Original Paper

Identification of O-GlcNAcylation Modification in Diabetic Retinopathy and Crosstalk with Phosphorylation of STAT3 in Retina Vascular Endothelium Cells

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Key Words

O-GlcNAcylation • Diabetic retinopathy • Phosphorylation • STAT3 • Apoptosis

Abstract

Background/Aims: Recently, we observed an increase in O-GlcNAc (O-linked-β-N-acetylglucosamine) modification, and signal transducer and activator of transcription proteins 3 (STAT3) expression in primary retinal vascular endothelial cells (RVECs) under high glucose conditions and tissues altered by diabetic retinopathy (DR). In this study, we focused on the correlations between O-GlcNAcylation and STAT3 phosphorylation, and their potential effects with regards to DR. **Methods:** Expression of O-GlcNAcylation and STAT3 were detected in DR-affected tissues and primary RVECs. The relationship between O-GlcNAcylation and STAT3 was further delineated by immunoprecipitation and Western blot analysis. Effects of O-GlcNAcylation on human RVEC apoptosis and involved protein expression were assayed with flow cytometry and Western blot. **Results:** Global O-GlcNAcylation and pSTAT3 levels were significantly elevated in diabetic rat retina and primary RVECs under high glucose conditions. *In vitro* assays demonstrated that the Tyr705 site was sensitive to high glucose. While O-GlcNAcylation inhibited p727STAT3 expression, augmented O-GlcNAcylation could balance p705STAT3 expression within relatively high levels corresponding to vascular endothelial growth factor (VEGF) changes. Immunoprecipitation revealed that STAT3 was modified by O-GlcNAcylation and phosphorylation simultaneously. Next, we observed that overexpression of O-GlcNAcylation could relieve human RVEC apoptosis related to the JAK2-Tyr705STAT3-VEGF pathway. **Conclusion:** O-GlcNAcylation could relieve RVECs apoptosis through the STAT3 pathway in DR, and O-GlcNAcylation combined with STAT3 phosphorylation might open up new insights into the mechanisms of DR and other diabetic complications.

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Introduction

Instances of diabetic retinopathy (DR) are increasing at an alarming rate, becoming a leading cause for visual impairment and blindness in diabetic patients worldwide [1, 2]. Visual loss primarily occurs due to increased permeability of retinal vessels (diabetic macular edema) [3] and retinal neovascularization (proliferative diabetic retinopathy) [4, 5]. As DR pathogenesis is multifactorial

[6-8], precise molecular mechanisms therein are still not well understood. However, blood-retina barrier (BRB) impairment is a common pathological change, as hyperglycemia induces retina vessel endothelial cell loss, particularly in the early phases of the disease [9]. Protecting retina vessel endothelial cells during hyperglycemia is vital to reducing DR.

Previous studies have reported that altered O-GlcNAcylation is a complication of insulin resistance, leading to diabetic pathologies [10, 11] such as DR [12]. O-GlcNAcylation is one of the most common post-translational protein modifications, dynamically regulated by a single pair of enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) [13]. OGT catalyzes O-GlcNAcylation, and OGA facilitates hydrolytic cleavage of the post-translational modification. O-GlcNAcylation participates in various biological functions including transcription, translation, protein degradation, cell cycle control, and apoptosis [13]. Previous reports have implicated O-GlcNAcylation in neurological degenerative diseases, and cardiovascular and cerebrovascular diseases, as well as type 2 diabetes [14]. Recent investigation of this modification in DR [15, 16], revealed its contribution to increased vascular endothelial growth factor (VEGF) expression [12], retinal neovascularization [17], reactive oxygen species (ROS) generation [18], and BRB impairment [19], mostly in the late stages of the disease. The effects and underlying mechanisms of O-GlcNAcylation on endothelial cells, however, remain poorly understood. In the present study, we focus on the underlying targets of O-GlcNAcylation, such as signal transducer and activator of transcription proteins 3 (STAT3), to investigate mechanisms and roles of O-GlcNAcylation in DR, particularly in the early stages of the disease.

STAT3 is an important participant in tumor angiogenesis [20] and the expression of anti-apoptotic proteins [21, 22] in DR [23]. STAT3 activation is also one of the main facilitators of BRB breakdown through increased VEGF expression [24] and downregulation of endothelial tight junction protein expression [25]. STAT3 proteins are targets of phosphorylation [24] focused on the sites of Tyr705 and Ser727 [26]. Tyr705 phosphorylation is mediated by the Janus kinase (JAK) family, while Ser727 phosphorylation is catalyzed by extracellular signal-regulated kinases (ERKs).

Previous reports have shown a negative relationship between p705STAT3 and p727STAT3 [18, 27]. Tyr705 phosphorylation is essential for STAT3 activation during cell apoptosis through the JAK-p705STAT3 pathway [28]. Whether STAT3 is modified by O-GlcNAcylation, and the effect of O-GlcNAcylation on STAT3 activity and retinal vascular endothelial cells, however, has yet to be defined. We are the first to investigate the relationship between O-GlcNAcylation and STAT3 phosphorylation in DR and its effect on retinal vascular endothelium cell apoptosis, which could provide new insights into DR disease mechanisms.

Materials and Methods

Reagents

Reagents used are as follows: HIF1 α antibody (Bethyl Laboratories, Inc., Montgomery, TX, USA); OGT antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); CTD110.6 antibody, anti-STAT3, anti-pSTAT3, anti-p705STAT3, anti-p727STAT3, anti-pJAK2, anti-JAK2, α -Tubulin, and β -actin purchased from Cell Signaling Technology (Danvers, MA, USA); anti-von Willebrand Factor antibody (vWF) (Abcam, Cambridge, MA, USA; VWF, ab6994); Dulbecco's modified Eagle's medium (DMEM; high glucose medium, 4.5 g/L glucose, and low glucose medium, 1 g/L glucose) (Gibco, Carlsbad, CA, USA); 4',6'-diamidino-2-phenylindole hydrochloride (DAPI), Thiamet G, Alloxan, AG490, Cucurbitacin I, and bovine serum albumin (BSA) (Sigma); plastic tissue culture flasks (Costar, Cambridge, MA, USA); and immobilon-NC transfer membrane (Millipore, Billerica, MA, USA). A Leica confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) was used for scanning.

Human specimens

Vitreous humor samples were collected from 10 patients with proliferative diabetic retinopathy (PDR) during initial pars plana vitrectomy. Control samples were obtained from 10 retinal detachment (RD) patients without diabetes. All patients included in this study signed an informed consent form before surgery. Undiluted vitreous samples (0.5 - 1.0 mL) were aspirated under standardized conditions, immediately transferred to sterile tubes, and stored at -80 °C prior to analysis.

