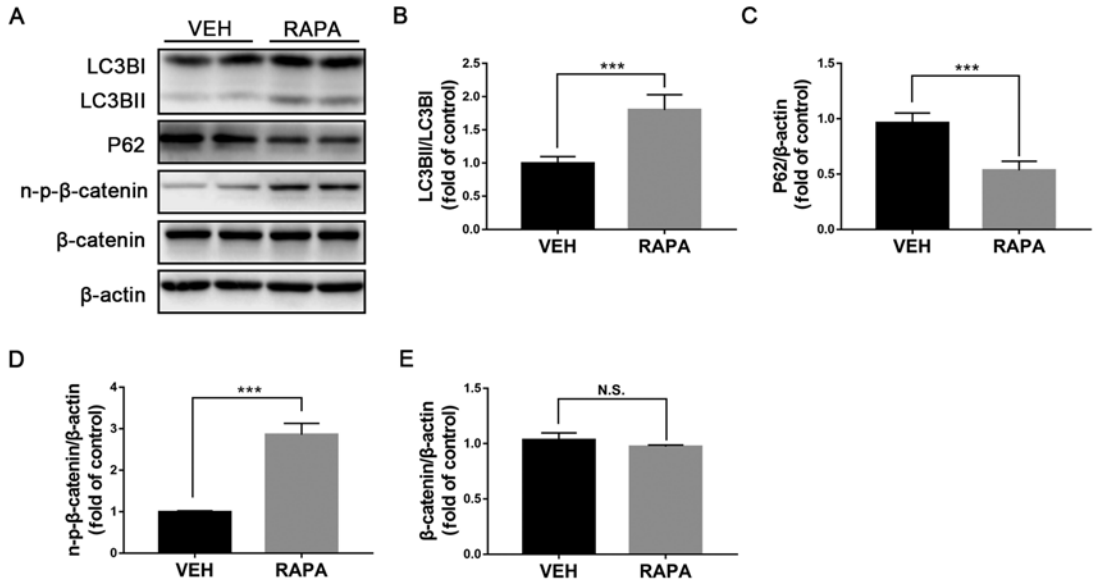

Figure 4. Induction of autophagy by RAPA upregulates Wnt signaling in rMC-1 cells. rMC-1 cells were treated with VEH or RAPA (4 μM) for 24 h. (A) Expression of LC3BI/II, P62, n-p-β-catenin and β-catenin was evaluated by western blotting. (B) Protein levels of LC3BI/II were quantified by densitometry and LC3BII/LC3BI was calculated and compared. (C-E) Expression of (C) P62, (D) n-p-β-catenin and (E) β-catenin was evaluated and quantified by densitometry and normalized to β-actin levels. All data are representative of three independent experiments. ***P<0.001. n-p-β-catenin, non-phosphorylated-β-catenin; N.S., non-significant; LC3B, microtubule-associated protein 1A/1B-light chain 3; VEH, vehicle; RAPA, rapamycin.

