Potentials of Gene Therapy for Diabetic Retinopathy: The Use of Nucleic Acid Constructs Containing a TXNIP Promoter

Lalit PS[1,2]*, Thangal Y[1], Fayi Y[1] and Takhellambam SD[1]

[1]Department of Anatomy and Cell Biology, Wayne State University School of Medicine, USA

[2]Department of Ophthalmology, Wayne State University School of Medicine, USA

*Corresponding author: Lalit P Singh, Department of Anatomy and Cell Biology and Ophthalmology, Wayne State University School of Medicine, Detroit, MI 48201, USA, Fax: 1-313-577-3125; E-mail: plsingh@med.wayne.edu

Abstract

Diabetic retinopathy (DR) is considered as a chronic eye disease leading to blindness. DR is associated with hyperglycemia-induced oxidative stress, chronic low-grade inflammation and premature cell death. DR affects retinal capillaries, neuroretina and the retinal pigment epithelium. Recently, the thioredoxin-interacting protein TXNIP has been shown as a pro-oxidative stress, pro-inflammatory and pro-apoptotic protein, highly induced by diabetes and high glucose in all cells examined including the retina. TXNIP's actions involve binding to and inhibition of anti-oxidant and thiol-reducing capacities of thioredoxins (Trx) causing cellular oxidative stress and apoptosis. Trx1 is found in the cytosol and nucleus while Trx2 is the mitochondrial isoform. Several studies provided evidence that knockdown of TXNIP by siRNA or chemical blockade ameliorates early abnormalities of DR including endothelial dysfunction, pericyte apoptosis, Müller cell gliosis and neurodegeneration. Therefore, TXNIP is considered a potential target for preventing or slowing down the progression of DR. We recently proposed that nucleic acid constructs containing a proximal TXNIP promoter linked to a redox gene or shRNA that reduces oxidative stress and inflammation may be used to treat DR. The TXNIP promoter is sensitive to hyperglycemia therefore can drive expression of the linked gene or shRNA under high glucose environment such as seen in diabetes while remaining unresponsive at physiological glucose levels. Such a TXNIP-promoter linked gene or shRNA construct can be delivered to the retina by using adeno-associated viral vectors including AAV2 and AAV2/8 or an appropriate carrier via the intravitreal or sub retinal delivery for long-term gene therapies in DR.

Keywords: Diabetic retinopathy; TXNIP promoter; Redox protein; shRNA; Gene therapy

Abbreviations: DR: Diabetic Retinopathy; iBRB: Inner Blood Retinal Barrier; PRE: Retinal Pigment Epithelium; TXNIP: Thioredoxin-Interacting Protein; IGF: Insulin-Like Growth Factor; PDI: Protein Disulfide Isomerase

Diabetic Retinopathy

Diabetic retinopathy (DR) is the number one cause of blindness among the working population both in developed and developing countries, however, there is no

effective treatment at present. Whether it is Type 1 (due to insulin deficiency) or Type 2 (insulin resistance) diabetes, chronic hyperglycemia leads to cellular oxidative stress, low grade inflammation and premature cell death in multiple organs. As the number of people living with obesity increases in high population countries such as China and India, the incidence of diabetes and hypertension are also considered to increase tremendously and the associated complications including DR. Therefore, there is an urgent need for developing new and innovative methods to combat this devastating disease. Initially, DR was considered a microvascular disease causing endothelial cell dysfunction, pericyte apoptosis and drop out that lead to inner blood retinal barrier (iBRB) breakdown and leakage. Endothelial dysregulation and expression of membrane adhesion molecules such as ICAM1 and VCAM1 result in leukocyte adhesion and retinal inflammation [1,2]. As the disease progresses, there is microaneurism and fragile new blood vessel formation (neovascularization) and edema. In addition, recent studies have shown that there is an early neuroinflammation, neurodegeneration and Müller glial activation in DR [3,4]. Furthermore, the retinal pigment epithelium (RPE) that separates neuroretina from the fenestrated choriocapillaris and forms the outer BRB may also be compromised [5]. RPE function is critical for photoreceptor metabolism and phagocytosis of the photoreceptor outer segment and recycling of retinoic acid (conversion of All-*trans* retinol to 11-*cis* retinal) for photo transduction. Therefore, DR may be redefined as a neurodegenerative disease as well. Current treatments include intravitreal injection of anti-VEGF antibodies in late stage proliferative DR [6]. Nonetheless, the effectiveness of this treatment is limited.

