

# STAT1-mediated Bim expression promotes the apoptosis of retinal pericytes under high glucose conditions

ES Shin<sup>1</sup>, Q Huang<sup>1,4</sup>, Z Gurel<sup>1</sup>, TL Palenski<sup>1</sup>, I Zaitoun<sup>1</sup>, CM Sorenson<sup>2</sup> and N Sheibani<sup>1,3</sup>\*

Hyperglycemia impacts different vascular cell functions and promotes the development and progression of various vasculopathies including diabetic retinopathy. Although the increased rate of apoptosis in pericytes (PCs) has been linked to increased oxidative stress and activation of protein kinase C- $\delta$  (PKC- $\delta$ ) and SHP-1 (Src homology region 2 domain-containing phosphatase-1) tyrosine phosphatase during diabetes, the detailed mechanisms require further elucidation. Here we show that the rate of apoptosis and expression of proapoptotic protein Bim were increased in the retinal PCs of diabetic Akita/+ mice and mouse retinal PCs cultured under high glucose conditions. Increased Bim expression in retinal PCs under high glucose conditions required the sustained activation of signal transducer and activator of transcription 1 (STAT1) through production of inflammatory cytokines. PCs cultured under high glucose conditions also exhibited increased oxidative stress and diminished migration. Inhibition of oxidative stress, PKC- $\delta$  or Rho-associated protein kinase I/II was sufficient to protect PCs against apoptosis under high glucose conditions. Furthermore, PCs deficient in Bim expression were protected from high glucose-mediated increased oxidative stress and apoptosis. However, only inhibition of PKC- $\delta$  lowered Bim levels. *N*-acetylcysteine did not affect STAT1 levels, suggesting that oxidative stress is downstream of Bim. PCs cultured under high glucose conditions disrupted capillary morphogenesis of retinal endothelial cells (ECs) in coculture experiments. In addition, conditioned medium prepared from PCs under high glucose conditions attenuated EC migration. Taken together, our results indicate that Bim has a pivotal role in the dysfunction of retinal PCs under high glucose conditions by increasing oxidative stress and death of PCs.

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Dysfunction in retinal blood vessel and pathological neovascularization causes blindness in patients with diabetes.<sup>1,2</sup> Mechanisms of vascular dysfunction in diabetes have been the subject of numerous studies. A causal link between diabetic hyperglycemia and the development of retinal vascular complications is established, considering that tight glucose control in diabetic patients reduces the progression of the disease.<sup>3</sup> Elucidating mechanisms for dysfunction of retinal vascular cell under high glucose conditions is necessary to develop new therapeutics for diabetic retinopathy.

Pericytes (PCs), smooth muscle-like cells that envelope capillaries provide vessel stability and control endothelial cell (EC) proliferation and survival. PCs have attracted much attention since the inception of studies to understand the pathogenesis of diabetic retinopathy. Alterations in the interactions between PCs and ECs have crucial roles in the

development of diabetic retinopathy.<sup>4</sup> Unfortunately, very little is known about the nature of these interactions, and how they are altered in diabetes.

PC loss is considered a hallmark of early diabetic retinopathy, which can result in focal EC proliferation associated with microaneurysm formation.<sup>5</sup> Recent studies suggest that loss of PCs contributes not only to the vasodynamic changes in the early stages of diabetic retinopathy but also to neovascularization in proliferative diabetic retinopathy.<sup>6</sup> In addition, several studies indicate that retinal PCs selectively degenerate to form PC ghosts, while ECs remain relatively constant, or pathologically proliferate when neovascularization forms.<sup>6–8</sup> Although apoptosis of vascular cells is documented in the early stages of diabetic retinopathy, we believe that PCs sense and respond to hyperglycemia differently compared with ECs.

<sup>1</sup>Department of Ophthalmology and Visual Sciences, University of Wisconsin School of Medicine and Public Health, Madison, WI 53792, USA; <sup>2</sup>Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, WI 53792, USA and <sup>3</sup>Mchpherson Eye Research Institute, University of Wisconsin School of Medicine and Public Health, Madison, WI 53792, USA

\*Corresponding author: N Sheibani, Department of Ophthalmology and Visual Sciences, University of Wisconsin School of Medicine and Public Health, 600 Highland Avenue, K6/458 CSC, Madison, WI 53792-4673, USA. Tel: +1 608 263 3345; Fax: +1 608 265 6021; E-mail: nsheibanikar@wisc.edu

<sup>4</sup>Current address: Department of Ophthalmology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

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**Abbreviations:** DHE, dihydroethidium; EC, endothelial cell; ERK, extracellular signal-related kinase; HG, high glucose; IL-1 $\beta$ , interleukin-1 $\beta$ ; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; NAC, *N*-acetylcysteine; NG, normal glucose; PBS, phosphate-buffered saline; PC, pericytes; PDGFR- $\beta$ , platelet-derived growth factor receptor- $\beta$ ; PKC, protein kinase C; ROCK, Rho-associated protein kinase; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SHP-1, Src homology region 2 domain-containing phosphatase-1; STAT, signal transducer and activator of transcription; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TUNEL, terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling

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The BCL-2 protein family regulates apoptosis by maintaining mitochondrial homeostasis through a balanced activity of pro- and antiapoptotic family members.<sup>9</sup> Among BCL-2 family of proteins, Bim is a proapoptotic protein with only one Bcl-2 homology (BH3) domain. Bim is required for the activation of cell death pathways mediated by other Bcl-2 proapoptotic family members, namely BAX and BAK.<sup>10</sup> Bim expression is increased in the neuroretina of diabetic patients compared with non-diabetics.<sup>11</sup> However, the role of Bim in the development and progression of diabetic retinopathy has not been addressed previously.

We recently described a novel method for isolation and culture of retinal PCs from wild-type and transgenic mice.<sup>12</sup> Here we demonstrate that mouse retinal PCs not only exhibited higher rates of apoptosis, as shown in PCs from other species,<sup>13,14</sup> but also exhibited attenuation of migration under high glucose conditions. High glucose also had a significant impact on production of reactive oxygen species (ROS) and interaction of ECs and PCs during capillary morphogenesis. Furthermore, we uncovered that increased Bim expression required sustained activation of signal transducer and activator of transcription 1 (STAT1) through increased production of inflammatory cytokines. Furthermore, PCs prepared from Bim  $-/-$  mice were protected from high glucose ill effects. Collectively, our results suggest a pivotal role for Bim expression and/or activation in the dysfunction of PCs under high glucose conditions.