Experimental animals

We used Sprague-Dawley (SD) rats as an animal model for diabetes. Age-matched, female SD rats (6–8 weeks) weighing between 200 to 250 g were obtained from Shanghai Laboratory Animal Center, CAS, in Shanghai, China. Animals were housed under standard animal care conditions (20 - 22°C; humidity 40 - 60%) with a 12 h/12 h light/dark cycle.

Prior to STZ injection, rats were weighed, and baseline blood glucose was measured via tail vein using the OneTouch Glucose Monitoring System (Johnson & Johnson, New Brunswick, NJ, USA). Rats were administered 50 mg/kg of streptozotocin (STZ) in 10 mM sodium citrate, intraperitoneally. Blood glucose was measured after 3 days, a blood glucose concentration of ≥ 16.7 mmol/L was considered to be diabetic. All procedures were approved by the Animal Ethics Committee of Tongji University, Shanghai, China. Eyes were enucleated and retina tissues immediately dissected; then kept at -80°C for further analysis.

Isolation and Culture of Primary Retinal Vascular Cells

Primary bovine retinal vascular endothelium cells (BRVECs) were cultured as previously described [16]. Human retinal vascular endothelium cells (HRVECs) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and passaged as described previously [16]. These cells were confirmed by the cell-specific marker vWF. All experiments were carried out in accordance with the Association for Research in Vision and Ophthalmology Statement.

Treatment with Thiamet G and Alloxan

O-GlcNAcylation is the post-translational modification of serine (Ser) or threonine (Thr) residues manipulated by OGT and OGA. Total levels of O-GlcNAcylation were downregulated by 2.5 mM Alloxan and upregulated by 2.5 μ M Thiamet G for 24 h.

Treatment with AG490 and Cucurbitacin I

HRVECs were treated with 80 μ M AG490 for 24 h and 0.25 μ M Cucurbitacin I for 2 h, then cells were lysed for Western blot (WB) analysis. AG490, a janus kinase 2 (JAK2) inhibitor, was used to reduce JAK2 activity. Cucurbitacin I (JSI-124) was used to reduce STAT3 phosphorylation both in p727STAT3 and p705STAT3.

Western Blot Analysis

Equal amounts (30 μ g) of proteins from cell extracts and animal retina samples were separated on a 10% acrylamide gel and subsequently transferred onto nitro-cellulose (NC) membranes at 200 mA for 1.5 h. After blocking in 5% (w/v) BSA for 1 h at room temperature (RT), blots were incubated overnight at 4°C with primary rabbit monoclonal antibody against anti-STAT3, anti-pSTAT3, anti-p705STAT3, anti-p727STAT3, anti-pJAK2, anti-JAK2, CTD110.6, α -Tubulin, and β -actin (all antibodies were used at a 1:1000 dilution). Blots were washed with TBS-T (0.1% Tween-20 in TBS) 3 times prior to incubation with the secondary antibody for 1 h at RT. The membrane was washed in TBS-T buffer and visualized using Odyssey (LI-COR Biosciences, Lincoln, NE, USA). Densitometric analysis was performed using Image J software (version 1.43, Broken Symmetry Software, Bethesda, MD, USA). For each experiment, the measurements were repeated 3 times.

Co-immunoprecipitation (IP)

Immunoprecipitation of STAT3 was carried out using anti-STAT3 antibody conjugated to agarose beads. The collected lysate (equivalent to 500 μ g total protein) was incubated with 1 μ g of anti-STAT3 antibody conjugated to agarose beads overnight at 4°C with gentle shaking. After washing the resin three times with lysis buffer, the beads were incubated with 40 μ L 1 $\times$ SDS-PAGE loading buffer and then centrifuged at 2,000g for 1 min to collect eluted antigen. The eluent was run on Tris-Glycin 10% gradient gels, and analyzed by WB as described above.

Adversely, immunoprecipitation of O-GlcNAcylation protein was carried out using the 10 μ L wheat germ agglutinin (WGA) beads (Santa Cruz Biotechnology) overnight at 4°C with gentle shaking, then washed, centrifuged, and analyzed by WB as described above.

Annexin V and PI double staining by flow cytometry

HRVECs were incubated with 2.5 μ M Thiamet G for 24 h and 0.25 μ M Cucurbitacin I for 2 h. Cells were suspended in Annexin V binding buffer (BD Biosciences) at a concentration of 1×10^6 cells/mL. Annexin V-FITC (BD Biosciences) was then added, followed by incubation in a 100 μ L cell suspension for 15 min. Propidium iodide (PI) was spiked into 400 μ L Annexin V binding buffer and added to the cell suspension immediately, then analyzed on a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Statistical Analysis

Experiments were repeated at least 3 times. Quantitative results were expressed as mean $\pm$ SEM. ANOVA and t-tests were used for statistical analysis, with $p < 0.05$ considered significant.

All procedures used in this study were approved by the Medical Ethics Committee of the Shanghai Tenth People's Hospital. Principles of human subject research and cell research were conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients.

Results

Elevated OGT expression in the vitreous humors of patients with PDR

We investigated the OGT expression in PDR and control vitreous samples (RD patients). Results showed increased OGT expression in PDR vitreous samples compared with controls (Fig. 1B, $p < 0.01$), indicating increased O-GlcNAcylation levels in PDR patients.

Expression of O-GlcNAcylation and pSTAT3 in diabetic rat retina tissues and primary BRVECs in high glucose

We detected expression of O-GlcNAcylation and pSTAT3 in diabetic rat retinal tissues and primary BRVECs in a high glucose environment. Increased O-GlcNAcylation and elevated pSTAT3 expression were detected in diabetic rat retinas (Fig. 2B, $^1_{vs}^2$, $p < 0.05$; Fig. 2D, $^1_{vs}^2$, $p < 0.05$). Similarly, increased O-GlcNAcylation and pSTAT3 expression were observed in primary BRVECs under high glucose conditions (Fig. 3B, $^1_{vs}^2$, $p < 0.05$).

Next, we treated primary BRVECs with advanced glycation end products (AGEs) for 24 h to simulate a pathological condition, to investigate the effects thereof on O-GlcNAcylation and pSTAT3 expression. As shown in Fig. 3, AGEs significantly elevated O-GlcNAcylation and pSTAT3 levels (Fig. 3B, $^3_{vs}^2$, $p < 0.05$; Fig. 3D, $^3_{vs}^2$, $p < 0.05$). These results demonstrate elevated pSTAT3 expression consistent with augmented O-GlcNAcylation in high glucose combined with AGEs.

O-GlcNAcylation levels in primary BRVECs under hypoxia and high glucose

As AGEs might directly influence O-GlcNAcylation expression or endothelial cell apoptosis [29], we utilized Thiamet G, an OGA inhibitor, to increase O-GlcNAcylation levels in BRVECs, and Alloxan, an OGT inhibitor, to decrease O-GlcNAcylation levels, to investigate the effects of O-GlcNAcylation on pSTAT3 expression.

