

GLUT1 activity contributes to the impairment of PEDF secretion by the RPE

Sofia M. Calado,^{1,2} Liliana S. Alves,^{2,3} Sónia Simão,^{2,4} Gabriela A. Silva²

¹PhD Program in Biomedical Sciences, Department of Biomedical Sciences and Medicine, University of Algarve, Campus Gambelas, Faro, Portugal; ²CEDOC, NOVA Medical School | Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria, Lisboa, Portugal; ³ProRegeM PhD Program, NOVA Medical School/Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria, Lisboa, Portugal; ⁴Centre for Biomedical Research (CBMR), University of Algarve, Campus Gambelas, Faro, Portugal

Purpose: In this study, we aimed to understand whether glucose transporter 1 (GLUT1) activity affects the secretion capacity of antiangiogenic factor pigment epithelium-derived factor (PEDF) by the RPE cells, thus explaining the reduction in PEDF levels observed in patients with diabetic retinopathy (DR).

Methods: Analysis of GLUT1 expression, localization, and function was performed in vitro in RPE cells (D407) cultured with different glucose concentrations, corresponding to non-diabetic (5 mM of glucose) and diabetic (25 mM of glucose) conditions, further subjected to normoxia or hypoxia. The expression of PEDF was also evaluated in the secretome of the cells cultured in these conditions. Analysis of GLUT1 and PEDF expression was also performed in vivo in the RPE of Ins2^{Akita} diabetic mice and age-matched wild-type (WT) controls.

Results: We observed an increase in GLUT1 under hypoxia in a glucose-dependent manner, which we found to be directly associated with the translocation and stabilization of GLUT1 in the cell membrane. This stabilization led to an increase in glucose uptake by RPE cells. This increase was followed by a decrease in PEDF expression in RPE cells cultured in conditions that simulated DR. Compared with non-diabetic WT mice, the RPE of Ins2^{Akita} mice showed increased GLUT1 overexpression with a concomitant decrease in PEDF expression.

Conclusions: Collectively, our data show that expression of GLUT1 is stimulated by hyperglycemia and low oxygen supply, and this overexpression was associated with increased activity of GLUT1 in the cell membrane that contributes to the impairment of the RPE secretory function of PEDF.

Diabetic retinopathy (DR), a blood–retinal barrier disorder, is the main complication of diabetes and the leading cause of blindness in working-age adults [1]. The major pathological features at advanced stages of the disease are the abnormal neovascularization due to hypoxia and blood leakage as a result of inner blood–retinal barrier breakdown [1,2]. The blood–retinal barrier (BRB) is responsible for the homeostasis of the neuroretina and is composed of two structures: the inner BRB (iBRB), formed by tight junctions between the endothelial cells of the retinal vessels, and the outer BRB (oBRB), formed by intercellular tight junctions in the RPE monolayer [3-5].

Most of the studies on the pathophysiology of DR focused on the iBRB breakdown and neuroretina damage [6-9], with little attention to the effects of diabetes on the oBRB and RPE cells. As the RPE is responsible, among others, for the transport of nutrients, such as glucose, ions, and water, and the

secretion of factors crucial for the homeostasis of the neuroretina such as the pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF) [2,10], the role of the RPE in DR is worth investigating. The healthy eye is characterized by low levels of angiogenic VEGF and high levels of antiangiogenic factors, such as PEDF [5]. This balance is disrupted by ischemia during the pathogenesis of DR, increasing the ratio of angiogenic to antiangiogenic factors and promoting abnormal neovascularization in the retina [5]. During ischemia, increasing levels of the heterodimeric hypoxia-inducible factor-1 (HIF-1) are detected [11,12]. Both HIF-1 subunits are constitutively expressed, but in normoxia conditions, the HIF-1 α subunit is rapidly degraded by an oxygen-dependent mechanism [13]. However, in a hypoxic environment both HIF-1 subunits form dimers and translocate to the nucleus, where they can induce the transcription of a wide range of genes [14-16], including *VEGF* (Gene ID: 7422; OMIM: 192240) [17], *EPO* (Gene ID: 2056; OMIM: 133170) [18], and the *glucose transporter 1 (GLUT1)*; Gene ID: 6513; OMIM: 138140) [19].

GLUT1 is one of the 12 GLUT isoforms described [20], composed of a glycoprotein with 12 transmembrane domains

Correspondence to: Gabriela A. Silva, CEDOC, NOVA Medical School | Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria 130, 1169-056, Lisboa, Portugal; Phone +351 21 880 3109; FAX +351 21 880 3010, email: gabriela.silva@nms.unl.pt

and a single N-glycosylation site. GLUT1 transports glucose bidirectionally across the cell membrane based on a concentration gradient [20,21]. In the retina, as in the brain, glucose is the only fuel source for cells, and in both tissues, glucose is transported to cells exclusively through the GLUT1 transporter [20,22].

In this study, we aimed to establish a correlation between RPE, GLUT1, and their role in diabetic retinopathy. Our hypothesis is that under diabetic conditions, where there is an increase in glucose and local hypoxia, the expression of GLUT1 in RPE cells is increased, and that can affect the secretory function of the RPE, namely, the production of PEDF. This change in PEDF secretion can lead to an imbalance between VEGF and PEDF, contributing to diabetic retinopathy. To test our hypothesis, we studied the effect of hyperglycemia and hypoxia on the cellular localization and expression of GLUT1 and PEDF expression on RPE cells and further confirmed our findings in a mouse model of diabetic retinopathy.