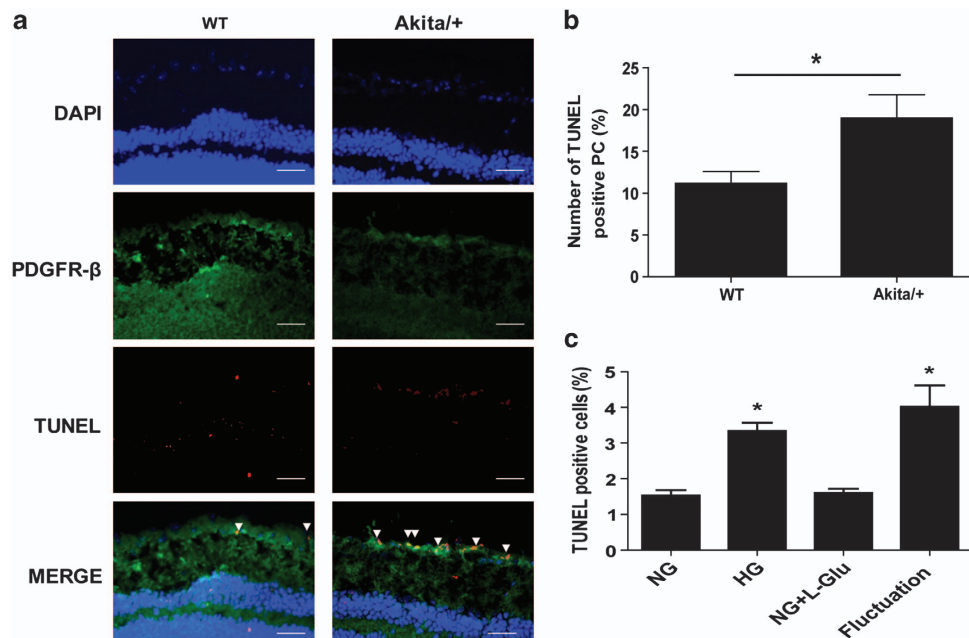
## Results

**Apoptosis of PCs was increased in the retina of diabetic mice and under high glucose conditions.** Apoptosis of PCs in mouse retina was determined in wild-type and

Ins2Akita/+ mice with 7 months of diabetes. Akita/+ mice carry a mutation in their insulin gene and develop diabetes by 4 weeks of age. Diabetes is more prominent in male mice. These mice do exhibit many of the early non-proliferative change in their retina. In immunofluorescence staining for terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) (apoptotic cells) and platelet-derived growth factor receptor- $\beta$  (PDGFR- $\beta$ ) (PCs), an increase in TUNEL-positive PCs in diabetic retinas was observed compared with wild-type mice (Figure 1a). Quantification for TUNEL-positive PCs is shown in Figure 1b.

We next determined the impact of high glucose on retinal PC apoptosis in culture. The effect of high glucose on mouse retinal PCs has not been previously addressed. Retinal PCs were cultured in medium under normal, high glucose or osmolarity control for 5 days as detailed and previously used by us and others.<sup>15–17</sup> Consistent with previous studies,<sup>13</sup> mouse retinal PCs cultured under high glucose conditions exhibited a significantly higher rate of apoptosis compared with cells cultured under normal glucose or osmolarity control (Figure 1c). A similar rate of apoptosis to that observed under high glucose conditions was noted in PCs cultured under fluctuating glucose conditions (Figure 1c).

**Bim level was increased in retinas of diabetic mice.** We next examined whether expression of Bcl-2 family member Bim was altered in retinas of diabetic Ins2Akita/+ mice. Bim is a proapoptotic member of the Bcl-2 family with significant role in retinal vascular homeostasis.<sup>18</sup> The expression level of Bim in mouse retina was determined in wild-type and Ins2Akita/+ mice. In immunofluorescence staining for Bim and PDGFR- $\beta$ , an increase in Bim retinas was observed in



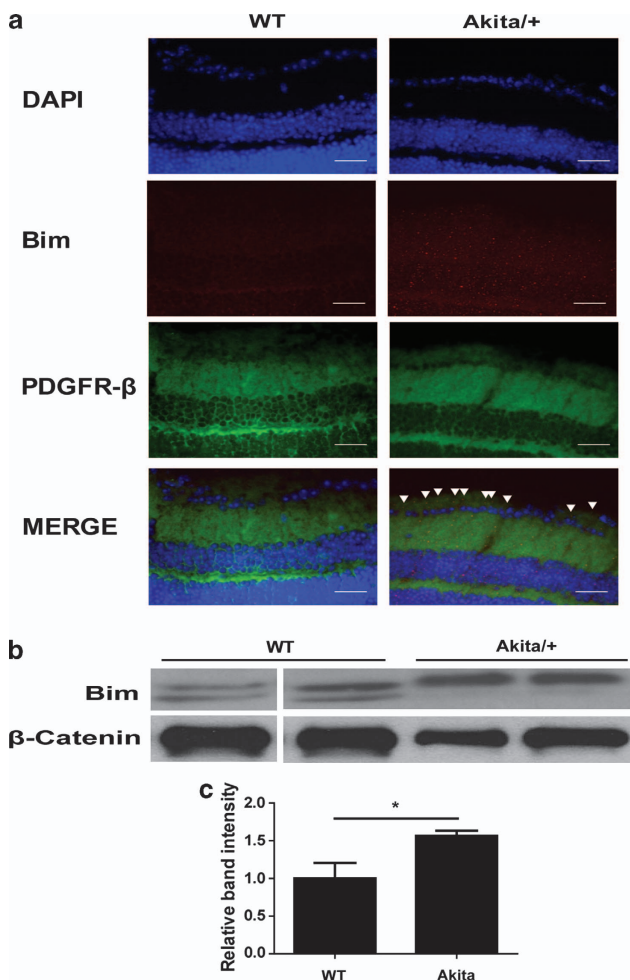
**Figure 1** Diabetes induces apoptosis of PCs in retinal vasculature. (a) Retinal frozen sections were prepared from wild-type and Akita/+ mice. Double immunofluorescence staining was performed for PDGFR- $\beta$  and TUNEL. Arrowheads indicate TUNEL- and PDGFR- $\beta$ -positive cells in the superficial layer of the retina. Bar indicates 20  $\mu$ m. (b) Quantification of TUNEL and PDGFR- $\beta$ -positive cells is shown. Data are presented as mean  $\pm$  S.E.M. ( $n = 7$  for wild-type and  $n = 8$  for Akita/+ mice;  $*P < 0.05$  compared with wild-type mice). (c) The effects of high glucose on apoptosis of retinal PCs. Retinal PCs cultured under high glucose conditions exhibited a significantly higher rate of apoptosis. NG: normal glucose condition; HG: high glucose condition. Similar results were observed under fluctuation conditions ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG or fluctuation))

diabetic compared with wild-type mice (Figure 2a). In superficial layer of retina, the expression level of Bim was increased in PCs. To further confirm increased Bim expression in diabetic mice retina, lysates from retinas were analyzed by western blotting. Bim level was significantly elevated in the retina of *Ins2Akita/+* mice compared with wild-type mice (Figures 2b and c). In addition, the diabetic retina predominantly expressed the extra long form of Bim (BimEL), while non-diabetic retina expressed the shorter isoforms of Bim (BimL and BimS). Furthermore, BimEL is generally the prominent isoform when Bim level is upregulated.<sup>19</sup>