TXNIP and Redox Regulation

Thioredoxin-Interacting Protein (TXNIP) is strongly induced by diabetes and high glucose in all tissues examined so far including pancreatic beta cells, the renal and retinal cells, muscle, heart and brain [7-11]. TXNIP is inhibited by insulin and insulin-like growth factor (IGF) 1 while high glucose and its metabolites induce its expression. Therefore, both in Type 1 and Type 2 diabetes, whether it is due to insulin insufficiency or resistance, there is sustained hyperglycemia in the body. Sustained TXNIP up regulation leads to cellular oxidative stress, inflammation, and premature cell death leading to disease initiation and progression [12]. TXNIP's action involves binding to thioredoxin (Trx) at its redox active site (^{32}CXXC^{35}) via disulfide linkage (Trx1 thiol ^{32}S-S^{247} TXNIP) and inhibits the anti-oxidant and thiol-reducing capacities thereby causing protein redox imbalance and

cellular oxidative and nitrosative stresses [12]. Of the thioredoxins, Trx1 is found in the cytosol and nucleus while Trx2 is the mitochondrial isoform. TXNIP also interacts with thioredoxin domain containing proteins such as protein disulfide isomerase (PDI) as well as redox independently with VEGF receptors [13,14]. In the nucleus, Trx1 provides a reducing environment for transcription factors to maintain them in an active form. The presence of TXNIP in the nucleus could trap Trx1 and oxidation of nuclear transcription factors. Furthermore, TXNIP is present in the mitochondrion in stressed cells and interaction with Trx2 [15,16]. Both Trx1 and Trx2 are known to bind to and trap ASK1 in an inactive state. TXNIP binding to Trx1 or Trx2 can dislodge ASK1 and cause cell death [15].

As mentioned above, TXNIP also interacts with PDI in ER and therefore causes ER stress and evokes an ER-stress response. (UPR^{er}) [13,14]. Irreversible or uncontrolled UPR^{er} activates the NLRP3 inflammasome, which activates caspase 1 and cleaves pro-IL-1β to mature IL-1β, which is a pro-inflammatory cytokine [17]. IL-1β is considered to activate several downstream cytokines such as TNF-α, IL-6 and CXC chemokines causing chronic low-grade inflammation and cell death. Furthermore, we have recently shown that TXNIP is also involved in mitophagy dysregulation and NLRP3 activation and inflammation [8,9,18]. These findings put TXNIP at the very heart of diabetes and its complications including DR. Till today, there is no effective drug to inhibit TXNIP directly. However, diabetes and anti-hypertensive drugs such as metformin and verapamil are known to inhibit TXNIP [19,20]. We also have shown that azaserine, which inhibits the hexosamine biosynthesis pathway and the production of UDP-GlcNAc, also reduces TXNIP expression under high glucose environment [8-10]. Furthermore, Akt and AMPK both phosphorylate TXNIP at Ser308 and enhance its degradation [21,22]. TXNIP itself can cause Akt oxidation (particularly cysteine residues at 296 and 310) and inhibit its activity thereby causing insulin signal defects [23]. Therefore, in the absence of insulin or insulin resistance; TXNIP up regulation may also alter Akt activity while sustaining the TXNIP protein level under high glucose conditions.

Transcriptional Regulation of the *TXNIP* Gene

The human *TXNIP* gene (ID 10628) contains 8 exons and has a 4206 nucleotides long cDNA (NC_000001.11) and the protein is a 395 amino acid polypeptide (XP_016855574.1). The corresponding molecular weight

Lalit PS, et al. Potentials of Gene Therapy for Diabetic Retinopathy: The Use of Nucleic Acid Constructs Containing a TXNIP Promoter. J Ophthalmol 2018, 3(2): 000147.