METHODS

Cell lines: D407, a human RPE cell line [23] used in the *in vitro* experiments, was kindly provided by Dr. Jean Bennett of the University of Pennsylvania. Cells were kept in culture in a humid chamber with 5% CO₂ at 37 °C and were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich, St. Louis, MO) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich), 1% glutamine (Sigma-Aldrich), and 5% fetal bovine serum (Sigma-Aldrich). Culture medium was changed every 2 days. For the experiments on the glucose effect, cells were cultured in 22.1 cm² plates (TPP, Trasadingen, Switzerland) for 3 days either in DMEM containing 5 mM D-glucose (to simulate normoglycemia) or in DMEM with 25 mM of D-glucose (to simulate hyperglycemia). Cells were also grown in DMEM containing 5 mM of D-glucose in which mannitol was added up to a final concentration of 25 mM. Mannitol was chosen as an osmotic control because it is a carbohydrate with no biologic activity and cannot be used by the cells as a source of energy [7,24]. Hypoxia was induced by the addition of desferrioxamine [25,26] (DFO, Sigma-Aldrich) to the culture media at a final concentration of 100 μM. DFO is an iron chelating agent often used as a hypoxia-mimetic agent to stabilize HIF-1α [25]. Under normoxic conditions and in the presence of iron, HIF-1α is hydroxylated and subsequently degraded by the proteasome. In the presence of DFO, the iron required for the enzymatic activity of prolyl hydroxylases is removed by its chelating capacity, allowing HIF-1α to be stabilized and dimerize with its β-subunit, originating a functional complex

that is translocated to the nucleus [25]. After 16 h of incubation with DFO, cells were collected for western blot and immunocytochemistry for GLUT1.

Detection of GLUT1 expression by western blot: Whole cell proteins were extracted in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA) supplemented with a protease inhibitor cocktail (Roche, Basel, Switzerland). For isolation of the membrane/soluble fractions, cells were extracted in cold homogenization buffer (20 mM HEPES pH 7.4; 1 mM EDTA; 250 mM sucrose) containing a protease inhibitor cocktail. The lysate was cleared by centrifugation at 4 °C for 10 min at 1,137 ×g. The supernatant was centrifuged at 100,000 ×g for 1 h at 4 °C, and the pellet corresponding to the membrane fraction was resuspended in buffer (10 mM HEPES pH 7.4; 250 mM sucrose) supplemented with a protease inhibitor cocktail. Protein content was measured with the Bradford assay, and the samples were stored at -80 °C. Thirty micrograms of each extract were separated in a denaturing 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, Little Chalfont, UK) and blocked using Superblock Blocking buffer (Thermo Scientific) containing 0.1% of Tween-20 (Sigma-Aldrich) for 1 h at room temperature. GLUT1 antibody (ab32551; Abcam, Cambridge, UK) was incubated overnight at 4 °C (1:2,000 dilution) and β-actin (A5441, Sigma-Aldrich) was incubated for 1 h at room temperature (1:10,000 dilution). The membrane was probed with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature, and the immunoreactive bands were detected with chemiluminescence, using an ECL Plus kit (Amersham).

Cellular localization of GLUT1 with immunocytochemistry: The cells were fixed in ice-cold methanol for 10 min, washed twice in PBS (1X; 137 mM NaCl, 10 mM NaPO₄, 2.7 mM KCl, 2 mM KPO₄, pH 7.4), and blocked in 1% goat serum/PBS at room temperature for 1 h. Incubation with the GLUT1 antibody (1:250) was performed for 1 h at room temperature, followed by a wash step and incubation with the secondary antibody (Alexa Fluor® 594; 1:500; Life Technologies, Waltham, MA) at room temperature for 1 h. Coverslips were mounted on glass slides with Fluoromount G (SouthernBiotech, Birmingham, AL) containing 4',6-diamidino-2-phenylindole (DAPI). Images were obtained using an AxioVision microscope with a 63× objective, using the appropriate filter sets (Axio Observer Z2, Zeiss, Oberkochen, Germany).

Glucose consumption assay: For determining glucose consumption by glucose depletion from the culture medium, D407 cells were seeded at a density of 7.0 × 10⁵ cells/well in

six-well flat-bottom tissue culture plates and maintained for 72 h in culture medium containing 25 mM of D-glucose. A sample of culture medium was collected from each well, and the glucose concentration was determined spectrophotometrically using the Glucose (GO) Assay Kit (Sigma-Aldrich), following the manufacturer's instructions. Culture medium with 25 mM of D-glucose was used as control.

PEDF detection in the culture medium of the RPE cells: The cells were grown as previously described, and 16 h before the supernatant was collected, the cells were washed with PBS and 1 ml of culture medium without serum was added. PEDF levels were detected with western blot in the culture medium. Briefly, the supernatant was collected, and four volumes of ice-cold acetone were added. After an incubation period of 30 min at -20°C , the supernatant was centrifuged for 10 min at $13,000 \times g$ and decanted. The pellet containing the precipitated proteins was resuspended in 1X Sample Buffer. Protein content was measured with the Bradford assay, and 30 μg of each extract were separated in a denaturing 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad, Berkeley, CA). Equal amounts of protein were loaded in the gel, determined by Ponceau S staining of the membranes before blocking. Western blot was performed as described previously, using a PEDF antibody (07-280, Merck Millipore, Billerica, MA; 1:1,000).

Animals: For the in vivo experiments, male C57BL/6 (wild-type) and C57Bl/6 Ins2^{Akita} (diabetic) mice (The Jackson Laboratory, Farmington, CT) 2, 4, 7, and 10 months after the onset of hyperglycemia (2 months after birth) were used. The animals were housed under controlled temperatures and a 12 h:12 h light-dark cycle with food and water ad libitum. Diabetic phenotype was confirmed 2 months after birth by measuring blood glucose levels in a drop of blood from a tail puncture (Freestyle Precision, Abbot, Lake Bluff, IL), with animals used in this study exhibiting blood glucose ≥ 500 mg/dl. All experimental procedures were performed according to the Portuguese and European Laboratory Animal Science Association (FELASA) Guide for the Care and Use of Laboratory Animals, the European Union Council Directive 2010/63/EU for the Use of Animals in Research, and the guidelines of the Association for Research in Vision and Ophthalmology (ARVO) for the Use of Animals in Ophthalmic and Vision Research. Animals were humanely euthanized by cervical dislocation and the eyes enucleated. The RPE was isolated by dissection of the eyeball and homogenized in ice-cold RIPA buffer.

Statistical analysis: All experiments were performed in triplicate, and the results expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed by

using a two-way ANOVA, followed by a Tukey's or Sidak's multiple comparisons test or an unpaired t-test, depending on the experiment. The data were analyzed using GraphPad Prism software. A p value of less than 0.05 was considered statistically significant.