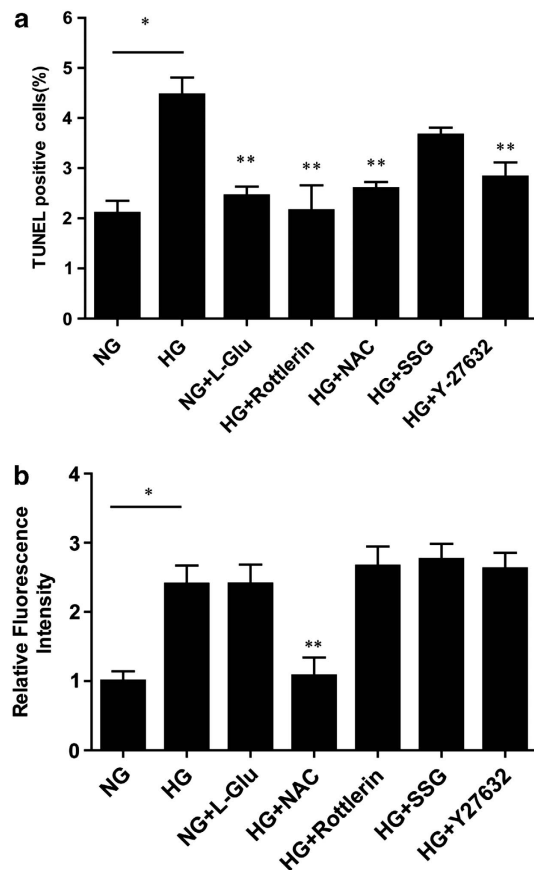
**Oxidative stress is an upstream effector of high glucose-mediated PC dysfunction.** Exposure of perivascular supporting cells to high glucose generally results in increased oxidative stress and apoptosis,<sup>13,20,21</sup> perhaps through

activation of protein kinase C- $\delta$  (PKC- $\delta$ ) promoting diabetic retinopathy.<sup>13</sup> To determine whether increased oxidative stress and/or activation of PKC- $\delta$  contributes similar to high glucose-induced apoptosis of mouse retinal PCs, we evaluated the rate of apoptosis in the presence of the *N*-acetylcysteine (NAC), an antioxidant with significant inhibitory effect on pathogenesis of diabetic retinopathy,<sup>22,23</sup> or Rottlerin. Rottlerin is a PKC- $\delta$ -specific inhibitor with an IC<sub>50</sub> of 3–6  $\mu$ M for PKC- $\delta$ .<sup>24,25</sup> Rottlerin inhibits apoptosis of smooth muscle cells in response to oxidative stress.<sup>26</sup> Incubation with Rottlerin or NAC protected PCs from high glucose-mediated apoptosis (Figure 3a). Inhibition of SHP-1 (Src homology region 2 domain-containing phosphatase-1), as demonstrated previously,<sup>13</sup> was also protective, although to a lesser extent (Figure 3a).

RhoA/Rho-associated protein kinase (ROCK) signaling pathway has an important role in regulation of angiogenesis,



**Figure 2** Bim expression was increased in the retina of diabetic mice. (a) Retinal frozen sections were prepared from wild-type and *Akita/+* mice. Double immunofluorescence staining was performed for PDGFR- $\beta$  and Bim. Arrowheads indicate Bim- and PDGFR- $\beta$ -positive cells in the superficial layer of retina. Bar indicates 20  $\mu$ m. (b) Expression of Bim in the retina of wild-type and *Akita/+* mice was examined by western blot analysis. The antibody to  $\beta$ -catenin was used for loading control. (c) Quantification of band intensity in western blots ( $n \geq 4$ ; \* $P \leq 0.05$  (wild type versus *Akita/+*))



**Figure 3** Signaling pathways involved in high glucose (HG)-mediated apoptosis and increased ROS production in PCs. (a) An increase in the rate of apoptosis was observed in PCs cultured under HG compared with normal glucose (NG) or osmolarity control (NG + L-Glu) conditions. Incubation of PCs under HG with NAC (antioxidant), Rottlerin (PKC- $\delta$  inhibitor) or Y-27632 (ROCK I/II inhibitor) reduced the rate of apoptosis. SSG (SHP-1 inhibitor) had a modest effect ( $n \geq 3$ ; \* $P \leq 0.05$  (NG versus HG); \*\* $P \leq 0.05$  (HG versus NG + L-Glu or HG + inhibitors)). (b) Retinal PCs cultured under HG conditions exhibited a significantly elevated level of ROS compared with NG or NG + L-Glu conditions. Incubation of PCs under HG with NAC reduced the level of ROS. Inhibition of PKC- $\delta$ , SHP-1 and ROCK I/II had no significant effect on ROS levels under HG conditions ( $n \geq 30$ ; \* $P \leq 0.05$  (NG versus HG); \*\* $P \leq 0.05$  (HG versus HG + NAC))

and its opposition by mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) signaling promotes EC survival and sprouting during angiogenesis.<sup>27–29</sup> Very little is known about the contribution of RhoA/ROCK pathway to the function of PCs. We next determined the impact of ROCK I/II inhibitor, Y-27632, on PC apoptosis under high glucose conditions. Inhibition of ROCK I/II protected PCs from high glucose-induced apoptosis (Figure 3a). Thus, activation of RhoA/ROCK pathway makes a significant contribution to the proapoptotic phenotype of retinal PCs under high glucose conditions.

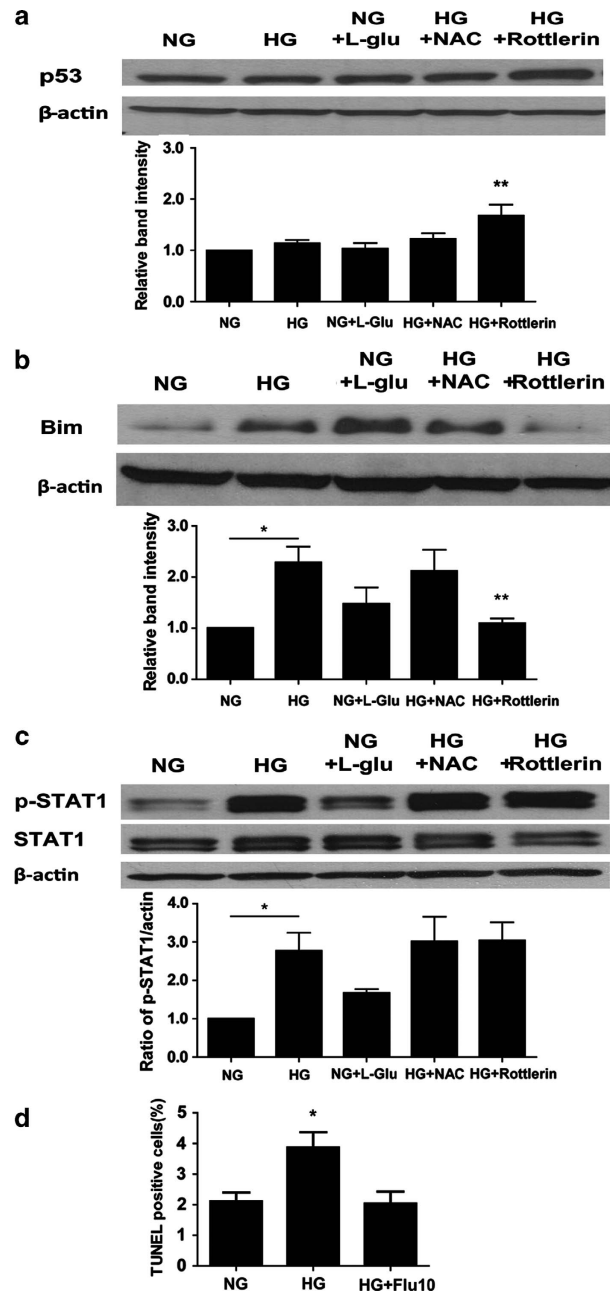
To examine the effect of high glucose condition on oxidative stress in PCs, the level of ROS was measured by dihydroethidium (DHE) staining. High glucose condition indeed elevated ROS levels in PCs. Osmolarity control conditions also increased ROS levels in PCs. This is consistent with a previous report showing that hyperosmotic stress elevates ROS levels.<sup>30</sup> NAC inhibited ROS production under high glucose condition. In contrast, Rottlerin, sodium stibogluconate (SSG; an SHP-1-specific inhibitor<sup>31</sup>) and Y-27632 did not affect ROS production in PCs under high glucose conditions (Figure 3b). Thus, oxidative stress is an upstream effector of high glucose-mediated PC dysfunction.