In addition to hyperglycemia, hypoxia caused by capillary abnormalities is another vital factor to DR [30]. We cultured primary BRVECs in high glucose combined with hypoxic

Fig. 1. OGT expression in vitreous humors from patients with PDR. Upregulated OGT expression in PDR patients. Protein lysates from patients' vitreous humors were analyzed by Western blot. (A) The first two lanes show the immunoblots of OGT expression in controls (retinal detachment), and the latter two in PDR (proliferative diabetic retinopathy) patients. (B) OGT levels in each lane were quantified using Image J software.

Compared to controls, OGT levels were elevated significantly in PDR samples ($p < 0.01$). (1: vitreous humors in RD patients, 2: vitreous humors in PDR patients).

Fig. 2. Expression of O-GlcNAcylation and pSTAT3 in rat retinal tissues. CTD110.6 antibody was used to estimate O-GlcNAcylation levels in rat retinal tissues. Results show elevated O-GlcNAcylation and pSTAT3 levels in diabetic rat retina tissues. (A) Expression of O-GlcNAcylation and (C) pSTAT3 were determined by Western blot. Compared with age-matched controls, O-GlcNAcylation and pSTAT3 levels were elevated in diabetic rat retinas. (Fig. 2B, $1^2_{VS} 3$, $p < 0.05$; Fig. 2D, $1^2_{VS} 3$, $p < 0.05$). (1: Control rat, 2: Diabetic rat 1 month post-STZ, 3: Diabetic rat 2 month post-STZ).

Fig. 3. Expression of CTD110.6 and pSTAT3 in primary BRVECs in high glucose. Primary BRVECs were treated with high glucose (4.5 g/L) for 72 h and combined with advanced glycation end products (AGEs) for 24 h. O-GlcNAcylation and pSTAT3 levels were elevated in primary BRVECs in high glucose and high glucose combined with AGEs. (A, C) Western blots show O-GlcNAcylation and pSTAT3 expression. Compared with normal glucose, high glucose elevated O-GlcNAcylation and pSTAT3 expression (Fig. 3B, 2^1_{VS} , $p < 0.05$; Fig. 3D, 2^1_{VS} , $p < 0.05$). AGEs treatment increased O-GlcNAcylation and pSTAT3 expression significantly (Fig. 3B, 3^2_{VS} , $p < 0.05$; Fig. 3D, 3^2_{VS} , $p < 0.05$). (1: NG (1 g/L), 2: HG (4.5 g/L), 3: HG combined with AGEs).

environment (3% O₂). Our results showed that O-GlcNAcylation levels increased significantly in high glucose compared with normal glucose (Fig. 4B, 1^2_{VS} , $p < 0.05$), and that the elevation was more pronounced when combined with hypoxia (Fig. 4B, 6^2_{VS} , $p < 0.05$), which could be manipulated by Thiamet G and Alloxan treatment.

Effects of O-GlcNAcylation on p727STAT3 and p705STAT3 expression

To investigate the specific effects of STAT3 sites involved in DR under high glucose conditions, levels of different STAT3 sites were explored. High glucose increased p705STAT3 expression (Fig. 5C, 6^3_{VS} , $p < 0.05$), but had no effect on p727STAT3 levels (Fig. 5B, 6^3_{VS} , $p > 0.05$). Low oxygen alone did not affect p727STAT3 or p705STAT3 expression (Fig. 5B, 3^1_{VS} , $p > 0.05$; Fig. 5C, 3^1_{VS} , $p > 0.05$), but combined with high glucose was found to upregulate p705STAT3, although it did not significantly affect p727STAT3 (Fig. 5C, 4^1_{VS} , $p < 0.01$; Fig. 5B, 4^1_{VS} , $p > 0.05$). This led us to conclude that Tyr705 is the site sensitive to high glucose, suggesting that Tyr705 is a site of importance for DR.

Augmentation of O-GlcNAcylation by Thiamet G increased p705STAT3 expression under normal glucose (Fig. 5C, 2^1_{VS} , $p < 0.05$), but this modification downregulated p705STAT3 expression in high glucose (Fig. 5C, 7^6_{VS} , $p < 0.01$). Even the downregulated p705STAT3 levels, however, were still much higher than in a normal glucose environment (Fig. 5C, 7^3_{VS} , $p < 0.01$); as such, O-GlcNAcylation was shown to balance p705STAT3 expression within relatively high levels under high glucose conditions.

Fig. 4.

O-GlcNAcylation expression in primary BRVECs in high glucose and hypoxia. Hypoxia combined with high glucose increased O-GlcNAcylation expression in primary BRVECs. (A) O-GlcNAcylation expression assayed

by Western blot. Levels of O-GlcNAcylation were determined by measuring each whole lane. Primary BRVECs were treated with 2.5 μM Thiamet G and 2.5 mM Alloxan for 24 h under high glucose in low O₂ (3%) conditions. (B) Thiamet G increased O-GlcNAcylation expression significantly (Fig. 4B, ³_{vs}⁵, $p < 0.05$), and Alloxan decreased O-GlcNAcylation levels under the same conditions (Fig. 4B, ⁴_{vs}⁵, $p < 0.05$). High glucose and hypoxia increased O-GlcNAcylation (Fig. 4B, ¹_{vs}², $p < 0.05$; Fig. 4B, ¹_{vs}⁵, $p < 0.05$), but O-GlcNAcylation was elevated even higher in high glucose combined with hypoxia (Fig. 4B, ⁶_{vs}², $p < 0.05$; Fig. 4B, ⁶_{vs}⁵, $p < 0.05$). (1: Normal O₂ and NG (1 g/L), 2: Normal O₂ and HG (4.5 g/L), 3: Low O₂ (3%) and Thiamet G, 4: Low O₂ (3%) and Alloxan, 5: Low O₂ (3%) and NG, 6: Low O₂ (3%) and HG).

Fig. 5. Effects of O-GlcNAcylation on p727STAT3 and p705STAT3 expression.