RESULTS

Hypoxia induces overexpression of GLUT1 in RPE cells: To evaluate the effects of glucose and ischemia in GLUT1 expression within the oBRB, we used an in vitro setup with human RPE cells. D407 cells were cultured either with 5 mM of D-glucose (corresponding to normoglycemia) or 25 mM of D-glucose (corresponding to hyperglycemia) and further subjected to hypoxia by the addition of DFO, a chelating agent that induces hypoxia by inhibiting HIF-1 α degradation at the proteasome [25,26]. We confirmed that DFO does not induce cell death at the final concentration of 100 μM (data not shown).

Figure 1 shows no differences at the protein level in cells in normoglycemic conditions under normoxia (N), when compared with hypoxia (H). However, there is a significant increase in GLUT1 protein in the cells cultured with 25 mM of D-glucose in hypoxia compared with the cells cultured in normoxia, showing a direct effect of the glucose concentration on GLUT1 expression.

GLUT1 translocation to the cell membrane of RPE cells increases in response to hypoxia: To determine whether the increase in GLUT1 expression observed in Figure 1 corresponded to an increase in the transport of glucose, we first determined the cellular localization of GLUT1 in human RPE cells with immunocytochemistry. The results showed a significant increase in the accumulation of GLUT1 in the membrane of the cells under hypoxia compared with the cells under normoxia (Figure 2). Again, we observed GLUT1 staining to be stronger in the cells in hypoxia and hyperglycemia compared with the cells in hypoxia and normoglycemia.

We confirmed these results with western blot analysis of the membrane and soluble fractions of RPE cells cultured in high glucose either in normoxia or hypoxia (Figure 3). In Figure 3, it is clear the marked increase of the GLUT1 transporter in the membrane of cells in hypoxia compared to normoxia, which correlates with the results shown in Figure 2. We could not observe an increase in GLUT1 in the soluble fraction of cells in hypoxia, which was expected since GLUT1 is a membrane transporter.

Glucose consumption is affected by hypoxia: Based on the finding that GLUT1 expression is increased in the cell

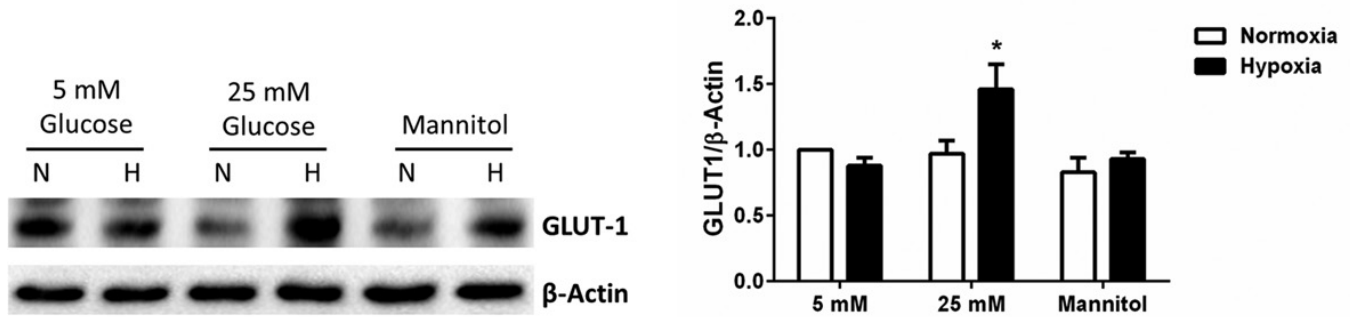


Figure 1. Effect of glucose and hypoxia on GLUT1 expression. Western blot analysis of GLUT1 in D407 RPE cells cultured under normoxia (N) and hypoxia (H) conditions and different concentrations of glucose in the culture medium: 5 mM of D-glucose (corresponding to normoglycemia), 25 mM of D-glucose (corresponding to hyperglycemia), and 25 mM of mannitol (osmolarity control). Quantitative data were obtained by normalization with β-actin bands. n = 4. *p<0.05 represents a significant difference in GLUT1 levels in cells cultured under hypoxia with high glucose concentration medium, determined with Tukey's multiple comparisons test.