#### High glucose enhanced Bim expression in retinal PCs.

The BCL-2 protein family members have important roles in regulation of apoptosis and angiogenesis.<sup>18,32</sup> We also assessed p53 expression under various glucose conditions. The p53 expression was minimally affected in PCs cultured under various glucose conditions (Figure 4a). Although incubation of PCs with NAC under high glucose conditions had no significant effect on p53 expression, incubation of PCs with PKC- $\delta$  inhibitor resulted in a modest but significant increase in p53 expression under high glucose conditions.

We observed a significant increase in Bim expression of PCs cultured under high glucose conditions compared with normal glucose or osmolarity control (Figure 4b). Although incubation of PCs with NAC in high glucose did not have a significant effect on Bim expression, inhibition of PKC- $\delta$  reduced Bim expression to levels comparable to that seen in cells cultured in normal glucose. Expression of Bim in pancreatic  $\beta$ -cells is regulated by the activation of STAT1.<sup>33,34</sup> To elucidate the mechanism(s) for the upregulation of Bim expression, we examined the level of active and total STAT1 in PCs under various glucose conditions. The level of phosphorylated (p)-STAT1 in PCs cultured under high glucose conditions was significantly increased compared with normal glucose or osmolarity control (Figure 4c). Inhibition of ROS or PKC- $\delta$  under high glucose condition did not affect the level of p-STAT1.

We next examined if inhibition of STAT1 protects PCs from apoptosis induced by high glucose conditions. Fludarabine is a purine analog and potent inhibitor of STAT1 activity.<sup>35</sup> Inhibition of STAT1 by fludarabine protected PCs against apoptosis under high glucose condition (Figure 4d). Thus, activation of STAT1, perhaps by production of inflammatory cytokines including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) in PCs under high glucose conditions may occur. In fact, Supplementary Figures 1a and b show that incubation of PCs under high glucose conditions resulted in



**Figure 4** Impact of high glucose (HG) on proapoptotic pathways. (a) Expression of p53 was confirmed by western blot analysis. The quantitative assessment of the data is also shown. No significant change in p53 expression was observed. NAC had no effect on the level of p53, while inhibition of PKC- $\delta$  caused a modest but significant increase in p53 level ( $n \geq 3$ ;  $*P \leq 0.05$  (normal glucose (NG) versus HG);  $**P \leq 0.05$  (HG versus HG + Rottlerin)). (b) HG resulted in a significant increase in Bim expression compared with NG or osmolarity control (NG + L-Glu). Although incubation of PCs under HG with NAC had no significant effect on Bim expression, inhibition of PKC- $\delta$  (Rottlerin) resulted in significant decrease in Bim expression. (c) HG increased p-STAT1 level significantly compared with NG or NG + L-Glu. NAC and Rottlerin did not affect p-STAT1 level under HG condition. Graphs of band intensities from three independent experiments are also shown ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG);  $**P \leq 0.05$  (HG versus HG + Rottlerin)). (d) Fludarabine, an inhibitor for STAT1, reduced the rate of apoptosis in PCs against apoptosis. HG condition increased the rate of apoptosis compared with NG and incubation of PCs with fludarabine under HG condition decreased the rate of apoptosis compared with HG conditions ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG and HG versus HG + Flu10))

increased expression of TNF- $\alpha$  compared with normal glucose conditions.

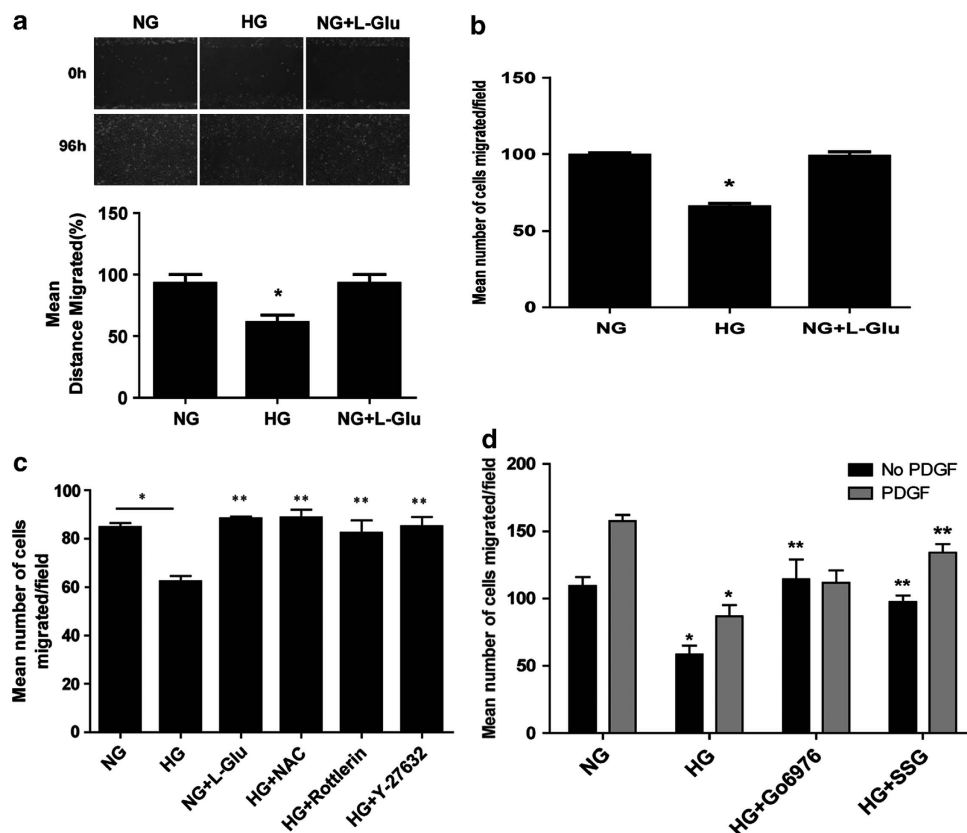
We next examined which cytokines is required for PC apoptosis. Supplementary Figure 1C shows incubation of PCs with TNF- $\alpha$ -induced PC apoptosis, whereas monocyte chemoattractant protein-1 (MCP-1) and IL-1 $\beta$  did not have any effect. The effect of inflammatory cytokines on the activation of STAT1 was then examined in PCs incubated with TNF- $\alpha$  and IL-1 $\beta$  under normal glucose conditions. TNF- $\alpha$  and IL-1 $\beta$  increased p-STAT1 level in retinal PCs (Supplementary Figure 1D). Thus, incubation of PCs under high glucose conditions results in the production of inflammatory cytokines, including TNF- $\alpha$ , and sustained activation of STAT1 driving Bim expression.

**High glucose inhibited retinal PC migration.** Migration of PCs is essential during vascular development and stabilization of newly formed vessels.<sup>36</sup> Alteration in migration of PCs may also contribute to pathogenesis of DR.<sup>37</sup> The impact of high glucose on migration of retinal PCs has not been previously addressed. We next assessed the migratory

properties of PCs in transwell and scratch wound assays. We observed decreased PC migration under high glucose conditions compared with normal or control conditions in both scratch wound (Figure 5a) and transwell (Figure 5b) migration assays.