While high glucose elevated p705STAT3 expression, O-GlcNAcylation regulated p705STAT3 within certain levels and negatively regulated p705STAT3 expression. (A) Expression of O-GlcNAcylation as determined by Western

blot. HRVECs were cultured in high glucose (4.5 g/L) and low O₂ (3%) conditions. (B) High glucose had no effect on p727STAT3 expression (Fig. 5B, ⁶_{vs}³, $p > 0.05$). (C) but increased p705STAT3 expression significantly (Fig. 5C, ⁶_{vs}³, $p < 0.05$). High glucose combined with low oxygen upregulated p705STAT3 expression, but had no significant effect on p727STAT3 expression (Fig. 5B, ³_{vs}⁴, $p > 0.05$; Fig. 5C, ³_{vs}⁴, $p < 0.01$). Augmented O-GlcNAcylation by Thiamet G upregulated p705STAT3 expression in normal glucose (Fig. 5C, ²_{vs}¹, $p < 0.05$), but downregulated p705STAT3 expression under high glucose (Fig. 5C, ⁶_{vs}⁷, $p < 0.01$), although it remained higher than in normal glucose (Fig. 5C, ³_{vs}⁷, $p < 0.01$). O-GlcNAcylation negatively regulated p727STAT3 levels under high glucose conditions (Fig. 5B, ⁶_{vs}⁷, $p < 0.05$; ⁵_{vs}⁴, $p < 0.01$). (1: Low O₂ (3%) and NG (1 g/L), 2: Low O₂ combined with NG and Thiamet G, 3: Normal O₂ and NG, 4: Low O₂ and HG (4.5 g/L), 5: Low O₂ combined with HG and Alloxan, 6: Normal O₂ and HG, 7: Normal O₂ combined with HG and Thiamet G).

On the other hand, p727STAT3 expression was depressed by O-GlcNAcylation under high glucose conditions. While augmented O-GlcNAcylation decreased p727STAT3 levels significantly (Fig. 5B, ⁷_{vs}⁶, $p < 0.05$), downregulated O-GlcNAcylation increased p727STAT3 expression (Fig. 5B, ⁵_{vs}⁴, $p < 0.01$). So while O-GlcNAcylation appeared to inhibit p727STAT3 expression, it regulated p705STAT3 expression within relatively high levels under high glucose or high glucose combined with hypoxia.

Fig. 6. pJAK2 expression under different O-GlcNAcylation concentrations. pJAK2 expression was elevated by augmented O-GlcNAcylation in hypoxic and high glucose conditions. (A) O-GlcNAcylation and pJAK2

expression as determined by Western blot. (B) Compared with normal conditions, pJAK2 expression was elevated under hypoxic and high glucose conditions (Fig. 6B, ⁷vs³, $p < 0.01$). While augmented O-GlcNAcylation increased pJAK2 expression under hypoxic and high glucose conditions (Fig. 6B, ²vs³, $p < 0.05$), O-GlcNAcylation had no significant effect on pJAK2 expression under hypoxic conditions alone (Fig. 6B, ⁴vs⁶, $p > 0.05$; ⁵vs⁶, $p > 0.05$). (1: Low O₂ (3%) combined with HG (4.5 g/L) and Thiamet G (5 μM), 2: Low O₂ combined with HG and Thiamet G (2.5 μM), 3: Low O₂ and HG, 4: Low O₂ combined with NG (1 g/L) and Thiamet G, 5: Low O₂ combined with NG and Alloxan, 6: Low O₂ and NG, 7: Normal O₂ and NG).

Fig. 7. Crosstalk between O-GlcNAcylation with STAT3 in high glucose. Immunoprecipitation of STAT3 and O-GlcNAcylation proteins were carried out by Protein A agarose beads and WGA beads. (A) STAT3 expression as determined by Western blot of cell lysis. (B) We precipitated O-GlcNAc modified proteins from HRVECs under normal glucose, high glucose, and augmented O-GlcNAcylation conditions. Results showed STAT3 expression in precipitated O-GlcNAc modified proteins; STAT3 levels increased in high glucose and augmented O-GlcNAcylation. (C) We then used Protein A agarose beads to precipitate STAT3 proteins, and detected O-GlcNAcylation expression in precipitated STAT3 proteins. (1: NG (1 g/L), 2: HG (4.5 g/L), 3: HG and augmented O-GlcNAcylation).

Expression of pJAK2 under different O-GlcNAcylation conditions

Tyr705 phosphorylation is mediated by JAK2, so we examined pJAK2 levels to investigate different mechanisms of O-GlcNAcylation on p705STAT3 expression. Compared with normal conditions, pJAK2 expression was elevated during hypoxia combined with high glucose (Fig. 6B, ⁷vs³, $p < 0.01$). Augmented O-GlcNAcylation can upregulate pJAK2 expression under these same parameters (Fig. 6B, ²vs³, $p < 0.05$). However, upregulated O-GlcNAcylation by Thiamet G or downregulated O-GlcNAcylation by Alloxan had no significant effects on pJAK2 expression during hypoxia alone (Fig. 6B, ⁴vs⁶, $p > 0.05$; ⁵vs⁶, $p > 0.05$). High glucose was thereby determined to be the parameter in which pJAK2 expression was affected by

Fig. 8. STAT3 inhibition by Cucurbitacin I in HRVECs. Cucurbitacin I was used to inhibit pSTAT3 expression. (A, B) Western blot detected pSTAT3 and STAT3 levels in each lane. Levels of pSTAT3 were quantified by pSTAT3/STAT3. (C) Compared with normal glucose, high glucose increased pSTAT3 expression (Fig. 8C, ²_{vs}¹, $p < 0.01$). While Cucurbitacin I inhibited pSTAT3 and STAT3 expression in a time-dependent manner (Fig. 8C, ²_{vs}³ ⁴_{vs}⁵, $p < 0.01$), DMSO had no effect on pSTAT3 expression (Fig. 8D, ²_{vs}³ ⁴_{vs}⁵, $p > 0.05$). (C: 1: NG (1 g/L), 2: HG (4.5 g/L) without Cucurbitacin I, 3: HG and Cucurbitacin I (1 h), 4: HG and Cucurbitacin I (3 h), 5: HG and Cucurbitacin I (6 h)).

O-GlcNAcylation. Enhanced O-GlcNAcylation can still increase pJAK2 expression under high glucose conditions (Fig. 6B, ¹_{vs}³, $p < 0.05$), which is inconsistent with p705STAT3 expression patterns, so p705STAT3 expression may be influenced by other factors.

Crosstalk between O-GlcNAcylation and STAT3 in high glucose

To delineate the correlations between O-GlcNAcylation and STAT3, immunoprecipitation of STAT3 and O-GlcNAcylation proteins was carried out by Protein A agarose beads and WGA beads. We detected STAT3 expression in precipitated O-GlcNAc-modified proteins, and increased STAT3 levels in high glucose coupled with augmented O-GlcNAc modification compared with normal glucose (Fig. 7B).

We also detected O-GlcNAcylation expression in precipitated STAT3 proteins by anti-CTD110.6 antibody (Fig. 7C). These co-immunoprecipitation results illustrate that STAT3 is modified both by O-GlcNAcylation and phosphorylation.

pSTAT3 inhibition by Cucurbitacin I in HRVECs

As Tyr705 is sensitive to high glucose, and could be balanced by O-GlcNAcylation at a certain level, we focused our further studies on Tyr705 changes. We used Cucurbitacin I to inhibit pSTAT3 expression to investigate the role of these Tyr705 sites.