Copyright© Lalit PS, et al.

is ~48-50 kilo daltons. The respective mouse and rat gene IDs are: 56338 and 117514. The factors that are involved in transcription of the *TXNIP* gene have been shown to include MondoA/MLX, ChREBP and co-factor p300 [24,25]. TXNIP level is found generally low under normal glucose conditions while high glucose and its metabolites, such as glucose-6-phosphate, Fructose-6-phosphate and Glucosamine-6-phosphate, strongly stimulate TXNIP transcription and translation. The withdrawal of glucose from the medium in *in vitro* cultures restores TXNIP to the basal level [12]. Insulin and IGF-1 also inhibit high glucose-induced TXNIP mRNA expression however their effects last for a few hours only if hyperglycemia persists. Therefore, under diabetic conditions, when there is insulin deficiency (Type1) or resistance (Type2), hyperglycemia persists and TXNIP remains up regulated. We have also shown that the TXNIP promoter exists as an opened and poised chromatin configuration [11] that high glucose activates TXNIP expression within minutes and remains up regulated as long as hyperglycemia is maintained [12]. Histone deacetylase inhibitors such as trichostatin A (TSA) up regulates TXNIP expression while 5-aza-2'-deoxycytidine, an inhibitor of the DNA methyltransferase, does not inhibit TXNIP expression [11] suggesting that TXNIP expression is regulated mainly by histone acetylation activity involving p300 histone acetyltransferase under diabetic conditions [11,25]. In addition, we showed that double-stranded short RNAs targeted to the sense DNA of the TXNIP promoter also inhibits TXNIP expression most probably via chromatin closing because diabetes or hyperglycemia-induced p300 binding to the proximal TXNIP promoter is prevented [11]. These data suggested that TXNIP promoter is highly sensitive to hyperglycemia and that it can be operably linked to drive expression of a gene or shRNA of interest in order to up regulate or down regulate in diabetes. This is a simple idea but innovative because such a nucleic acid construct bearing the TXNIP promoter linked to a gene or a shRNA((including TXNIP shRNA itself – a concept of fire fights fire (F3) approach)) will be induced when there is high glucose environment in the system (Figure 1) while maintaining a basal level under physiological glucose conditions. Such a nucleic acid construct could be used for gene therapies targeting disease causing or associated genes including those that cause cellular oxidative stress, inflammation and apoptosis or genes involved in diabetes and age-related neurodegenerative diseases including insulin, redox proteins and neurotrophic factors (Figure 2A). Thus far, many gene therapy ideas fail because of the lack of a proper physiological or pathological regulatory mechanism(s) when introduced into the system.
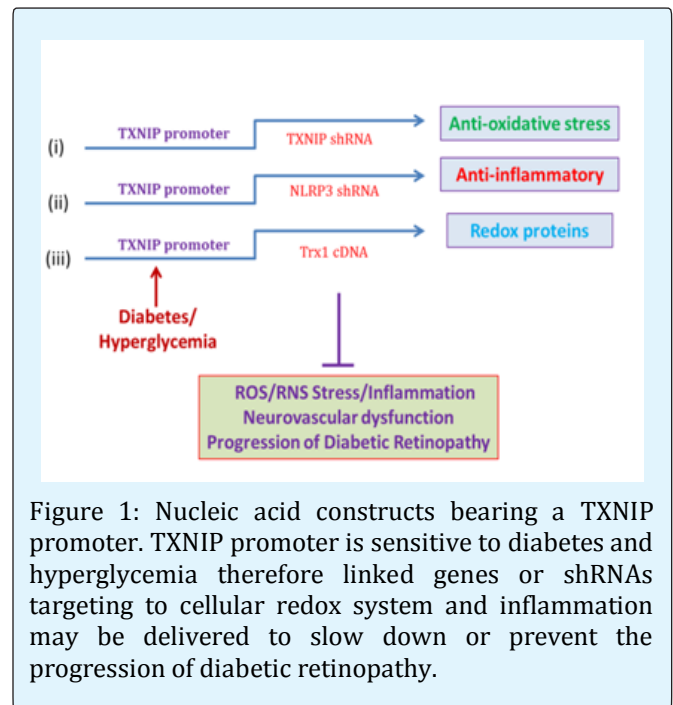

Figure 1: Nucleic acid constructs bearing a TXNIP promoter. TXNIP promoter is sensitive to diabetes and hyperglycemia therefore linked genes or shRNAs targeting to cellular redox system and inflammation may be delivered to slow down or prevent the progression of diabetic retinopathy.