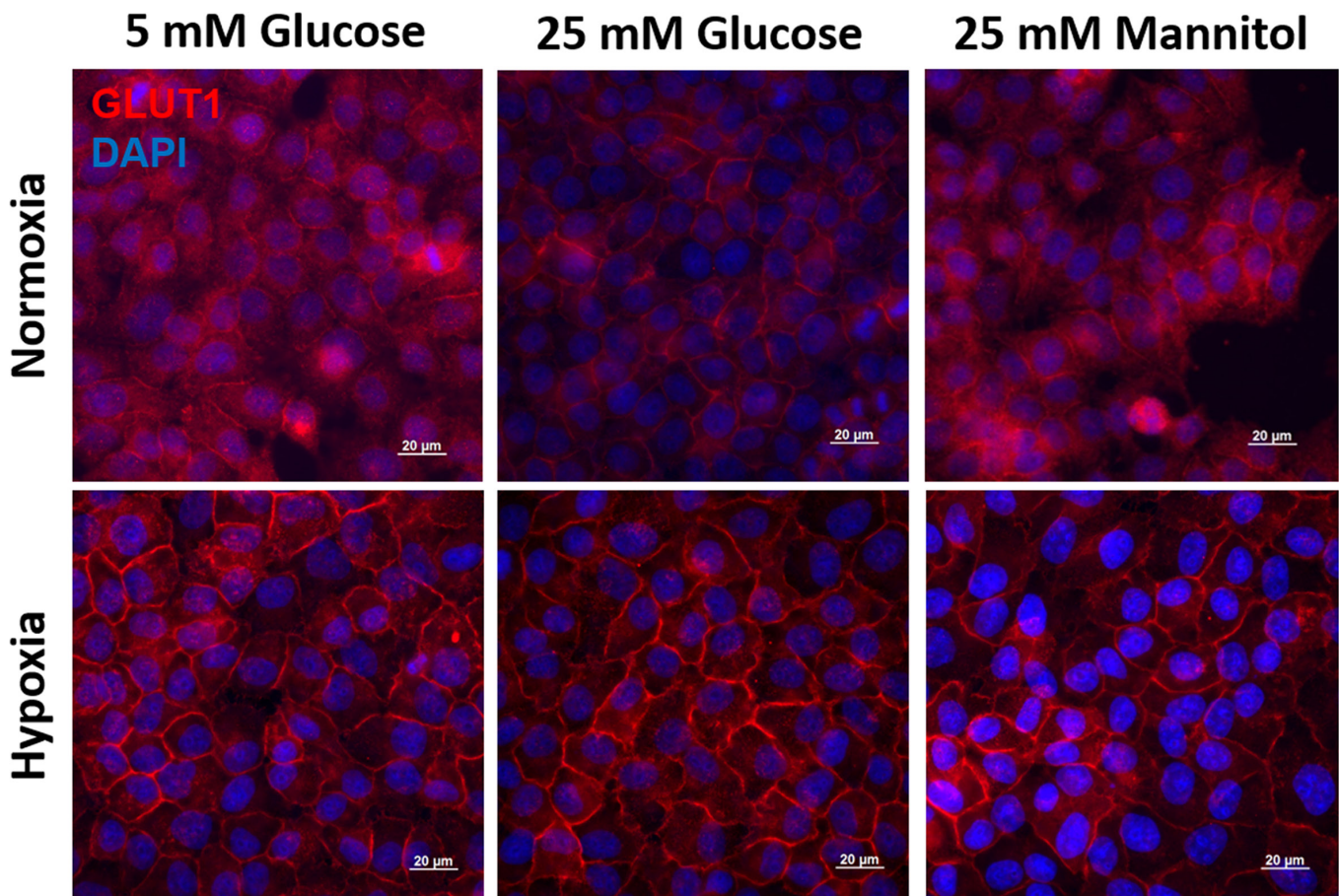


Figure 2. Immunocytochemistry for GLUT1 in RPE cells. D407 cells were cultured with different concentrations of glucose and subjected to hypoxia and normoxia. Staining for GLUT1 (red) shows higher intensity in the cell membrane of cells subjected to hypoxia. 4',6-diamidino-2-phenylindole (DAPI; blue) represents the nuclei. Magnification = 630X, scale bar = 20 μM.

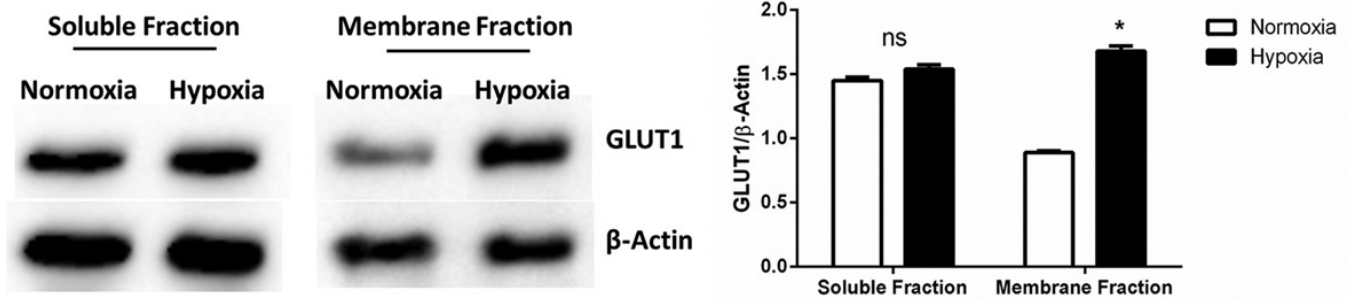


Figure 3. GLUT1 expression is stabilized in the cell membrane in response to hypoxia. GLUT1 protein levels in the soluble and membrane fraction of D407 cells cultured with 25 mM of D-glucose and subjected to normoxia and hypoxia, show a marked increase in GLUT1 expression in the membrane of cells cultured in hypoxic conditions. n = 3. *p<0.05 symbolizes a significant increase in GLUT1 expression in the membrane fraction of the cells cultured under hypoxia with high glucose concentration medium, determined with Sidak’s multiple comparisons test.

membrane of the RPE cells subjected to hypoxia, it was necessary to determine if this translates into increased glucose uptake by RPE cells. GLUT1 activity was measured by glucose consumption through glucose depletion in the culture medium. We found a significant increase in glucose consumption induced by hypoxia (Figure 4), with the cells consuming 60% of the glucose present in the culture medium compared to the 40% consumption in normoxia. This shows a marked effect of hypoxia on glucose consumption in diabetic retinopathy conditions.

Secretory function of RPE cells is impaired by high glucose and hypoxia: One of the main functions of RPE cells is the secretion of multiple trophic factors essential for the

maintenance and integrity of the neuroretina and choriocapillaries [2]. One of these factors is PEDF, a neurotrophic and antiangiogenic factor responsible for protecting neurons from ischemia-induced apoptosis [27] and inhibiting endothelial cell proliferation caused by VEGF [28]. We evaluated the expression of PEDF in RPE cells cultured as described previously and found a significant decrease in PEDF levels for conditions where cells were cultured in hyperglycemia (25 mM glucose) and hypoxia (H; Figure 5). This result shows that in diabetic conditions there is a decrease in the secretion of PEDF, which contributes to the disruption of the balance between the antiangiogenic and angiogenic factors, as observed in human diabetic retinas [29,30].