HRVECs were treated with 20 μ M Cucurbitacin I under high glucose for 1, 3, or 6 h, with DMSO as negative control. Cells were lysed and subjected to WB analysis using specific antibody against pSTAT3. Cucurbitacin I was shown to inhibit pSTAT3 and STAT3 expression in a time-dependent manner (Fig. 8C, ²_{vs}³ ⁴_{vs}⁵, $p < 0.01$), while DMSO had no influence on pSTAT3 or STAT3 expression (Fig. 8D, ²_{vs}³ ⁴_{vs}⁵, $p < 0.05$).

Effects of O-GlcNAcylation and pSTAT3 on HRVEC apoptosis in high glucose

To investigate the effects of O-GlcNAcylation and pSTAT3 on HRVEC apoptosis, we used Annexin V and PI double staining to assess cell apoptotic states. Compared with normal glucose, high glucose increased the percentage of apoptotic cells (Fig. 9, ³_{vs}², $p < 0.01$), and augmented O-GlcNAcylation (adding Thiamet G) rescued HRVEC apoptosis (Fig. 9, ¹_{vs}², $p < 0.05$), indicating a protective role. pSTAT3 inhibition by Cucurbitacin I increased cell apoptosis (Fig. 9, ⁶_{vs}², $p < 0.001$); but this increase could be partially mitigated by enhanced O-GlcNAcylation (Fig. 9, ⁴_{vs}⁶, $p < 0.01$). These results suggest that augmented O-GlcNAcylation can reduce cell apoptosis through the p705STAT3 pathway.

Effects of O-GlcNAcylation and pSTAT3 on cleaved caspase-3 expression in high glucose

Caspase-3 is the primary facilitator of programmed cell death, so we assayed cleaved caspase-3 expression to confirm the effects of O-GlcNAcylation and pSTAT3 on HRVEC

Fig. 9. Apoptosis analysis of HRVECs by Annexin V and PI double staining. Augmented O-GlcNAcylation could rescue HRVECs apoptosis through STAT3 pathway. Apoptotic cells were represented as the percentage of Annexin V single-positive plus Annexin V/PI double-positive cells. The bottom right quadrant represents Annexin V-FITC-stained cells (early-phase apoptotic cells), and top right quadrant represents PI and Annexin V-FITC double stained cells (late-phase apoptotic/necrotic cells). Compared with normal glucose, high glucose correlated with a higher percentage of apoptotic cells (Fig. 9, $3^{\text{vs}2}$, $p < 0.01$), which could be rescued by augmented O-GlcNAcylation (Fig. 9, $1^{\text{vs}2}$, $p < 0.05$). Cucurbitacin1 increased cell apoptosis (Fig. 9, $6^{\text{vs}2}$, $p < 0.001$), but this effect was partially counteracted by Thiamet G (Fig. 9, $4^{\text{vs}6}$, $p < 0.01$). (1: C2 HG (4.5 g/L) and Thiamet G, 2: E HG, 3: A NG (1 g/L), 4: D2 HG combined with Thiamet G and Cucurbitacin I, 5: HG combined with Thiamet G and DMSO, 6: HG and Cucurbitacin I).

Fig. 10. Expression of cleaved caspase-3 under different pSTAT3 conditions. Cucurbitacin I treatment increased cleaved caspase-3 expression. 10 μ M, 20 μ M, and 30 μ M Cucurbitacin I was independently added to co-cultured HRVECs in high glucose for 3 h. (A) Expression of pSTAT3 and cleaved

caspase-3 as determined by Western blot. (B) Cucurbitacin I downregulated pSTAT3 and STAT3 expression in a concentration-dependent manner (Fig. 10B, 1 vs 3 vs 4 vs 5 , $p < 0.01$), and also increased cleaved caspase-3 levels significantly (Fig. 10C, 1 vs 3 vs 4 vs 5 , $p < 0.01$). (1: HG (4.5 g/L), 2: NG (1 g/L), 3: HG and 10 μ M Cucurbitacin I, 4: HG and 20 μ M Cucurbitacin I, 5: HG and 30 μ M Cucurbitacin I).

Fig. 11. Expression of VEGFA under different O-GlcNAcylation and pSTAT3 conditions. (A) Expression of VEGFA as determined by Western blot under different treatments. Alloxan or OGT siRNA were used to

downregulate O-GlcNAcylation expression, and Thiamet G was used to augment O-GlcNAcylation levels in HRVECs. (B) Increased VEGFA expression was detected in high glucose (Fig. 11B, 5 vs 6 , $p < 0.05$), and OGT inhibition (using Alloxan or OGT siRNA) (Fig. 11B, 3 vs 5 , $p < 0.05$; Fig. 11C, 1 vs 5 , $p < 0.05$) or Cucurbitacin I decreased VEGFA expression significantly (Fig. 11B, 4 vs 5 , $p < 0.05$). Compared with the high glucose group, augmented O-GlcNAcylation decreased VEGFA expression (Fig. 11B, 2 vs 5 , $p < 0.05$), but levels remained much higher than in the normal group (Fig. 11B, 2 vs 6 , $p < 0.05$). (1: NG and OGT siRNA, 2: HG and Thiamet G, 3: HG and Alloxan, 4: HG and Cucurbitacin I, 5: HG, 6: NG).

apoptosis. 10 μ M, 20 μ M, and 30 μ M Cucurbitacin I were independently added to high glucose levels for 3 h. Inhibition of STAT3 induced by Cucurbitacin I treatment downregulated pSTAT3 and STAT3 expression, but increased cleaved caspase-3 levels in a concentration-dependent manner (Fig. 10B, 1 vs 3 vs 4 vs 5 , $p < 0.01$; Fig. 10C, 1 vs 3 vs 4 vs 5 , $p < 0.01$). Increased cleaved caspase-3 expression induced by pSTAT3 inhibition was consistent with the flow cytometry results that O-GlcNAcylation affected HRVEC apoptosis through the p705STAT3 pathway.

VEGFA expression under different O-GlcNAcylation conditions

VEGF is downstream of STAT3, and acts as the main subsite contributing to DR formation. We detected an increase of VEGFA expression in high glucose, but augmentation of O-GlcNAcylation downregulated VEGFA (Fig. 11B, 2 vs 5 , $p < 0.05$), although it was still higher than in normal glucose conditions (Fig. 11B, 2 vs 6 , $p < 0.05$). This result was consistent with previous data that p705STAT3 expression patterns are regulated by augmented O-GlcNAcylation under high glucose conditions.