We next determined the downstream events that may contribute to the PC migratory defect under high glucose conditions. PCs incubated under high glucose conditions, in the presence of NAC, showed migration similar to that observed in PCs incubated under normal glucose or osmolarity control (Figure 5c). Similar results were observed in the presence of PKC- $\delta$  and ROCK I/II inhibitor (Figure 5c). Furthermore, retinal PCs cultured under normal glucose responded to promigratory activity of PDGF-BB, while basal and PDGF-BB-mediated migration of PCs under high glucose conditions was attenuated (Figure 5d). In contrast, PDGF stimulated the migration of PCs in high glucose in the presence of NAC (data not shown).

The activation of PKC- $\alpha$  also negatively impacts migration of SMC in response to PDGF.<sup>38</sup> We also determined whether activation of PKC- $\alpha$  contributes to high glucose-mediated



**Figure 5** Attenuation of PC migration under high glucose (HG) conditions. (a) In scratch wound assays, a significant portion of wound remained uncovered in PCs cultured under HG compared with normal glucose (NG) or osmolarity control (NG + L-Glu) conditions. The quantitative assessment of the data is shown below ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG or HG versus NG + L-Glu)). (b) A significantly lower number of PCs cultured under HG conditions migrated through the transwell membrane compared with PCs cultured under NG or NG + L-Glu conditions ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG or HG versus NG + L-Glu)). (c) Incubation of PCs cultured under HG with NAC restored basal migration. Incubation of retinal PCs under HG conditions with Rottlerin (PKC- $\delta$  inhibitor) or Y-27632 (ROCK I/II inhibitor) restored basal migration to levels seen in PCs cultured under NG or NG + L-Glu conditions ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG),  $**P \leq 0.05$  (HG versus NG + L-Glu or HG + NAC or HG + Rottlerin or HG + Y-27632)). (d) Incubation of PCs with PDGF-BB enhanced their migration under NG conditions. However, PDGF-BB had a minimal effect on migration of PCs cultured under HG. Although PKC- $\alpha$  inhibitor (Gö6976) restored basal migration of PCs cultured in HG, it did not restore migratory response to PDGF-BB. The inhibition of SHP-1 (SSG) in PCs cultured in HG restored both basal and PDGF-stimulated migration ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG),  $**P \leq 0.05$  (HG versus HG + Gö6976 or HG + SSG))

inhibition of retinal PC migration. Although inhibition of PKC- $\alpha$  restored basal migration of PCs under high glucose conditions, it minimally affected their PDGF-mediated migration (Figure 5d). Activation of SHP-1 tyrosine phosphatase is recently shown to impact negatively PDGF receptor signaling in retinal PCs under high glucose conditions.<sup>13</sup> Inhibition of SHP-1 activity using the SHP-1-specific inhibitor SSG<sup>31,39,40</sup> restored both basal and PDGF-mediated migration of PCs under high glucose conditions (Figure 5d).

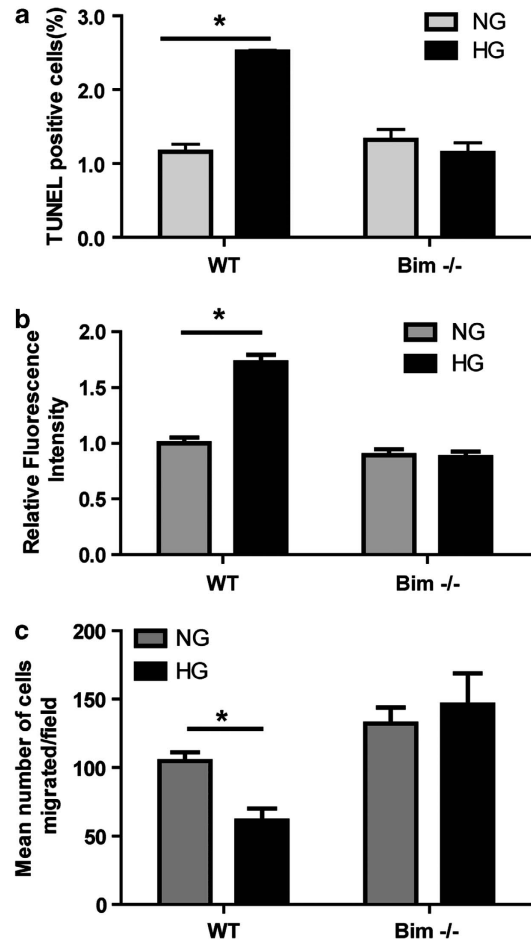
**Bim -/- PCs are protected from high glucose-mediated oxidative stress and apoptosis.** Retinal PCs isolated from Bim -/- mice were used to investigate the function of PCs under normal or high glucose conditions. Apoptosis of Bim -/- PCs was not affected under high glucose conditions, whereas high glucose increased the apoptosis rate of wild-type PCs (Figure 6a). As indicated above, increased ROS production by high glucose is an upstream event in inducing apoptosis of PCs. High glucose condition did not impact ROS production in Bim -/- PCs, whereas ROS production in wild-type PCs was increased under high glucose conditions (Figure 6b).

Migratory property is crucial for the function of PCs in maintaining vascular structure and function. The migration of wild-type PCs was attenuated under high glucose condition compared with normal glucose conditions. However, the migration of Bim -/- PCs under high glucose conditions was not altered (Figure 6c). Thus, Bim expression may promote PC dysfunction under high glucose conditions.

**PCs incubated under high glucose inhibited capillary morphogenesis and migration of ECs.** We recently showed that inflammatory mediators have a significant impact on capillary morphogenesis of retinal ECs.<sup>41</sup> We next determined the effects of conditioned medium collected from PCs cultured under various glucose conditions on migration of retinal EC. Conditioned medium from PCs under high glucose conditions inhibited migration of ECs compared with PC conditioned medium under normal glucose or control (Figure 7a).

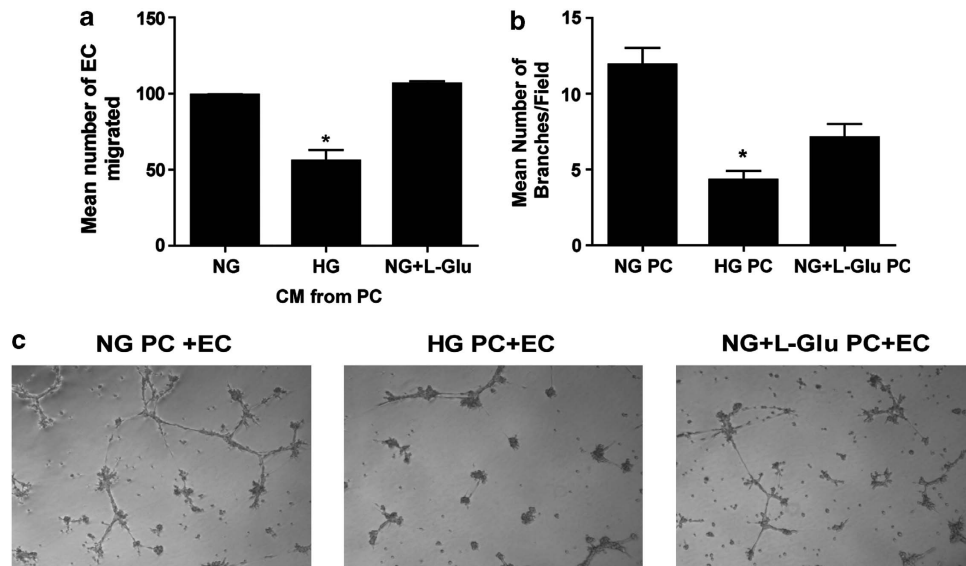
ECs undergo capillary morphogenesis when grown on Matrigel, which mimics the late stage of angiogenesis.<sup>42</sup> PCs were incubated under various glucose conditions and used in coculture experiments with retinal ECs. When ECs were mixed with PCs cultured under high glucose conditions, capillary morphogenesis of ECs was significantly inhibited compared with PCs cultured under normal glucose conditions (Figure 7b). Representative images of capillary morphogenesis are shown in Figure 7c.