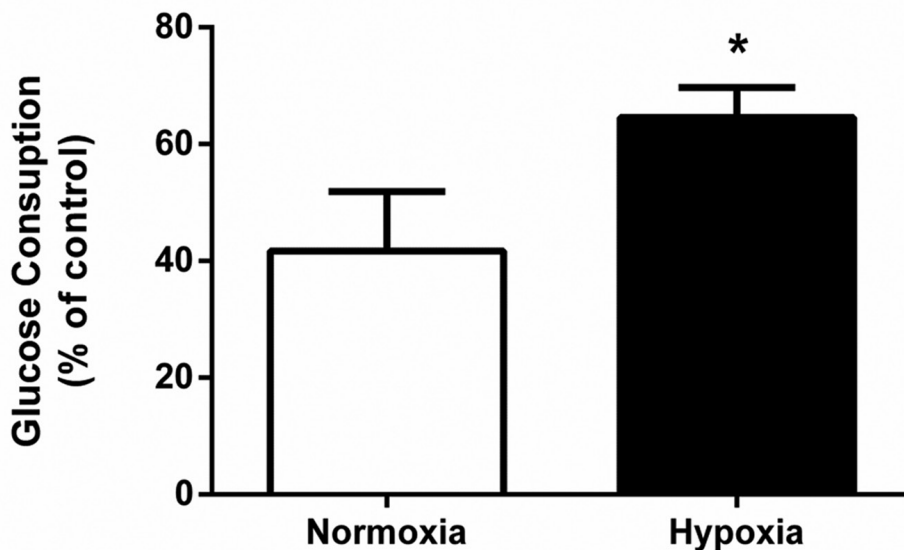


Figure 4. Glucose consumption by RPE cells. Glucose depletion from the culture medium was increased for cells cultured under hypoxia, compared with cells in normoxia. The results are expressed as percentage of the control (culture medium). n = 6. *p<0.05 denotes a significant difference in glucose consumption by cells in hypoxia compared with cells in normoxia, determined with the two-tailed t test.

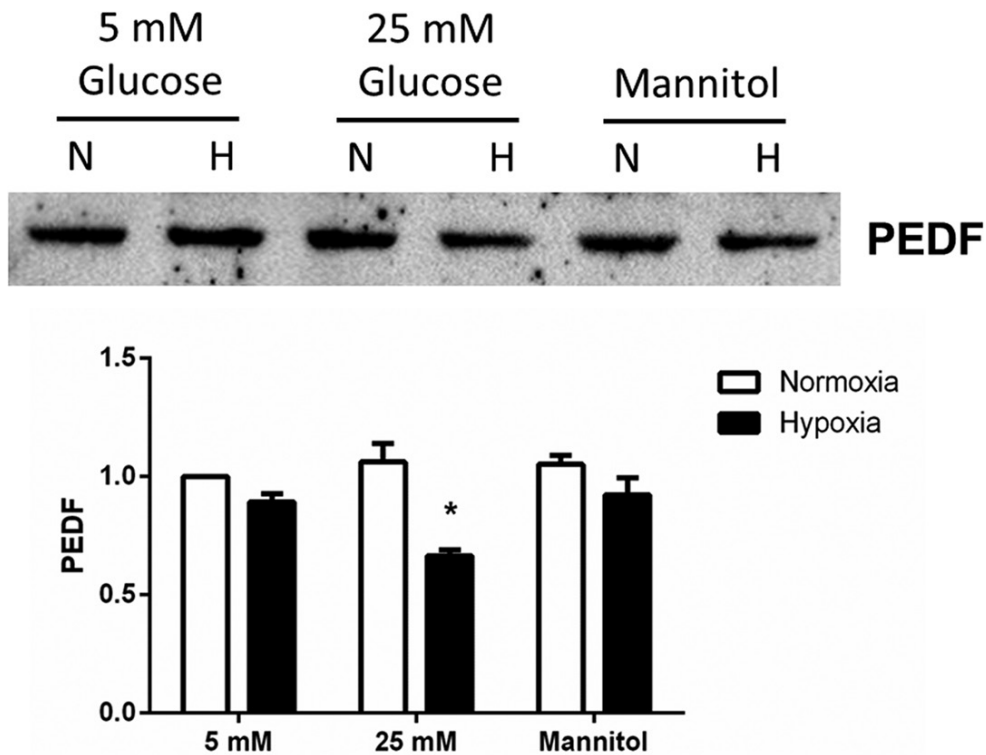


Figure 5. Effects of glucose and hypoxia in PEDF secretion by RPE cells. Western blot analysis of pigment epithelium-derived factor (PEDF) secretion in D407 cells cultured under normoxia (N) and hypoxia (H) conditions and different concentrations of glucose in the culture medium: 5 mM of D-glucose (corresponding to normoglycemia), 25 mM of D-glucose (corresponding to hyperglycemia), and mannitol (osmolarity control), $n = 4$. * $p < 0.05$ represents a significant decrease in PEDF secretion by the RPE cells cultured under hypoxia with high glucose concentration medium, determined with Tukey's multiple comparisons test.

GLUT1 and PEDF expression is altered in the RPE of diabetic mice: To confirm the validity of our in vitro findings, we analyzed the expression of GLUT1 and PEDF in the RPE of wild-type and Ins2^{Akita} diabetic mice (Figure 6). For all time points (2, 4, 7, and 10 months after the onset of hyperglycemia), GLUT1 expression was significantly increased in the retina of diabetic mice compared with age-matched wild-type animals. Additionally, we found a marked decrease in PEDF levels in the RPE of the diabetic mice, especially at later ages. These in vivo results corroborate our in vitro results in which we found an increase in GLUT1 (Figure 1) and a decrease in PEDF secretion by RPE cells cultured in conditions simulating DR (Figure 5).

DISCUSSION

DR is one of the most frequent complications of diabetes mellitus, affecting about 90% of patients with type 1 diabetes [31]. It is known that hyperglycemia and ischemia are key factors for the progression of the disease [5]; however, the mechanism by which hyperglycemia contributes to the development of the disease remains unclear [1]. DR is traditionally characterized as a blood-retinal barrier disorder, in which the leakage of blood content, due to pathological neovascularization, is the main feature of the disease [1]. Although the iBRB breakdown has been extensively investigated, the

effects of diabetes on the RPE cells composing the oBRB are still not well-known [2]. The RPE monolayer is extremely important to maintain the homeostasis of the neural retina [2] suggesting that its impairment can compromise the retinal function.