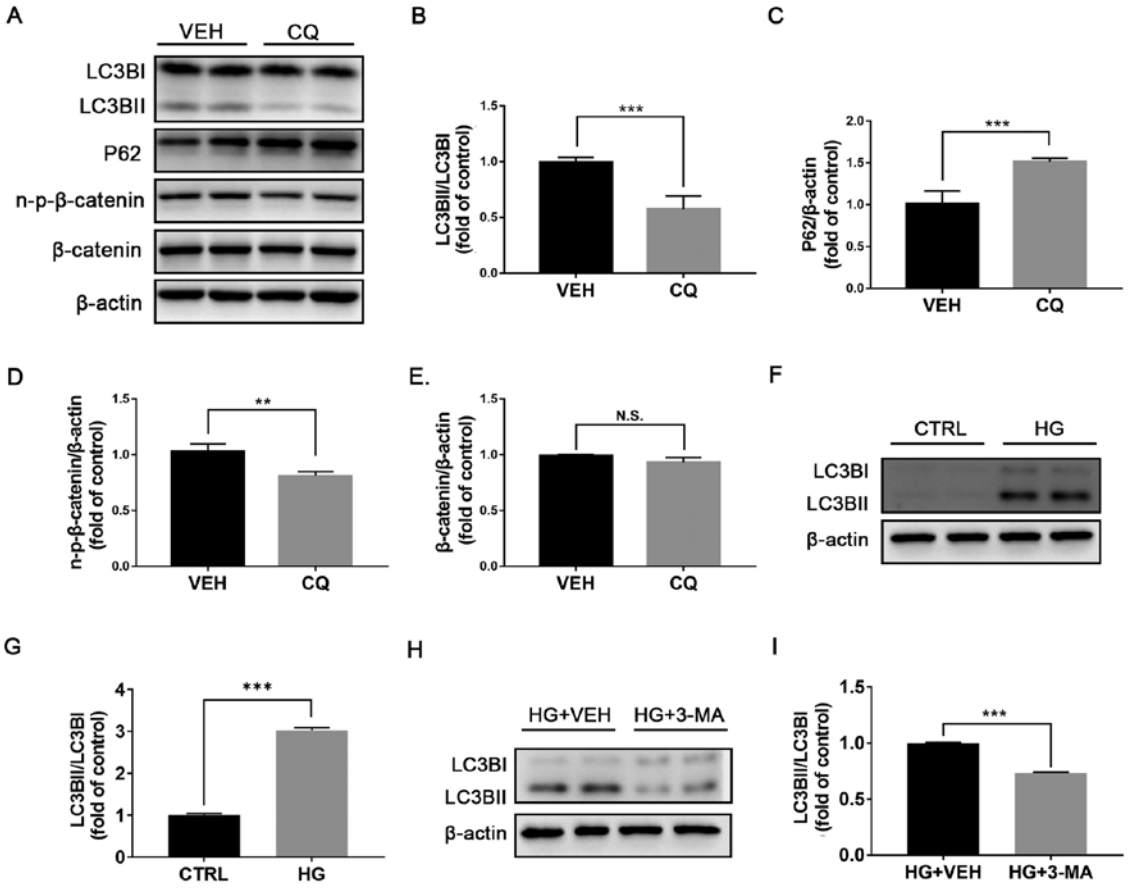

Figure 5. Inhibition of autophagy by CQ suppresses Wnt signaling in rMC-1 cells. rMC-1 cells were treated with VEH or CQ (1 μM) for 24 h. (A) Expression of LC3BI and LC3BI/II, P62, n-p-β-catenin and β-catenin was evaluated by western blotting. (B) Protein levels of LC3BI/II were quantified by densitometry and LC3BII/LC3BI was calculated and compared. Protein levels of (C) P62, (D) n-p-β-catenin and (E) β-catenin were quantified by densitometry and normalized to β-actin levels. (F amd G) Expression of LC3BI and LC3BII was evaluated by western blotting and quantified by densitometry. LC3BII/LC3BI was calculated and compared between CTRL rMC-1 cells and HG-treated cells. (H and I) Expression of LC3BI and LC3BII was evaluated by western blotting and quantified by densitometry. LC3BII/LC3BI was calculated and compared between rMC-1 cells treated with HG+VEH or HG+3-MA. All data are representative of three independent experiments. **P<0.01 and ***P<0.001. n-p-β-catenin, non-phosphorylated-β-catenin; N.S., non-significant; LC3B, microtubule-associated protein 1A/1B-light chain 3; VEH, vehicle; CQ, chloroquine; HG, high glucose; 3-MA, 3-methyladenin; CTRL, control.

Discussion

Wnt signaling is involved in the physiological and pathological processes of a variety of diseases, including DR. The present study demonstrated that autophagy could positively regulate Wnt signaling in the retina of diabetic mice. Furthermore, pharmacological manipulation of autophagy could modulate Wnt signaling in the retina and retinal cells. To the best of our knowledge, the present study was the first to report the potential regulatory role of autophagy on Wnt signaling in the retina of diabetic mice. This study revealed a novel mechanism of Wnt signaling upregulation in DR by which autophagy may positively regulate Wnt signaling, indicating a possible new therapeutic target for DR via autophagy modulation.