OGT inhibition (using Alloxan or OGT siRNA) and pSTAT3 inhibition (using Cucurbitacin I) decreased VEGFA expression significantly (Fig. 11B, 4 vs 5 , $p < 0.05$; 1 vs 5 , $p < 0.05$). These results suggest that augmented O-GlcNAcylation is able to maintain a certain level of VEGFA via the p705STAT3-VEGFA pathway.

Discussion

Hyperglycemia is one of the most important risk factors for DR, but the specific molecular mechanisms involved remain poorly understood. Previous studies observed increased O-GlcNAc modification in diabetic complications [31-33], and our present study detected elevated O-GlcNAcylation in PDR vitreous samples, diabetic rat retinas, and primary RVECs in high glucose conditions. In further experiments, we purified O-GlcNAcylated proteins to explore potential targets and underlying mechanisms related to endothelial apoptosis.

STAT3 factors influence endothelial function in DR [34, 35]. Thus, we focused on the relationship between O-GlcNAcylation and STAT3 phosphorylation, and changes to specific STAT3 sites under different O-GlcNAcylation levels. Our results showed an increased expression of O-GlcNAcylation and pSTAT3 both *in vivo* and *in vitro* in high glucose alone and when combined with hypoxia. We used Thiamet G, an OGA inhibitor, and Alloxan, an OGT inhibitor, to alter O-GlcNAcylation levels and explore the effects of O-GlcNAcylation on STAT3 phosphorylation.

Our results also demonstrated increased p705STAT3 expression under high glucose conditions, with no significant effect on p727STAT3 expression, suggesting Tyr705 is the site sensitive to high glucose. O-GlcNAcylation negatively affected p727STAT3 expression and regulated p705STAT3 expression within certain levels. The results showed that augmented O-GlcNAcylation upregulated p705STAT3 in normal glucose, but downregulated p705STAT3 in high glucose, although levels remained higher than in normal glucose. In addition, a negative relationship between p705STAT3 and p727STAT3 has been reported previously [27]. While expression of p727STAT3 decreased, p705STAT3 levels increased; this also contributed to total STAT3 activity.

O-GlcNAcylation and phosphorylation are thought to be complementary processes, namely in the Yin and Yang theory [33]. However, the relationship between O-GlcNAcylation and phosphorylation is more complicated than simple “competitive inhibition”. There are at least four different dynamic interactions between O-GlcNAcylation and O-phosphorylation: first, they competitively modify the same sites, such as in the mER-beta protein [36]; second, they competitively modify adjacent sites, such as in the C/EBP beta protein [37]; third, they co-modify the same protein at same time, such as in IRS-1 factors [38]; and fourth, modification and competitive decoration can coexist simultaneously, such as in CaMKIV proteins [39]. To explore the correlations of O-GlcNAcylation and STAT3, we investigated the expression of STAT3 and O-GlcNAcylation in precipitated proteins using immunoprecipitation.

We observed STAT3 expression in O-GlcNAcylated proteins and O-GlcNAcylation in precipitated STAT3 proteins. Our results suggest that STAT3 is co-modified by phosphorylation and O-GlcNAcylation simultaneously. As O-GlcNAcylation negatively influences pSTAT3Ser727 expression, and regulated pSTAT3Tyr705 expression within relatively high levels, we supposed that phosphorylation and O-GlcNAcylation might co-modify the Tyr705 site and compete for the Ser727 site. The interaction between Ser727 and Tyr705 sites, and O-GlcNAcylation combined with phosphorylation, can produce a great deal of molecular diversity that plays an important role both in physiological and pathological conditions.

In early DR, HRVEC apoptosis mainly contributes to BRB breakdown [9]. We observed the protective effects of O-GlcNAcylation on HRVEC apoptosis under high glucose conditions in a previous study [18]. According the influence of STAT3 on cell apoptosis, we supposed that O-GlcNAcylation might rescue cell apoptosis through the STAT3 pathway. To examine the role of STAT3 on HRVEC apoptosis and the underlying mechanisms involved therein, we applied the phosphorylation inhibitor Cucurbitacin I to downregulate pSTAT3 expression (both at Tyr705 and Ser727), and OGA inhibitor and OGT inhibitor to regulate O-GlcNAcylation levels.

The present study showed increased HRVEC apoptosis in high glucose, which could be reversed by augmented O-GlcNAcylation. Conversely, pSTAT3 inhibition by Cucurbitacin I induced higher percentages of cell apoptosis and increased cleaved caspase-3 expression in a concentration-dependent manner. While pSTAT3 inhibition increased cell apoptosis, augmented O-GlcNAcylation partially counteracted this adverse effect, defining an anti-apoptotic role for O-GlcNAcylation on HRVEC related to the STAT3 pathway.

The Tyr705 site is the target of JAK2 [40, 41], and confers protective effects on cell activity by increasing DNA binding affinity [42]. Increased STAT3 activity can promote protein dimerization and translocation to regulate expression of critical genes such as cell survival

factors [42-44], signaling pathways [45, 46], and MnSOD activity in mitochondria [47]. We determined JAK2 expression provided insight into the mechanisms of O-GlcNAcylation on p705STAT3 expression. Similarly, we detected elevated pJAK2 expression in high glucose conditions corresponding to p705STAT3 expression. Augmented O-GlcNAcylation increased pJAK2 expression in high glucose, however, which was not entirely consistent with p705STAT3 changes. We theorized that O-GlcNAcylation might affect p705STAT3 expression by changing phosphorylation levels directly rather than through the pJAK2 pathway.

VEGF is downstream of STAT3 [48] and its presence has been implicated in retinal macular edema, neovascularization, and vitreous hemorrhage. While VEGF has always been considered a risk factor in DR [7, 49], VEGF is also necessary for vascular endothelial cell survival under pressure [50] and human embryo retina development [51]. Our results showed that high glucose upregulated VEGFA expression, which could be partially mitigated by augmented O-GlcNAcylation consistent with p705STAT3 changes. In addition, pSTAT3 inhibition and decreased O-GlcNAcylation significantly downregulated VEGFA expression. Thus, a relatively high VEGFA regulated by O-GlcNAcylation might exert a protective effect on retinal endothelial cells via the p705STAT3-VEGFA pathway. Many factors can affect VEGF expression, however, particularly under high glucose conditions, and the exact effects of specific STAT3 sites on VEGF expression and cell apoptosis might be explored in the future using site mutation.

Our project detected a functional relationship between O-GlcNAcylation and STAT3 phosphorylation, demonstrating that STAT3 is the target of O-GlcNAcylation. Furthermore, our results illustrated Tyr705 is sensitive to high glucose. O-GlcNAcylation could regulate p705STAT3 expression within relatively high levels, and partially mitigated HRVEC apoptosis induced by pSTAT3 inhibition and high glucose. We propose that O-GlcNAcylation protects HRVECs through the p705STAT3-VEGF pathway. Although Tyr705 is more sensitive to high glucose, Ser727 can be significantly negatively regulated by O-GlcNAcylation, and we recommend that the function of Ser727 in diabetic complications be explored in future research.