**Altered intracellular signaling pathways in retinal PCs cultured under high glucose conditions.** Increased apoptosis of PCs under high glucose conditions is attributed to altered intracellular signaling pathways with important roles in cell survival, including Src/phosphoinositide 3 kinase, Akt, and MAPKs. We determined the activities of selected signaling molecules and pathways that impact PC function. Src phosphorylation was decreased in PCs under high glucose conditions compared with normal glucose or osmolarity control (Supplementary Figure 2A).



**Figure 6** Bim expression is essential for high glucose (HG)-mediated apoptosis and ROS production of PCs. (a) Bim -/- PCs were resistant to HG-induced apoptosis. Wild-type (WT) and Bim -/- PCs were incubated under normal glucose (NG) or HG conditions for 5 days. HG did not increase the rate of apoptosis in Bim -/- PCs ( $n \geq 3$ ;  $*P \leq 0.05$  (LG versus HG)). (b) Bim -/- PCs generated less ROS under HG conditions ( $n \geq 100$ ;  $*P \leq 0.05$  (NG versus HG)). (c) Migration of Bim -/- PCs was not affected under HG conditions, whereas the migration of WT PCs was attenuated under HG condition ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG))

Supplementary Figure 2B also shows decreased activation of Akt1 in PCs under high glucose and osmolarity control conditions compared with retinal PCs under normal glucose conditions. These results imply that osmotic challenge attenuates the activation of Akt1 in PCs as shown by others.<sup>17</sup> In addition, activation of MAPK/ERKs, downstream effectors of Src kinase pathway, was also downregulated in retinal PCs under high glucose and osmolarity control compared with normal conditions (Supplementary Figure 2C). The levels of phosphorylated MAPK/p38 were not altered in PCs under high glucose conditions (Supplementary Figure 2D). In contrast, MAPK/c-Jun N-terminal kinase 1 (JNK1), whose activation is related to stress stimuli and increased apoptosis, was upregulated in PCs under high glucose conditions compared with normal glucose or control (Supplementary Figure 2E). Thus, high glucose conditions modulate various intracellular signaling pathways with significant impact on PC proliferation, migration and apoptosis.



**Figure 7** Incubation of PCs under high glucose (HG) conditions impacts their effect on EC function. (a) Conditioned medium was prepared from PCs incubated under normal glucose (NG), HG or control for 5 days. The effects of these conditioned medium on the migration of retinal EC was determined in a transwell assay. Please note a significant decrease in migration of retinal EC incubated with conditioned medium from PCs cultured in HG ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG and HG versus osmolarity control (NG + L-Glu))). (b) Retinal PCs were incubated under various glucose conditions for 5 days and used to assess their impact on retinal EC capillary morphogenesis in coculture (1:1) experiments. The mean number of branch points in 10 high power fields ( $\times 100$ ) was determined. Please note a significant decrease in capillary morphogenesis of retinal ECs incubated with PCs under HG conditions ( $n \geq 3$ ;  $*P \leq 0.05$  (NG versus HG and HG versus NG + L-Glu))). Representative images for each condition are shown in (c)

## Discussion

Here we used mouse retinal PCs to further delineate their response to high glucose and to elucidate the molecular mechanisms involved. Apoptosis of PCs was significantly increased under high glucose conditions and in the retina of diabetic Akita/+ mice. We showed that high glucose conditions increased Bim expression in PCs through the sustained activation of STAT1. The activation of STAT1 was mediated by the increased production of inflammatory cytokines including TNF- $\alpha$  under high glucose conditions. Bim is a proapoptotic protein and its level is significantly elevated in the neuroretina of diabetic patients.<sup>11</sup> These results are consistent with increased Bim levels in the retina of diabetic Akita/+ mice and retinal PCs cultured under high glucose conditions reported here. Thus, increased Bim level in the retina, and more specifically in PCs, may be a crucial step in the development and progression of diabetic retinopathy.

To further demonstrate the important role of Bim in the apoptosis of PCs under high glucose conditions, we determined the apoptosis rate of retinal PCs prepared from Bim -/- mice under high glucose conditions. Bim deficiency protected PCs from apoptosis induced by high glucose conditions and reduced ROS levels. Thus, Bim expression contributes to ROS production under high glucose conditions and promotion of PC apoptosis. To elucidate how high glucose condition may increase Bim expression, we examined the level of p-STAT1 in PCs under various glucose conditions. STAT1 is a transcription factor and a known regulator of Bim expression, which is activated by proinflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$ , and

interferon- $\gamma$ .<sup>33,34</sup> We observed that high glucose conditions resulted in increased p-STAT1 level in PCs.

The increase in p-STAT1 level in PCs may result from increased level of proinflammatory cytokines produced by PCs under high glucose conditions. We observed that high glucose conditions significantly increased the mRNA level of TNF- $\alpha$  in PCs. High glucose condition is also shown to increase p-STAT1 level in renal tubular epithelial cell.<sup>43</sup> Although NAC treatment under high glucose decreased p-STAT1 level in tubular epithelial cell, NAC treatment under high glucose did not affect the p-STAT1 level in PCs. This discrepancy may be attributed to the differences in duration of NAC treatment. Huang *et al.*<sup>43</sup> treated renal tubular epithelial cells with NAC for 30 min under high glucose, but only that here PCs were exposed to NAC along with high glucose conditions for 5 days. Thus, chronic exposure to high glucose might contribute to sustained production of inflammatory mediators and activation of STAT1 in PCs and lack of response to NAC.

STAT1 has been considered as a drug target for vascular disease and fludarabine, a nucleoside analog, reduces STAT1 phosphorylation without affecting other STATs.<sup>44</sup> We observed the protective effect of fludarabine against apoptosis induced by high glucose condition in PCs. Fludarabine also decreased Bim expression under high glucose conditions in PCs (not shown). Considering STAT1-mediated Bim expression and the role of Bim in the dysfunction of PCs under high glucose conditions, compounds affecting STAT1 expression and/or activity may alleviate the symptom of diabetic retinopathy.

Bim expression level is also regulated by various microRNAs (miRs). miR-24 is shown to attenuate apoptosis of cardiomyocytes by reducing Bim expression.<sup>45</sup> In osteoblast,

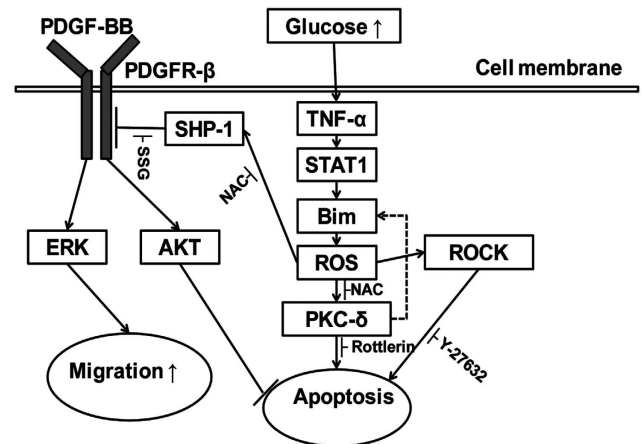
the miR cluster miR-17-92a inhibits apoptosis by reducing Bim expression level.<sup>46</sup> However, the effect of high glucose or diabetic condition on regulation of miRNAs including miR-17-92a and miR-24 in retinal PCs remains unknown and is subject of current investigation in our laboratory.