Nucleic Acid Constructs Containing a TXNIP Promoter

A complete cDNA sequence for rat Trx1 (or Txn1, NM_053800.3) was linked downstream to the rat TXNIP promoter (-1 to -1526) from the transcriptional start site in a pcDNA3.1 vector with Hygromycin B selection marker. The TXNIP promoter Trx1 pcDNA3.1 construct was transfected into a human retinal pigment epiethelial cell line ARPE-19 and stable transfectants were selected in the presence of Hygromycin. An empty vector was used as a control vector. Under these conditions, we observed that Trx1 mRNA expression is unchanged under low glucose (LG, 5.5 mM glucose) between control ARPE-19 and TXNIPpromoter-Trx1 expressing ARPE-19 cells (Figure 2B). However, under high glucose (HG, 25 mM) conditions, the expression of Trx1 mRNA is increased significantly (Figure 2C). Interesting, the stable expression of TXNIPpromoter-Trx1 also reduces TXNIP mRNA levels under high glucose conditions while it remained the same under low glucose (Figures 2D,2E). This observation suggests that the TXNIP promoter is operational and therefore Trx1 expression can be increased under high glucose conditions. Several redox proteins including Trx1, Trx2, SOD1 and SOD2 are known to be down regulated or dysfunctional in diabetes and neurodegenerative diseases [26,27] and causes cellular redox imbalance, cellular oxidative stress, inflammation, and cell death. Furthermore, TXNIP up regulation inhibits

Trx1 and Trx2 activities further causing ER-stress, mitochondrial dysfunction, and NLRP3 inflammasome assembly and chronic low grade inflammation [18,28]. Therefore, with a nucleic acid construct bearing the TXNIP promoter and a redox protein of choice can serve both purposes – one to increase the expression of the redox protein while down regulating TXNIP at the same time. The mechanism(s) for a TXNIP promoter driven gene (Trx1) down regulating TXNIP expression itself may be of several folds. Without giving a definitive mechanistic assurance at this time, a possibility exists that Trx1 over expression suppresses cellular oxidative stress and down regulates TXNIP protein. Alternatively, the transfected *TXNIP* gene promoter limits the availability of transcription factors and co-factors that are needed for the expression of the endogenous *TXNIP* gene. Further in-depth studies will clarify such a mechanism and others, if occur. Finally, various disease-associated genes and their promoters can also be identified and utilized based on the same principle of a TXNIP promoter-linked target gene or constructs bearing short activating or inhibiting RNAs targeted to promoters or noncoding RNAs such as microRNA and long noncoding RNAs. Such a treatment method, in addition to diabetic retinopathy, may particularly be suitable for blood borne and systemic inflammatory diseases as they will encounter hyperglycemic episodes after each meal that can activate the TXNIP-promoter and drive expression of a desired gene.
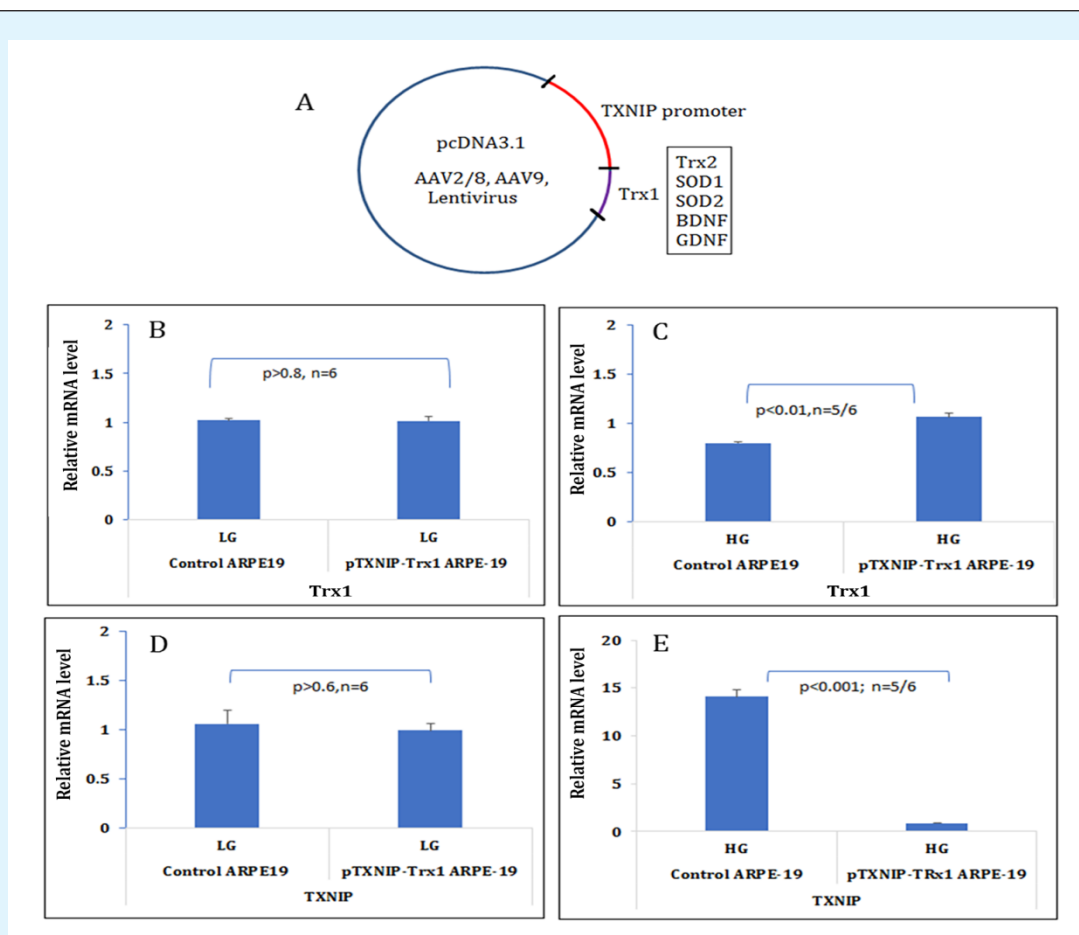