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Disclosure Statement

The authors declare to have no competing financial interests.

References

- 1 Ting DS, Cheung GC, Wong TY: Diabetic retinopathy: global prevalence, major risk factors, screening practices and public health challenges: a review. *Clin Exp Ophthalmol* 2016;44:260-277.
- 2 Hernández-Ramírez E, Sánchez-Chávez G, Estrella-Salazar LA, Salceda R: Nitrosative Stress in the Rat Retina at the Onset of Streptozotocin-Induced Diabetes. *Cell Physiol Biochem* 2017;42:2353-2363.
- 3 Sander B, Thornit DN, Colmorn L, Strøm C, Girach A, Hubbard LD, Lund-Andersen H, Larsen M: Progression of diabetic macular edema: correlation with blood retinal barrier permeability, retinal thickness, and retinal vessel diameter. *Invest Ophthalmol Vis Sci* 2007;48:3983-3987.
- 4 Ajlan RS, Silva PS, Sun JK: Vascular endothelial growth factor and diabetic retinal disease. *Semin Ophthalmol* 2016;31:40-48.
- 5 Zhang Y, Wang L, Zhang Y, Wang M, Sun Q, Xia F, Wang R, Liu L: Nogo-B Promotes Angiogenesis in Proliferative Diabetic Retinopathy via VEGF/PI3K/Akt Pathway in an Autocrine Manner. *Cell Physiol Biochem* 2017;43:1742-1754.

- 6 Qiu AW, Liu QH, Wang JL: Blocking IL-17A Alleviates Diabetic Retinopathy in Rodents. *Cell Physiol Biochem* 2017;41:960-972.
- 7 Ishimoto Y, Hirota-Takahata Y, Kurosawa E, Chiba J, Iwadata Y, Onozawa Y, Hasegawa T, Tamura A, Tanaka M, Kobayashi H: A Novel Natural Product-Derived Compound, Vestaine A1, Exerts both Pro-Angiogenic and Anti-Permeability Activity via a Different Pathway from VEGF. *Cell Physiol Biochem* 2016;39:1905-1918.
- 8 Zhou RM, Shen Y, Yao J, Yang H, Shan K, Li XM, Jiang Q, Yan B: Nmnat 1: a Security Guard of Retinal Ganglion Cells (RGCs) in Response to High Glucose Stress. *Cell Physiol Biochem* 2016;38:2207-2218.
- 9 Simon AM, Goodenough DA: Diverse functions of vertebrate gap junctions. *Trends Cell Biol* 1998;8:477-483.
- 10 Antonetti DA, Barber AJ, Khin S, Lieth E, Tarbell JM, Gardner TW: Vascular permeability in experimental diabetes is associated with reduced endothelial occludin content: vascular endothelial growth factor decreases occludin in retinal endothelial cells. *Penn State Retina Research Group. Diabetes* 1998;47:1953-1959.
- 11 Wright JN, Collins HE, Wende AR, Chatham JC: O-GlcNAcylation and cardiovascular disease. *Biochem Soc Trans* 2017;45:545-553.
- 12 Donovan K, Alekseev O, Qi X, Cho W, Azizkhan-Clifford J: O-GlcNAc modification of transcription factor Sp1 mediates hyperglycemia-induced VEGF-A upregulation in retinal cells. *Invest Ophthalmol Vis Sci* 2014;55:7862-7873.
- 13 Hart GW, Housley MP, Slawson C: Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature* 2007;446:1017-1022.
- 14 Zachara N, Akimoto Y, Hart GW: The O-GlcNAc Modification. *Essentials of Glycobiology* 2017;DOI: 10.1101/glycobiology.3e.019.
- 15 Mizutani M, Kern TS, Lorenzi M: Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *J Clin Invest* 1996;97:2883-2890.
- 16 Hirase T, Staddon JM, Saitou M, Ando-Akatsuka Y, Itoh M, Furuse M, Fujimoto K, Tsukita S, Rubin LL: Occludin as a possible determinant of tight junction permeability in endothelial cells. *J Cell Sci* 1997;110:1603-1613.
- 17 Gurel Z, Sieg KM, Shallow KD, Sorenson CM, Sheibani N: Retinal O-linked N-acetylglucosamine protein modifications: implications for postnatal retinal vascularization and the pathogenesis of diabetic retinopathy. *Mol Vis* 2013;19:1047-1059.
- 18 Liu GD, Xu C, Feng L, Wang F: The augmentation of O-GlcNAcylation reduces glyoxal-induced cell injury by attenuating oxidative stress in human retinal microvascular endothelial cells. *Int J Mol Med* 2015;36:1019-1027.
- 19 Xu C, Liu G, Liu X, Wang F: O-GlcNAcylation under hypoxic conditions and its effects on the blood-retinal barrier in diabetic retinopathy. *Int J Mol Med* 2014;33:624-632.
- 20 Gao P, Niu N, Wei T, Tozawa H, Chen X, Zhang C, Zhang J, Wada Y, Kapron CM, Liu J: The roles of signal transducer and activator of transcription factor 3 in tumor angiogenesis. *Oncotarget* 2017;8:69139-69161.
- 21 Sulkowska U, Wincewicz A, Kanczuga-Koda L, Koda M, Sulkowski S: Comparison of E-cadherin with STAT3 and apoptosis regulators: Bak and Bcl-xL in endometrioid adenocarcinomas of different ER-alpha immunoprofile. *Gynecol Endocrinol* 2017;22:1-4.
- 22 Zhang C, Deng Y, Lei Y, Zhao J, Wei W, Li Y: Effects of selenium on myocardial apoptosis by modifying the activity of mitochondrial STAT3 and regulating potassium channel expression. *Exp Ther Med* 2017;14:2201-2205.
- 23 Ye EA, Steinle JJ: miR-146a suppresses STAT3/VEGF pathways and reduces apoptosis through IL-6 signaling in primary human retinal microvascular endothelial cells in high glucose conditions. *Vision Res* 2017;139:15-22.
- 24 Al-Shabraway M, Bartoli M, El-Remessy AB, Ma G, Matragoon S, Lemtalsi T, Caldwell RW, Caldwell RB: Role of NADPH oxidase and Stat3 in statin-mediated protection against diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2008;49:3231-3238.
- 25 Yun JH, Park SW, Kim KJ, Bae JS, Lee EH, Paek SH, Kim SU, Ye S, Kim JH, Cho CH: Endothelial STAT3 Activation Increases Vascular Leakage Through Downregulating Tight Junction Proteins: Implications for Diabetic Retinopathy. *J Cell Physiol* 2017;232:1123-1134.
- 26 Zhang Y, Liu G, Dong Z: MSK1 and JNKs mediate phosphorylation of STAT3 in UVA-irradiated mouse epidermal JB6 cells. *J Biol Chem* 2001;276:42534-42542.
- 27 Andersson CX, Sopasakis VR, Wallerstedt E, Smith U: Insulin antagonizes interleukin-6 signaling and is anti-inflammatory in 3T3-L1 adipocytes. *J Biol Chem* 2007;282:9430-9435.
- 28 Sun Y, Zhou P, Chen S, Hu C, Bai Q, Wu H, Chen Y, Zhou P, Zeng X, Liu Z, Chen L: The JAK/STAT3 signaling pathway mediates inhibition of host cell apoptosis by Chlamydia psittaci infection. *Pathog Dis* 2017;75: doi: 10.1093/femsle/ftx088.