The retina is one of the most metabolically active tissues in the human body and glucose is the retina's only source of energy [32]. In the retina, glucose transport is exclusively mediated by GLUT1 [9]. To better characterize GLUT1 expression in conditions of DR, we devised a series of in vitro experiments using D407 cells, a spontaneously transformed human RPE cell line derived from a primary culture of human RPE cells [23]. These cells are extensively used as in vitro models and are suitable for studying molecular mechanisms of the RPE [33,34]. RPE cells were exposed to different concentrations of glucose to mimic normoglycemia (5 mM of D-glucose) and hyperglycemia (25 mM D-glucose). In addition, cells were also exposed to hypoxia to simulate retinal ischemia observed in patients with DR [5]. It was previously shown that hypoxia induces overexpression of GLUT1 in mouse fibroblasts in response to metabolic adaptation [19], but there was no evidence regarding changes in GLUT1 expression due to hypoxia in RPE cells. Our western blot analysis showed no differences in GLUT1 protein expression in the cells cultured with normoglycemic medium

(Figure 1). In contrast, it is possible to observe that hypoxia induces a significant increase in GLUT1 protein levels in the cells cultured with 25 mM of D-glucose compared with the control cells cultured in normoglycemic (5 mM of D-glucose) medium. This suggests that the diabetic environment induced by high glucose and hypoxia most likely contributes to the overexpression of GLUT1. Analysis of protein expression with immunocytochemistry shows an increase in GLUT1 staining in the cell membrane of the RPE cells in response to hypoxia (Figure 2). Similarly to the previous western blot results shown in Figure 1, the staining is stronger in cells cultured with 25 mM of D-glucose, which further supports the contribution of high glucose levels for the expression of GLUT1. To confirm this finding, we isolated the membrane and soluble fractions of D407 cells cultured with 25 mM of D-glucose. We found a marked increase in GLUT1 expression in the membrane fraction of cells in hypoxic conditions compared with cells cultured with the same glucose concentration in normoxia (Figure 3). Interestingly, the soluble concentration of GLUT1 is similar in both conditions, suggesting that the increase in GLUT1 expression observed in the whole cell lysates (Figure 1) is due to an increase in GLUT1 expression in the cell membrane (Figure 3). These results are in accordance with what was previously shown

for GLUT4 in cardiomyocytes, where hypoxia induces the translocation of this protein to the cell membrane [35]. It is known that in response to low levels of oxygen cells shift their glucose metabolism to anaerobic respiration, which is much less efficient [36]. The translocation of GLUT1 to the cell membrane can be a response to achieve a more effective glucose uptake in low oxygen conditions. To test this, we performed a glucose consumption assay in which the glucose remaining in the culture medium was measured in cells cultured under normoxia and hypoxia. Our results show that cells under hypoxia display higher glucose consumption compared with cells cultured under normoxia (Figure 4), showing an increase in glucose uptake by GLUT1 under hypoxia.

As previously stated, one of the main functions of RPE cells is their secretory capacity, responsible for producing and secreting a wide range of factors that support photoreceptors and guaranteeing optimal circulation and supply of nutrients [2,37]. One of those factors is PEDF, a neurotrophic and anti-angiogenic factor secreted by RPE cells that acts by inhibiting retinal endothelial cell growth and migration [38].

Several studies have shown an imbalance between VEGF and PEDF levels in the vitreous of diabetic patients, showing

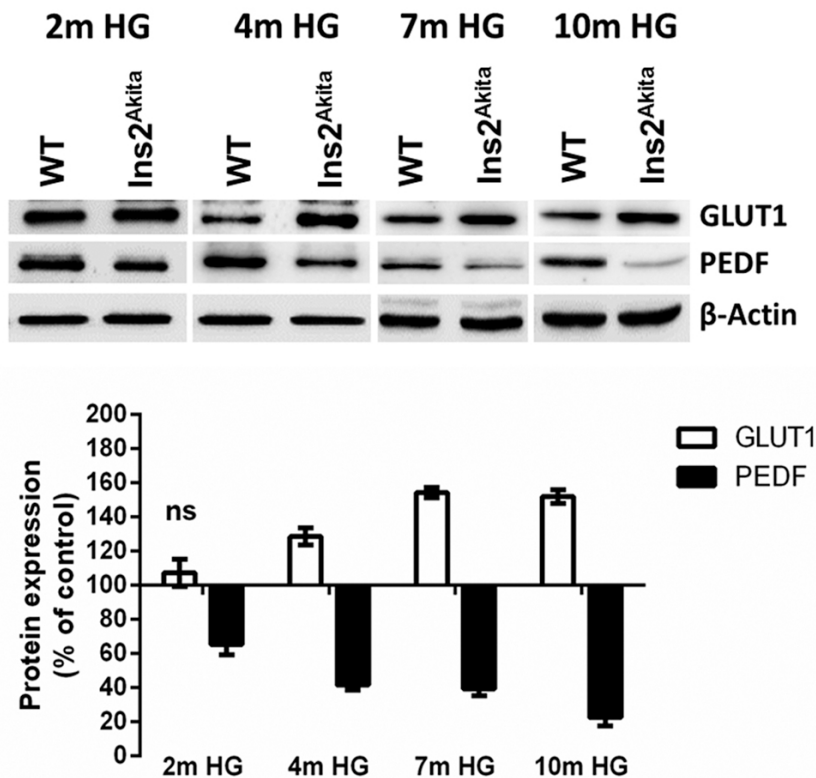


Figure 6. Secretory function of RPE is impaired in Ins2^{Akita} mice. Pigment epithelium-derived factor (PEDF) and GLUT1 protein expression was assessed with western blot of RPE tissue from diabetic mice with 2, 4, 7, and 10 months of hyperglycemia (HG) and compared with age-matched wild-type (WT) controls. Quantitative data were obtained by normalization of β-actin bands and represents GLUT1 and PEDF expression in Ins2^{Akita} related to WT (control). n = 6. ns represents non-significant data determined with Sidak's multiple comparisons test, p<0.05.

that the secretory function of RPE might be impaired [29,30,39]. A significant decrease in PEDF secreted by cells cultured with high glucose and subjected to hypoxia is observed in Figure 5, showing that the increase in glucose uptake by GLUT1 has a negative impact in the PEDF expression. Additionally, as we have previously shown, there is a concomitant increase in the expression of angiogenic VEGF and a decrease in the antiangiogenic isoform VEGF_{165b} [40,41].

Our in vitro findings were also confirmed in vivo in the RPE of the diabetic Ins2^{Akita} mice, compared with non-diabetic WT mice. The Ins2^{Akita} mouse is a non-obese model of type 1 diabetes that has been widely used as a model of DR [42-44]. This mouse model is considered a more reliable model to study diabetes mellitus complications as this model develops the disease spontaneously [40] in contrast with the Streptozotocin (STZ) models. In Ins2^{Akita} animals, hyperglycemia starts approximately at 4 weeks after birth, with retinal complications visible approximately 3 months after the onset of hyperglycemia, including vascular leakage, loss of pericytes, thickening of the inner retinal layers [42], and increase in angiogenic markers such as VEGF [44]. In the present study, we found a marked increase in GLUT1 expression in the RPE of diabetic mice, 4, 7, and 10 months after the onset of hyperglycemia, when compared with non-diabetic WT animals (Figure 6). This is consistent with the findings of Badr and coworkers in SZT-induced diabetic mice where the fraction of glucose entering the retina is higher across the RPE than across the iBRB [6].