Figure 2: A nucleic acid construct bearing TXNIP promoter linked with anti-oxidant Trx1cDNA (NM_053800.3) in pcDNA3.1 vector (synthesized by GenScript, Piscataway, NJ). Stable expression of this vector in a retinal pigment epithelial cell line, ARPE-19, leads to upregulation of Trx1 and down regulation of TXNIP under HG conditions, but not under LG.

Lalit PS, et al. Potentials of Gene Therapy for Diabetic Retinopathy: The Use of Nucleic Acid Constructs Containing a TXNIP Promoter. J Ophthalmol 2018, 3(2): 000147.

Copyright© Lalit PS, et al.

Vectors for Ocular Gene Delivery

A vector is a molecular device that will carry the gene into the cell and within the nucleus for transcription. Therefore, the choice of an appropriate vector for gene therapy is critical for a successful outcome. The retina being a part of the central nervous system is made up of neuroretina (consist of different types of neurons such as retinal ganglion, bipolar, amacrine and photoreceptor cells), RPE, astrocytes, microglia, Müller glia, and the capillaries [1-5]. Furthermore, the retina is a closed organ and a relatively immune privileged site therefore has several advantages for gene therapy for ocular diseases in that there is less systemic immune response and chances of cross contamination to other organs are minimal. In addition, genetic material needed for the therapy is small (less cost) when compared to a systemic delivery to other organs. Most gene therapy methods for retinal diseases have used adeno-associated viral vectors (AAVs) of different serotypes that transduce different cells [29,30]. These AAV vectors include AAV2, AAV5, AAV8, AAV9 and their modified versions [29], which transduce photoreceptor neurons and RPE particularly in the retina. Vectors that transduce to other neurons, Müller glia and other retinal cells are also developing.

Lentivirus is another vector that transfects non-dividing fully differentiated cells [31]. However, these vectors are incorporated into the genome while AAVs exist as an episome, not incorporated into the genome. For this reason, AAVs have been developed and tested in most laboratory experiments and clinical trials [28]. Two main routes for vector delivery to the back of the eye are the intravitreal and subretinal injections. Because of the blood retinal barrier, a systemic delivery for the retina is inefficient. However, in cases where the blood-retinal barrier has been broken down, a systemic administration of the vectors may reach the retina. Still then, vectors delivery to the retina have to cross the Müller glial end-feet, which are composed of extracellular matrix components including glycosaminoglycans secreted by Müller cells. Enzymatic thinning of the inner limiting membrane may enhance intravitreal delivery of vectors for gene therapy [32]. The second vector route is the subretinal delivery to the photoreceptors and RPE. Here again, there is an outer limiting membrane of the Müller glia end-feet, which needs to be crossed. Several studies have shown that the modified AAV2/8 vector may deliver gene constructs to several retinal cells. Choosing the right vector will be critical in further research and clinical trials in humans. This together with selection of cell type specific promoters for gene delivery will form the basis for further studies in ocular gene therapies.