The apoptosis of mouse retinal PCs cultured under high glucose conditions was also attenuated by the inhibition of downstream signaling events including PKC- $\delta$ , ROS production and ROCK I/II activity. The activation of these pathways under high glucose was previously shown to be responsible for PC dysfunction.<sup>13,47,48</sup> Only NAC reduced ROS levels in PCs under high glucose, whereas the inhibition of PKC- $\delta$ , ROCK I/II or SHP-1 were ineffective. These results suggest that ROS production is an upstream event leading to the apoptosis of PCs under high glucose conditions. However, incubation of PCs with NAC under high glucose conditions did not affect Bim expression. Thus, Bim activity is upstream of ROS production.

Very few studies have systemically examined retinal PC migration under high glucose conditions. This property of PCs plays critical roles during angiogenesis and during the integrity of vascular function. Under high glucose conditions, the migration of wild-type PCs was significantly decreased. In contrast, high glucose had no significant effect on migration of Bim -/- PCs. Taken together, these results suggest that high glucose impairs PC migration and is impacted by Bim expression. Incubation of PCs with NAC, Rottlerin, Y-27632 and SSG under high glucose conditions restored PC migration, confirming the role of oxidative stress, PKC- $\delta$ , ROCK I/II and SHP-1 in PC migration.

PDGF-BB is secreted by EC promoting the recruitment of PCs to newly formed blood vessels and their stabilization.<sup>49</sup> We showed that high glucose impaired PDGF-BB-mediated migration of PCs, which was reversed by inhibition of SHP-1. Thus, increased SHP-1 activity under high glucose conditions impairs not only PDGFR- $\beta$ -mediated survival but also migration of PCs by reducing PDGFR- $\beta$  activity.

Interactions between ECs and PCs are crucial for their survival and maintenance of vessel structure and function. To examine the effect of high glucose conditions on the interactions between retinal ECs and PCs, the effect of soluble factors released in the conditioned medium of PCs on migration of ECs was also investigated. Conditioned medium from PCs under high glucose conditions attenuated the migration of retinal ECs. We also examined the effect of PCs incubated under various glucose conditions on capillary morphogenesis of retinal ECs in coculture experiments. PCs are able to stabilize capillary tube formation by inhibiting tube regression in EC-PC cocultures.<sup>50</sup> PCs cultured under high glucose conditions attenuated capillary morphogenesis of retinal ECs. This can be attributed to inhibition of EC migration by PCs cultured under high glucose, as demonstrated here. In addition, high glucose conditions may also disturb the expression of other regulating factors including metalloproteinases and tissue inhibitor of metalloproteinase.<sup>51</sup> Thus, high glucose condition may affect secretion of soluble factors produced by PCs, including inflammatory cytokines that impact EC function as we recently demonstrated.<sup>41</sup> The identity of these factors and their regulation under various glucose conditions require further investigation.



**Figure 8** A summary of signaling pathways known to participate in response to high glucose. The exposure of PCs to high glucose results in increased Bim expression and oxidative stress, which lead to activation of SHP-1 and PKC- $\delta$ , and thus leading to enhanced apoptosis. Our results indicate that oxidative stress is upstream of SHP-1 and PKC- $\delta$  activation. In addition, we showed high glucose inhibited basal and PDGF-mediated migration of PCs and this can be reversed by NAC and inhibition PKC- $\delta$  or ROCK I/II

In summary, our results demonstrate that exposure of retinal PCs to high glucose results in increased Bim expression and oxidative stress, and reduced migration with a significant impact on their rate of apoptosis. We showed that these effects are associated with the activation of STAT1-mediated Bim expression axis in retinal PCs. A summary of how high glucose conditions affect signaling pathways involved in PC migration, proliferation, and apoptosis is shown in Figure 8. Collectively, we demonstrate that increased expression of Bim through the production of inflammatory cytokines and sustained activation of STAT1 is a significant pathway in the modulation of PC function under high glucose conditions. Thus, targeting Bim expression and/or activity may provide a more effective modality for treatment of diabetic retinopathy.

#### Materials and Methods

**Animals.** All experiments were carried out in accordance with and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin School of Medicine and Public Health. Akita/+ heterozygous (Akita/+) male mice on C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The diabetic (Akita/+) mice used in the experiments described here were male owing to effectiveness and severity of the diabetic phenotype in males. The wild-type male mice were used as control. Genomic tail DNA isolation and PCR were performed using the PCR Kit (REExtract-N-Amp Tissue PCR Kit; Sigma, St. Louis, MO, USA), and the transgenic Ins2Akita/+ mice were identified by PCR screening. The amplified fragments were digested with Fnu4HI and resulting bands separated on 3.0% agarose gel, as recommended (Jackson Laboratories).

Immunohistochemical staining of the frozen sections. Frozen sections from mouse eyes were prepared as previously described by us.<sup>52</sup> Seven-month-old mice were used for frozen sections. Apoptotic cell death was assessed by TUNEL staining. TUNEL staining was performed using Click-iT TUNEL Alexa Fluor Imaging Assay (Invitrogen, Carlsbad, CA, USA). Sections were then incubated with rabbit anti-PDGFR $\beta$  antibody (eBiosciences, San Diego, CA, USA) (1:500 dilution prepared in blocking solution) or rabbit anti-Bim antibody (Cell Signaling, Danvers, MA, USA) (1:500 dilution prepared in blocking solution) overnight at 4 °C in a humid environment. After three washes in phosphate-buffered saline (PBS) 5 min each,



sections were incubated with appropriate CY3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Sections were counterstained with DAPI (Invitrogen) for staining of the nuclei for 15 min. Sections were washed three times with PBS, covered with PBS/glycerol (2 vol: 1 vol), and mounted with a coverslip. Sections were viewed by fluorescence microscopy, and images were captured in digital format using a Zeiss microscope (Carl Zeiss, Chester, VA, USA).

**Cell culture.** Mouse retinal PCs were isolated and cultured as previously described by us.<sup>12</sup> The medium contained either 5.7 mM D-glucose (physiological/normal glucose; LG) or 40.7 mM D-glucose (hyperglycemia/high glucose; HG), or 5.7 mM D-glucose and 35 mM L-glucose (control; LG + L-Glu), as described previously.<sup>15</sup> Cells were maintained in the growth medium for 5 days before each experiment, and the medium was changed every other day. The cells were also exposed to a cycle of high glucose for 2 days, normal glucose for 2 days, and then high glucose for 2 days before each experiment (fluctuation condition). Mouse retinal ECs were isolated and cultured as previously described by us.<sup>42</sup>