We found a significant decrease in PEDF expression in the RPE of the diabetic mice, showing a significant impairment of the neurotrophic secretory function of RPE immediately after the onset of the disease. These results together with our in vitro results and the work of others [42] confirm the imbalance between pro- and antiangiogenic factors in the retina in this diabetic mouse model. Although they observed no significant decrease in PEDF levels in the neuroretina (which excludes the RPE) of diabetic mice before 7 months of hyperglycemia (corresponding to 9 months of age), we found a significant decrease in PEDF expression by RPE cells 2 months after the onset of hyperglycemia [44]. This result points to the possibility that in DR the oBRB is affected before the iBRB.

Further studies will focus on determining whether this increase in GLUT1 expression and activity can explain the increase in levels of reactive oxygen species (ROS) [43] and advanced glycation end products (AGEs) [44]. Additionally, our laboratory is studying whether the well-studied upregulation of VEGF by ROS, AGEs [45], and hypoxia itself can contribute to the decrease in PEDF secretion by RPE cells,

thus promoting the imbalance between PEDF and VEGF that is visible in patients with DR.

ACKNOWLEDGMENTS

This work was supported by the Portuguese Foundation for Science and Technology (FCT) with individual grants to S.M. Calado (SFRH/BD/76873/2011), and S.Simão (SFRH/BPD/78404/2011), funding to G.A. Silva (EXPL-BIM-MEC-1433-2013) and Pest-OE/EQB/LA0023 /2013. G.A. Silva was also funded by PIRG05-GA-2009-249314-EyeSee. iNOVA4Health - UID/Multi/04462/2013, a program financially supported by Fundação para a Ciência e Tecnologia / Ministério da Educação e Ciência, through national funds and co-funded by FEDER under the PT2020 Partnership Agreement is acknowledged

REFERENCES

- Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. *Lancet* 2010; 376:124-36. [PMID: 20580421].
- Simo R, Villarroel M, Corraliza L, Hernandez C, Garcia-Ramirez M. The retinal pigment epithelium: something more than a constituent of the blood-retinal barrier—implications for the pathogenesis of diabetic retinopathy. *J Biomed Biotechnol* 2010; xxx:190724-[PMID: 20182540].
- Cunha-Vaz J. The blood-ocular barriers. *Surv Ophthalmol* 1979; 23:279-96. [PMID: 380030].
- Gottfried LF, Dean DA. Extracellular and Intracellular Barriers to Non-Viral Gene Transfer. 2013; xxx:2-13. .
- Farjo KM, Ma JX. The potential of nanomedicine therapies to treat neovascular disease in the retina. *J Angiogenes Res* 2010; 2:21-[PMID: 20932321].
- Badr GA, Tang J, Ismail-Beigi F, Kern TS. Diabetes downregulates GLUT1 expression in the retina and its microvessels but not in the cerebral cortex or its microvessels. *Diabetes* 2000; 49:1016-21. [PMID: 10866055].
- Duffy A, Liew A, O'Sullivan J, Avalos G, Samali A, O'Brien T. Distinct effects of high-glucose conditions on endothelial cells of macrovascular and microvascular origins. *Endothelium* 2006; 13:9-16. [PMID: 16885062].
- Knott RM, Robertson M, Muckersie E, Forrester JV. Glucose-mediated regulation of GLUT-1 and GLUT-3 mRNA in human retinal endothelial cells. *Biochem Soc Trans* 1996; 24:216S-[PMID: 8736874].
- Kumagai AK, Kang YS, Boado RJ, Pardridge WM. Upregulation of blood-brain barrier GLUT1 glucose transporter protein and mRNA in experimental chronic hypoglycemia. *Diabetes* 1995; 44:1399-404. [PMID: 7589845].
- Xu HZ, Song Z, Fu S, Zhu M, Le YZ. RPE barrier breakdown in diabetic retinopathy: seeing is believing. *J Ocul Biol Dis Infor* 2011; 4:83-92. [PMID: 23275801].