Conclusion

For a proof of concept, one can use lentivirus as well as AAV viruses to deliver genes to the retina. In our case, further studies with animal models will be conducted using AAV vectors as well as lentivirus bearing the TXNIP-promoter and gene of interest. TXNIP is induced by diabetes in all cells examined including retinal cells and causes cellular oxidative stress, inflammation, and apoptosis. Therefore, selecting a TXNIP promoter gene construct for the treatment of diabetes and its complications including diabetic retinopathy is innovative and appropriate. Gene therapy opens up possibilities for long-term treatment of diabetic retinopathy compared to what is currently being practiced for multiple injections of proteins, peptides, antibodies and drugs. We plan to exploit the TXNIP promoter to deliver genes and shRNAs that will restore cellular redox environment, reduce inflammation and prevent retinal cell death in diabetes and diabetic retinopathy [32].

Acknowledgement

References

1. Wong TY, Cheung CM, Larsen M, Sharma S, Simó R (2016) Diabetic retinopathy. Nat Rev Dis Primers 2: 16012.

2. Roy S, Kern TS, Song B, Stuebe C (2017) Mechanistic Insights into Pathological Changes in the Diabetic Retina: Implications for Targeting Diabetic Retinopathy. Am J Pathol 187(1): 9-19.

3. Abcouwer SF, Gardner TW (2014) Diabetic retinopathy: loss of neuroretinal adaptation to the diabetic metabolic environment. Ann N Y Acad Sci 1311: 174-190.

4. Du Y, Veenstra A, Palczewski K, Kern TS (2013) Photoreceptor cells are major contributors to diabetes-induced oxidative stress and local inflammation in the retina. Proc Natl Acad Sci U S A 110(41): 16586-16591.

5. Ponnalagu M, Subramani M, Jayadev C, Shetty R, Das D (2017) Retinal pigment epithelium-secretome: A

diabetic retinopathy perspective. Cytokine 95: 126-135.

6. Gupta A, Sun JK, Silva PS (2018) Complications of Intravitreous Injections in Patients with Diabetes. Semin Ophthalmol 33(1): 42-50.

7. Shalev A (2014) Mini review: Thioredoxin-interacting protein: regulation and function in the pancreatic β-cell. Mol Endocrinol 28(8): 1211-1220.

8. Cheng DW, Jiang Y, Shalev A, Kowluru R, Crook ED, et al. (2006) An analysis of high glucose and glucosamine-induced gene expression and oxidative stress in renal mesangial cells. Arch Physiol Biochem 112(4-5): 189-218.

9. Perrone L, Devi TS, Hosoya K, Terasaki T, Singh LP (2009) Thioredoxin interacting protein (TXNIP) induces inflammation through chromatin modification in retinal capillary endothelial cells under diabetic conditions. J Cell Physiol 221(1): 262-272.

10. Perrone L, Devi TS, Hosoya KI, Terasaki T, Singh LP (2010) Inhibition of TXNIP expression in vivo blocks early pathologies of diabetic retinopathy. Cell Death Dis 1(8): e65.

11. Devi TS, Lee I, Hüttemann M, Kumar A, Nantwi KD, et al. (2012) TXNIP links innate host defense mechanisms to oxidative stress and inflammation in retinal Müller glia under chronic hyperglycemia: implications for diabetic retinopathy. Exp Diabetes Res 2012: 438238.

12. Lee S, Min Kim S, Dotimas J, Li L, Feener EP, et al. (2014) Thioredoxin-interacting protein regulates protein disulfide isomerases and endoplasmic reticulum stress. EMBO Mol Med 6(6): 732-743.

13. Spindel ON, World C, Berk BC (2012) Thioredoxin interacting protein: redox dependent and independent regulatory mechanisms. Antioxid Redox Signal 16(6): 587-596.