**Apoptosis assays.** Cells (0.1 ml of  $1 \times 10^5$  cells per ml) were placed on fibronectin (2  $\mu$ g/ml)-coated 4-well chamber slides. After attachment, cells were cultured in the medium under normal glucose or high glucose conditions or high glucose conditions containing various treatments such as the antioxidant NAC (1 mM; Sigma), PKC- $\delta$  inhibitor Rottlerin (1  $\mu$ M; Sigma), ROCK1/2 inhibitor Y-27632 (10  $\mu$ M; Cayman Chemical, Ann Arbor, MI, USA), SHP-1 inhibitor SSG (1  $\mu$ M) (EMD, Gibbstown, NJ, USA) or fludarabine (10  $\mu$ M; Sigma) for 5 days. Apoptotic cell death was assessed using Click-iT TUNEL Alexa Fluor Imaging Assay (Invitrogen), as recommended by the manufacturer. Positive cells were counted under fluorescence microscope and the percentage of apoptotic cells relative to the total number of cells was calculated. Concentrations for various pharmacological inhibitors were determined based on minimal effect on cell viability during the 5-day exposure to high glucose concentrations. The effect of cytokines on apoptosis was determined by measuring caspase activation using Caspase-Glo 3/7-assay kit (Promega, Madison, WI, USA), as recommended by the supplier. The assay provides caspase-3/7 DEVD-aminoluciferin substrate and the caspase-3/7 activity was detected by luminescent signal. For the assay, retinal PCs were plated at  $8 \times 10^3$  cells per well of a 96-well plate and the next day incubated with 10 ng/ml TNF- $\alpha$ , IL-1 $\beta$  or MCP-1 for 24 h. Caspase activity was detected using a luminescent microplate reader (Victor2 1420 Multilabel Counter; Perkin-Elmer, Waltham, MA, USA). All samples were prepared in triplicate and repeated at least three times with similar results.

**Determination of oxidative stress.** Cells (0.1 ml of  $1 \times 10^5$  cells per ml) were plated on fibronectin (2  $\mu$ g/ml)-coated 4-well chamber slides. After attachment, cells were cultured in the medium under different glucose conditions for 5 days with/without various treatments as noted above. The level of cellular ROS was assessed using DHE staining (Invitrogen), as described previously.<sup>53</sup> For quantitative assessments, the images were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA). Values were obtained from each cells captured in five high power fields ( $\times 400$ ). More than 40 cells per each condition were analyzed.

**Transwell migration assays.** The impact of high glucose on migration of PCs and of conditioned medium collected from PCs under various glucose conditions on the migration of ECs was assessed using transwell assays as described in Scheef *et al*.<sup>12</sup> The method for determining the effect of PDGF-BB on the migration of PCs was also described by us.<sup>12</sup> To examine the migration of ECs,  $1 \times 10^5$  cells in 100  $\mu$ l of medium were added to the top of each transwell membrane. Transwell inserts were placed in 24-well dishes containing conditioned medium collected from PCs cultured under various glucose conditions for 5 days. Cells were allowed to migrate through the membrane for 3 h at 37 °C. The number of migrated cells was determined as described previously.<sup>15</sup>

**Scratch wound assays.** The migration of cells cultured under various glucose concentrations was also assessed by scratched wound assay as described.<sup>15</sup> Confluent monolayers of retinal PCs cultured in different glucose conditions for 5 days were wounded using a 1-ml micropipette tip and incubated with growth medium with appropriate glucose concentration. Wound closure was monitored by phase microscopy, and the wounds were photographed at 0, 24, 48 and 96 h. Each sample was performed in triplicate on at least three independent occasions using two different isolations of PCs, with similar results.

**Western blot analysis.** Approximately  $2 \times 10^5$  cells were plated in 60-mm plates and cultured in the growth medium containing different glucose concentrations for 5 days. For cytokine treatment, retinal PCs were incubated with 10 ng/ml TNF- $\alpha$ , IL-1 $\beta$  or 10 ng/ml TNF- $\alpha$  + 10 ng/ml IL-1 $\beta$  for 24 h after serum starvation for 24 h. The cells were washed two times with cold PBS, lysed in 0.1 ml of lysis buffer (20 mM Tris (pH 7.4), 2 mM EDTA, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1% Triton X-100, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail; Roche, Indianapolis, IN, USA) and briefly sonicated. The lysates were centrifuged and protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). For the retina lysate, the retinas were lysed in the same lysis buffer and prepared as described above for cells. The protein samples were mixed with appropriate volume of 6  $\times$  SDS sample buffer and analyzed using 4–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen). Proteins were transferred to nitrocellulose membrane, blocked and incubated with appropriate primary antibodies at 4 °C overnight. After washing, the blots were incubated with appropriate HRP-conjugated secondary antibodies and developed using ECL system (GE Healthcare, Piscataway, NJ, USA). The antibody to  $\beta$ -actin (Sigma) or  $\beta$ -catenin (BD Biosciences, San Jose, CA, USA) was used to verify equal protein loading. The following primary antibodies were used: p-STAT1 (Tyr701), STAT1, p-Src (Tyr418), p-p38 (Thr180/Tyr182), p38, p-Akt1 (Ser 473), Akt1, p-ERK and ERK (Cell Signaling, Danvers, MA, USA), p-JNK1 and JNK1 (R&D System, Minneapolis, MN, USA), Bim (Cell Signaling) and p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Capillary morphogenesis.** Capillary morphogenesis was performed as previously described by us.<sup>15</sup> Equal numbers of retinal pericytes and ECs ( $2 \times 10^5$ ) in 2 ml were applied to the Matrigel-coated plates (0.5 ml Matrigel per 35 mm dish), incubated at 37 °C and photographed after 18 h in digital format using a Nikon microscope (Nikon Instruments, Melville, NY, USA). For quantitative assessment of the data, the mean number of branch points was determined by counting the number of branch points in five high-power fields ( $\times 100$ ).

**RNA purification and real-time qPCR analysis.** The total RNA from PCs was extracted using mirVana PARIS Kit (Invitrogen). cDNA synthesis was performed from 1  $\mu$ g of total RNA using Sprint RT Complete-Double PrePrimed Kit (Clontech, Mountain View, CA, USA). One microliter of each cDNA (dilution 1:10) was used as template in qPCR assays, performed in triplicate of three biological replicates on Mastercycler Realplex (Eppendorf, Hauppauge, NY, USA) using the SYBR-Green qPCR Premix (Clontech). Amplification parameters were as follows: 95 °C for 2 min; 40 cycles of amplification (95 °C for 15 s, 60 °C for 40 s); dissociation curve step (95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s). Primer sequences for TNF- $\alpha$  were: 5'-ACCGTCAGCCGATTGCTAT-3' (forward) and 5'-TTGACGGCAGAGAGAGGTT-3' (reverse). For IL-1 $\beta$ , 5'-GTTCCATTAGACAACTGCCTACA-3' (forward) and 5'-CCGACAGCAGGAGCTTTT-3' (reverse). Standard curves were generated from known quantities for each of the target gene of linearized plasmid DNA. Ten times dilution series were used for each known target, which were amplified using SYBR-Green qPCR. The linear regression line for ng of DNA was determined from relative fluorescent units (RFU) at a threshold fluorescence value (Ct) to quantify gene targets from cell extracts by comparing the RFU at the Ct to the standard curve, normalized by the simultaneous amplification of *RpL13a*, a housekeeping gene. Primer sequences for *RpL13a* were: 5'-TCTCAAGGTTGTCGGCTGAA-3' (forward) and 5'-CCAGACGCCCCA GGTA-3' (reverse).

**Statistical analysis.** Statistical differences between control and treated samples were evaluated with Student's unpaired *t*-test (two-tailed) or one-way ANOVA with *post hoc* Dunnett's test for multiple comparisons. Mean  $\pm$  S.E. are shown. *P*-values  $\leq 0.05$  were significant.

### Conflict of Interest

The authors declare no conflict of interest.

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### Author contributions

ESS, QH and NS designed experiments and analyzed data. ESS, QH, ZG, TLP and IZ conducted experiments. CMS provided cells and animals. ESS, QH, CMS and NS contributed to writing the manuscript.

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