11. Hughes JM, Groot AJ, van der Groep P, Sersansie R, Vooijs M, van Diest PJ, Van Noorden CJ, Schlingemann RO, Klaassen I. Active HIF-1 in the normal human retina. *J Histochem Cytochem* 2010; 58:247-54. [PMID: 19901273].
12. Catrina SB, Okamoto K, Pereira T, Brismar K, Poellinger L. Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. *Diabetes* 2004; 53:3226-32. [PMID: 15561954].
13. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci USA* 1995; 92:5510-4. [PMID: 7539918].
14. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem* 1996; 271:32253-9. [PMID: 8943284].
15. Kallio PJ, Pongratz I, Gradin K, McGuire J, Poellinger L. Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc Natl Acad Sci USA* 1997; 94:5667-72. [PMID: 9159130].
16. Ke Q, Costa M. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* 2006; 70:1469-80. [PMID: 16887934].
17. Levy AP, Levy NS, Loscalzo J, Calderone A, Takahashi N, Yeo KT, Koren G, Colucci WS, Goldberg MA. Regulation of vascular endothelial growth factor in cardiac myocytes. *Circ Res* 1995; 76:758-66. [PMID: 7728992].
18. Semenza GL, Nejfelt MK, Chi SM, Antonarakis SE. Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. *Proc Natl Acad Sci USA* 1991; 88:5680-4. [PMID: 2062846].
19. Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. *J Biol Chem* 2001; 276:9519-25. [PMID: 11120745].
20. Shah K, Desilva S, Abbruscato T. The role of glucose transporters in brain disease: diabetes and Alzheimer's Disease. *Int J Mol Sci* 2012; 13:12629-55. [PMID: 23202918].
21. Bell GI, Kayano T, Buse JB, Burant CF, Takeda J, Lin D, Fukumoto H, Seino S. Molecular biology of mammalian glucose transporters. *Diabetes Care* 1990; 13:198-208. [PMID: 2407475].
22. Sone H, Deo BK, Kumagai AK. Enhancement of glucose transport by vascular endothelial growth factor in retinal endothelial cells. *Invest Ophthalmol Vis Sci* 2000; 41:1876-84. [PMID: 10845612].
23. Davis AA, Bernstein PS, Bok D, Turner J, Nachtigal M, Hunt RC. A human retinal pigment epithelial cell line that retains epithelial characteristics after prolonged culture. *Invest Ophthalmol Vis Sci* 1995; 36:955-64. [PMID: 7706045].
24. Ellis FW, Krantz JC. Sugar alcohols XXII. Metabolism and toxicity studies with mannitol and sorbitol in man and animals. *J Biol Chem* 1941; 141:147-54. .
25. Aprelikova O, Chandramouli GV, Wood M, Vasselli JR, Riss J, Maranchie JK, Linehan WM, Barrett JC. Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. *J Cell Biochem* 2004; 92:491-501. [PMID: 15156561].
26. Triantafyllou A, Liakos P, Tsakalof A, Georgatsou E, Simos G, Bonanou S. Cobalt induces hypoxia-inducible factor-1 alpha (HIF-1 alpha) in HeLa cells by an iron-independent, but ROS-, PI-3K- and MAPK-dependent mechanism. *Free Radic Res* 2006; 40:847-56. [PMID: 17015263].
27. Takita H, Yoneya S, Gehlbach PL, Duh EJ, Wei LL, Mori K. Retinal neuroprotection against ischemic injury mediated by intraocular gene transfer of pigment epithelium-derived factor. *Invest Ophthalmol Vis Sci* 2003; 44:4497-504. [PMID: 14507898].
28. Hutchings H, Maitre-Boube M, Tombran-Tink J, Plouet J. Pigment epithelium-derived factor exerts opposite effects on endothelial cells of different phenotypes. *Biochem Biophys Res Commun* 2002; 294:764-9. [PMID: 12061772].
29. Boehm BO, Lang G, Volpert O, Jehle PM, Kurkhaus A, Rosinger S, Lang GK, Bouck N. Low content of the natural ocular anti-angiogenic agent pigment epithelium-derived factor (PEDF) in aqueous humor predicts progression of diabetic retinopathy. *Diabetologia* 2003; 46:394-400. [PMID: 12687338].
30. Funatsu H, Yamashita H, Nakamura S, Mimura T, Eguchi S, Noma H, Hori S. Vitreous levels of pigment epithelium-derived factor and vascular endothelial growth factor are related to diabetic macular edema. *Ophthalmology* 2006; 113:294-301. [PMID: 16406543].
31. Garg S, Davis RM. Diabetic Retinopathy Screening Update. *Clin Diabetes* 2009; 2009:140-5. .
32. Chiu CJ, Taylor A. Dietary hyperglycemia, glycemic index and metabolic retinal diseases. *Prog Retin Eye Res* 2011; 30:18-53. [PMID: 20868767].
33. Reinisalo M, Putula J, Mannermaa E, Urtti A, Honkakoski P. Regulation of the human tyrosinase gene in retinal pigment epithelium cells: the significance of transcription factor orthodenticle homeobox 2 and its polymorphic binding site. *Mol Vis* 2012; 18:38-54. [PMID: 22259223].
34. Slomiany MG, Rosenzweig SA. IGF-1-induced VEGF and IGFBP-3 secretion correlates with increased HIF-1 alpha expression and activity in retinal pigment epithelial cell line D407. *Invest Ophthalmol Vis Sci* 2004; 45:2838-47. [PMID: 15277511].
35. Sun D, Nguyen N, Degrado TR, Schwaiger M, Brosius FC. Ischemia Induces Translocation of the Insulin-Responsive Glucose-Transporter Glut4 to the Plasma-Membrane of Cardiac Myocytes. *Circulation* 1994; 89:793-8. [PMID: 8313568].
36. Wenger RH. Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. *FASEB* 2002; 16:1151-62. [PMID: 12153983].

37. Strauss O. The retinal pigment epithelium in visual function. *Physiol Rev* 2005; 85:845-81. [PMID: 15987797].
38. Barnstable CJ, Tombran-Tink J. Neuroprotective and antiangiogenic actions of PEDF in the eye: molecular targets and therapeutic potential. *Prog Retin Eye Res* 2004; 23:561-77. [PMID: 15302351].
39. Ogata N, Nishikawa M, Nishimura T, Mitsuma Y, Matsumura M. Unbalanced vitreous levels of pigment epithelium-derived factor and vascular endothelial growth factor in diabetic retinopathy. *Am J Ophthalmol* 2002; 134:348-53. [PMID: 12208245].
40. Simão S, Bitoque DB, Calado SM, Silva GA. Oxidative stress modulates the expression of VEGF isoforms in the diabetic retina. *New Frontiers in Ophthalmology*. In press.
41. Calado SM, Diaz-Corrales F, Silva GA. pEPito-driven PEDF Expression Ameliorates Diabetic Retinopathy Hallmarks. *Hum Gene Ther Methods* 2016; [PMID: 26942449].
42. Barber AJ, Antonetti DA, Kern TS, Reiter CE, Soans RS, Kradly JK, Levison SW, Gardner TW, Bronson SK. The Ins2Akita mouse as a model of early retinal complications in diabetes. *Invest Ophthalmol Vis Sci* 2005; 46:2210-8. [PMID: 15914643].
43. Gastinger MJ, Singh RS, Barber AJ. Loss of cholinergic and dopaminergic amacrine cells in streptozotocin-diabetic rat and Ins2Akita-diabetic mouse retinas. *Invest Ophthalmol Vis Sci* 2006; 47:3143-50. [PMID: 16799061].
44. Han Z, Guo J, Conley SM, Naash MI. Retinal angiogenesis in the Ins2(Akita) mouse model of diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2013; 54:574-84. [PMID: 23221078].
45. Simão S, Bitoque DB, Calado SM, Silva GA. Oxidative stress modulates the expression of VEGF isoforms in the diabetic retina. *New Frontiers in Ophthalmology* In press.

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 14 July 2016. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.