14. Saxena G, Chen J, Shalev A (2010) Intracellular shuttling and mitochondrial function of thioredoxin-interacting protein. J Biol Chem 285(6): 3997-4005.

15. Devi TS, Somayajulu M, Kowluru RA, Singh LP (2017) TXNIP regulates mitophagy in retinal Müller cells under high-glucose conditions: implications for diabetic retinopathy. Cell Death Dis 8(5): e2777.

16. Lerner AG, Upton JP, Praveen PV, Ghosh R, Nakagawa Y, et al. (2012) IRE1α induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress. Cell Metab 16(2): 250-264.

17. Singh LP (2013) Thioredoxin Interacting Protein (TXNIP) and Pathogenesis of Diabetic Retinopathy. J Clin Exp Ophthalmol 5: 4.

18. Chai TF, Hong SY, He H, Zheng L, Hagen T, et al. (2012)A potential mechanism of metformin-mediated regulation of glucose homeostasis: inhibition of Thioredoxin-interacting protein (Txnip) gene expression. Cell Signal 24(8): 1700-1705.

19. Chen J, Cha-Molstad H, Szabo A, Shalev A (2009) Diabetes induces and calcium channel blockers prevent cardiac expression of proapoptotic thioredoxin-interacting protein. Am J Physiol Endocrinol Metab 296(5): E1133-1139.

20. Waldhart AN, Dykstra H, Peck AS, Boguslawski EA, Madaj ZB,et al. (2017) Phosphorylation of TXNIP by AKT Mediates Acute Influx of Glucose in Response to Insulin Cell Rep 19(10): 2005-2013.

21. Wu N, Zheng B, Shaywitz A, Dagon Y, Tower C, et al. (2013) AMPK-dependent degradation of TXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. Mol Cell 49(6): 1167-1175.

22. Ahmad F, Nidadavolu P, Durgadoss L, Ravindranath V (2014) Critical cysteines in Akt1 regulate its activity and proteasomal degradation: implications for neurodegenerative diseases. Free Radic Biol Med 74: 118-128.

23. Stoltzman CA, Kaadige MR, Peterson CW, Ayer DE (2011) MondoA senses non-glucose sugars: regulation of thioredoxin-interacting protein (TXNIP) and the hexose transport curb. J Biol Chem 286(44): 38027-38034.

24. Cha-Molstad H, Saxena G, Chen J, Shalev A (2009) Glucose-stimulated expression of Txnip is mediated by carbohydrate response element-binding protein, p300, and histone H4 acetylation in pancreatic beta cells. J Biol Chem 284(25): 16898-16905.

25. Newsholme P, Cruzat VF, Keane KN, Carlessi R, de Bittencourt PI Jr (2016) Molecular mechanisms of ROS production and oxidative stress in diabetes. Bio chem J 473(24): 4527-4550.

26. Yin F, Boveris A, Cadenas E (2014) Mitochondrial energy metabolism and redox signaling in brain aging and neurodegeneration. Antioxid Redox Signal 20(2): 353-371.

27. Singh LP, Devi TS, Yumnamcha T (2017) The Role of Txnip in Mitophagy Dysregulation and Inflammasome Activation in Diabetic Retinopathy: A New Perspective. JOJ Ophthalmol 4(4): pii: 555643.

28. Planul A, Dalkara D (2017) Vectors and Gene Delivery to the Retina. Annu Rev Vis Sci 3: 121-140.

29. Da Costa R, Röger C, Segelken J, Barben M, Grimm C, et al. (2016) A Novel Method Combining Vitreous Aspiration and Intravitreal AAV2/8 Injection Results in Retina-Wide Transduction in Adult Mice. Invest Ophthalmol Vis Sci 57(13): 5326-5334.

30. Bemelmans AP, Kostic C, Crippa SV, Hauswirth WW,et al. (2006) Lentiviral gene transfer of RPE65 rescues survival and function of cones in a mouse model of Leber congenital amaurosis. PLoS Med 3(10): e347.

31. Ochakovski GA, Bartz-Schmidt KU, Fischer MD (2017) Retinal Gene Therapy: Surgical Vector Delivery in the Translation to Clinical Trials. Front Neurosci 11: 174.

32. Pukhrambam LS (2017) Nucleic acid constructs including a Txnip promoter for the treatment of disease, US20170252376A1.