Wnt signaling has been reported to contribute to the pathogenesis of DR (5); however, the mechanism of Wnt signaling upregulation in DR remains unknown. Previous studies demonstrated that autophagy might have a regulator role in Wnt signaling. For example, a previous study showed that nutrition starvation, a commonly used condition that induces autophagy in cells, downregulates Wnt signaling in HEK-293T cells; while the inhibition of autophagy with 3-MA attenuates the inhibitory effect of nutrition starvation on Wnt signaling (17). Furthermore, previous studies reported that autophagy is induced in the retina of diabetic human and mice (13,14). In the present study, a regulatory role of autophagy on Wnt signaling in the retina of diabetic mice was demonstrated. Interestingly, unlike most of studies in cancer field which show a negative regulatory effect of autophagy on Wnt signaling (17,26), our findings suggested that autophagy may positively regulate Wnt signaling in the retina of diabetic mice. A possible mechanism of autophagy negatively regulating Wnt signaling is that the autophagy protein P62 could bind to Dishevelled 2 (Dvl2) and promote LC3-mediated autophagosome recruitment of Dvl2, thus accelerating Dvl degradation (17). However, only a few studies demonstrated that autophagy positively regulates Wnt signaling and described the possible underlying mechanism (27,28). Further investigation is required to determine the regulatory mechanisms of autophagy on Wnt signaling in the retina, especially in the diabetic retina.

Db/db mouse is a commonly used animal model of type 2 diabetes (29). Previous studies have shown that db/db mice display pathological features of DR, such as loss of pericytes and retinal capillary degeneration (30,31). In the present study, the ratio LC3BII/LC3BI and expression of Beclin-1 were increased, whereas P62 expression was decreased in the retinas of db/db mice, indicating an activation of autophagy in diabetic retinas. These findings were consistent with other studies reporting that autophagy is induced in DR (12-14). In addition, the expression of a key component of Wnt signaling, n-p-β-catenin, and of the downstream target gene of Wnt signaling, VEGF, were increased in the present study, suggesting that Wnt signaling is activated in db/db retinas. Activation of both autophagy and Wnt signaling in db/db retinas indicates a possible relationship between them. Furthermore, Wnt signaling was inhibited in db/db mice treated with the autophagy inhibitor 3-MA and activated after intraperitoneal injection of the autophagy activator rapamycin in normal C57BL/6J mice. These results suggested that autophagy could positively regulate Wnt signaling in diabetic retinas, and that modulating autophagy may control Wnt signaling in DR, which may serve as a potential therapeutic strategy for DR.

Müller cell, which is a major retinal glial cell type, covers the whole retina and interacts with many other types of cell in the retina, and serves therefore a crucial role in maintaining retinal hemostasis (32). The involvement of Müller cells in DR has been well studied. For example, Müller cells are activated in DR and produce inflammatory cytokines, resulting in cell apoptosis in diabetic retinas (23). Previous studies have reported that high glucose can promote mitochondrial dysfunction of Müller cells and induce cells apoptosis, contributing therefore to the pathogenesis of DR (23,33). Furthermore, other studies have demonstrated that high glucose could induce autophagy in Müller cells (14,34). Therefore, the present study used Müller cells to further investigate the relationship between autophagy and Wnt signaling. In this study, Wnt signaling was upregulated in rapamycin-treated Müller cells and inhibited in chloroquine-treated Müller cells. Interestingly, we found that chloroquine-induced changes in LC3II expression had a dose-dependent effect on Müller cells, and low dose of chloroquine could decrease LC3BII expression. Similar observation was found by Iwai-Kanai *et al* (35) in cardiac-derived myocytes. In addition, in the present study, high glucose could induce autophagy whereas 3-MA was capable of reversing the induction of autophagy in Müller cells. These findings demonstrated that manipulation of autophagy could modulate Wnt signaling in Müller cells, indicating autophagy may regulate Wnt signaling in Müller cells.

In the present study, the lack of immunostaining of autophagic markers in the retina was a limitation. Further investigation will explore the autophagic changes in specific retinal cells of diabetic retinas, especially in Müller cells.

In summary, the present study demonstrated that autophagy could positively regulate Wnt signaling in diabetic retinas. These findings revealed a potential role for autophagy in regulating Wnt signaling in DR, and modulation of autophagy may have therapeutic effects in DR.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Authors' contributions

SY, YZ, ZL and QC designed the experiments. SY, YZ, XW, XL, MW and RZ performed the experiments. SY, YZ and QC analyzed the data. ZL and QC supervised the experiments. SY, ZL and QC wrote the manuscript. QC revised the manuscript. SY and QC confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal protocols were approved by the Xiamen University Experimental Animal Ethics Committee (approval no. XMULA20190022).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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