- 29 Liu B, Li CP, Wang WQ, Song SG, Liu XM: Lignans Extracted from *Eucommia Ulmoides* Oliv. Protects Against AGEs-Induced Retinal Endothelial Cell Injury. *Cell Physiol Biochem* 2016;39:2044-2054.
- 30 Shao J, Yin Y, Yin X, Ji L, Xin Y, Zou J, Yao Y: Transthyretin Exerts Pro-Apoptotic Effects in Human Retinal Microvascular Endothelial Cells Through a GRP78-Dependent Pathway in Diabetic Retinopathy. *Cell Physiol Biochem* 2017;43:788-800.
- 31 Gurel Z, Zaro BW, Pratt MR, Sheibani N: Identification of O-GlcNAc Modification Targets in Mouse Retinal Pericytes: Implication of p53 in Pathogenesis of Diabetic Retinopathy. *PLoS One* 2014;9:e95561.
- 32 Sato T, Haimovici R, Kao R, Li AF, Roy S: Downregulation of connexin 43 expression by high glucose reduces gap junction activity in microvascular endothelial cells. *Diabetes* 2002;51:1565-1571.
- 33 Peterson SB, Hart GW: New insights: A role for O-GlcNAcylation in diabetic complications. *Crit Rev Biochem Mol Biol* 2016;51:150-161.
- 34 Yun JH, Park SW, Kim KJ, Bae JS, Lee EH, Paek SH, Kim SU, Ye S, Kim JH, Cho CH: Endothelial STAT3 Activation Increases Vascular Leakage through Downregulating Tight Junction Proteins: Implications for Diabetic Retinopathy. *J Cell Physiol* 2017;232:1123-1134.
- 35 Vanlandingham PA, Nuno DJ, Quiambao AB, Phelps E, Wassel RA, Ma JX, Farjo KM, Farjo RA: Inhibition of Stat3 by a Small Molecule Inhibitor Slows Vision Loss in a Rat Model of Diabetic Retinopathy. *Invest Ophthalmol Vis Sci* 2017;58:2095-2105.
- 36 Jahangir Z, Ahmad W, Shabbiri K: Alternate Phosphorylation/O-GlcNAc Modification on Human Insulin IRs: A Road towards Impaired Insulin Signaling in Alzheimer and Diabetes: A road. *Adv Bioinformatics* 2014;2014:324753.
- 37 Ma J, Hart GW: O-GlcNAc profiling: from proteins to proteomes. *Clin Proteomics* 2014;11:8.
- 38 Cheng X, Hart GW: Alternative O-glycosylation/O-phosphorylation of serine-16 in murine estrogen receptor beta: post-translational regulation of turnover and transactivation activity. *J Biol Chem* 2001;276:10570-10575.
- 39 Dias WB, Cheung WD, Wang Z, Hart GW: Regulation of calcium/calmodulin-dependent kinase IV by O-GlcNAc modification. *J Biol Chem* 2009;284:21327-21337.
- 40 Smet-Nocca C, Broncel M, Wieruszeski JM, Tokarski C, Hanouille X, Leroy A, Landrieu I, Rolando C, Lippens G, Hackenberger CP: Identification of O-GlcNAc sites within peptides of the Tau protein and their impact on phosphorylation. *Mol Biosyst* 2011;7:1420-1429.
- 41 McCowen KC, Chow JC, Smith RJ: Leptin signaling in the hypothalamus of normal rats *in vivo*. *Endocrinology* 1998;139:4442-4447.
- 42 Park OK, Schaefer LK, Wang W, Schaefer TS: Dimer stability as a determinant of differential DNA binding activity of Stat3 isoforms. *J Biol Chem* 2000;275:32244-32249.
- 43 Verma NK, Davies AM, Long A, Kelleher D, Volkov Y: STAT3 knockdown by siRNA induces apoptosis in human cutaneous T-cell lymphoma line Hut78 via downregulation of Bcl-xL. *Cell Mol Biol Lett* 2010;15:342-355.
- 44 Gritsko T, Williams A, Turkson J, Kaneko S, Bowman T, Huang M, Nam S, Eweis I, Diaz N, Sullivan D, Yoder S, Enkemann S, Eschrich S, Lee JH, Beam CA, Cheng J, Minton S, Muro-Cacho CA, Jove R: Persistent activation of stat3 signaling induces survivin gene expression and confers resistance to apoptosis in human breast cancer cells. *Clin Cancer Res* 2006;12:11-19.
- 45 Zhu WQ, Wang J, Guo XF, Liu Z, Dong WG: Thymoquinone inhibits proliferation in gastric cancer via the STAT3 pathway *in vivo* and *in vitro*. *World J Gastroenterol* 2016;22:4149-4159.
- 46 Ding C, Li L, Yang T, Fan X, Wu G: Combined application of anti-VEGF and anti-EGFR attenuates the growth and angiogenesis of colorectal cancer mainly through suppressing AKT and ERK signaling in mice model. *BMC Cancer* 2016;16:791.
- 47 Zhang SS, Wei JY, Li C, Barnstable CJ, Fu XY: Expression and activation of STAT proteins during mouse retina development. *Exp Eye Res* 2003;76:421-431.
- 48 Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL, Cheng JQ, Jove R, Yu H: Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. *Oncogene* 2005;24:5552-5560.
- 49 Wei J, Jiang H, Gao H, Wang G: Blocking Mammalian Target of Rapamycin (mTOR) Attenuates HIF-1 α Pathways Engaged-Vascular Endothelial Growth Factor (VEGF) in Diabetic Retinopathy. *Cell Physiol Biochem* 2016;40:1570-1577.
- 50 Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N: Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 1998;273:30336-30343.
- 51 Fan J, Ponferrada VG, Sato T, Vemaraju S, Fruttiger M, Gerhardt H, Ferrara N, Lang RA: Crim1 maintains retinal vascular stability during development by regulating endothelial cell VEGFA autocrine signaling. *Development* 2014;141:448-459.