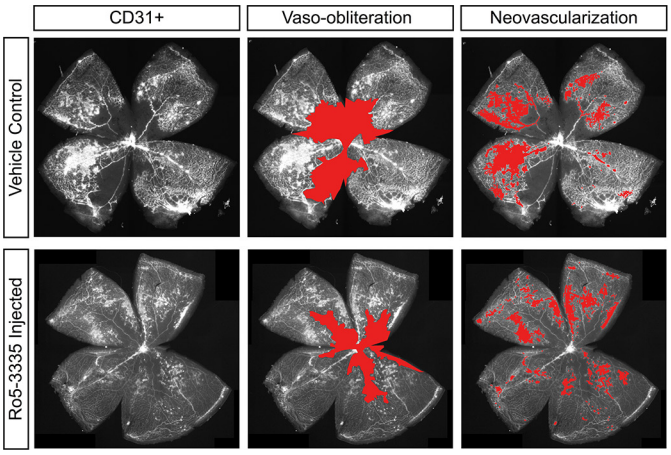
422 Diabetic Retinopathy: New Targets and Emerging Therapies
Wednesday, May 10, 2017 8:30 AM–10:15 AM
Exhibit/Poster Hall Poster Session
Program #/Board # Range: 4028–4051/B0001–B0024
Organizing Section: Retinal Cell Biology

Program Number: 4028 **Poster Board Number:** B0001
Presentation Time: 8:30 AM–10:15 AM
Secretogranin III as a novel disease-associated target for anti-angiogenic therapy of diabetic retinopathy
Wei Li[1, 4], Michelle LeBlanc[1, 4], Weiwen Wang[1], Xiuping Chen[1, 2], Nora B. Caberoy[3], Chen Shen[1], Yanli Ji[1], Hong Tian[4, 1], Hui Wang[5], Rui Chen[5]. [1]Ophthalmology, Bascom Palmer Eye Institute, Miami, FL; [2]Ophthalmology, Zhongshan Hospital, Shanghai, China; [3]School of Life Sciences, University of Nevada Las Vegas, Las Vegas, NV; [4]Everglades Biopharma, Miami, FL; [5]Baylor College of Medicine, Houston, TX.
Purpose: Vascular endothelial growth factor (VEGF) inhibitors have been approved for diabetic macular edema (DME) therapy but with limited efficacy. The purpose of this study is to identify unknown vascular permeability factors (VPFs) and develop novel therapies for diabetic retinopathy (DR).
Methods: We applied a new technology of ligandomics to diabetic or control mice for global identification of cell-wide retinal endothelial ligands. Quantitative comparison of the entire ligand profiles for diabetic vs. control retina systematically identified ligands with increased or decreased binding to diabetic endothelia. Identified ligands were independently verified as VPFs by *in vitro* endothelial permeability assay. Disease-associated angiogenic activity was verified by corneal pocket assay in diabetic or control mice. Monoclonal antibodies (mAb) were generated. Therapeutic agents were intravitreally injected to evaluate their ability to inhibit retinal vascular leakage using Evans blue assay.
Results: Comparative ligandomics systematically identified 353 ligands with increased binding (DR-high) to diabetic retinal endothelia and 105 ligands with decreased binding (DR-low). Secretogranin III (Scg3) was identified as a DR-high ligand with the highest binding activity ratio for diabetic vs. control retina (1,731:0, $p<0.0001$, c^2 test). Scg3 was upregulated in the vitreous fluid of diabetic mice only by 1.38-fold ($p<0.05$). We independently verified Scg3 as a novel VPF. Scg3 was corroborated as a DR-high ligand that selectively stimulated corneal angiogenesis in diabetic but not control mice. In contrast, VEGF induced angiogenesis in both diabetic and control mice. Scg3 induced angiogenesis through a VEGF-independent receptor pathway. Intravitreal injection of Scg3-neutralizing mAb significantly alleviated retinal vascular leakage in two independent mouse models of DR with high efficacy ($p<0.01$).
Conclusions: Scg3 was previously reported as a protein to regulate secretory granule biogenesis and has not hitherto been described as a cellular ligand. This study discovers Scg3 as a novel and highly disease-associated VPF and angiogenic factor. Scg3-neutralizing mAb ameliorates DR leakage with high efficacy, implicating its potential for DME therapy. These results also demonstrate the validity and utility of the comparative ligandomics for systematic mapping of therapeutic ligands.
Commercial Relationships: Wei Li; **Michelle LeBlanc**, Inventor - University of Miami (P), Cofounder - Everglades Biopharma (S); **Weiwen Wang**, Inventor - University of Miami (P); **Xiuping Chen**, None; **Nora B. Caberoy**, None; **Chen Shen**, None; **Yanli Ji**, None; **Hong Tian**, Co-founder - Everglades Biopharma (S), Spouse - Everglades Biopharma (S); **Hui Wang**, None; **Rui Chen**, None
Support: NIH R01GM094449; R21EY027065; AHA 16PRE27250308

Program Number: 4029 **Poster Board Number:** B0002
Presentation Time: 8:30 AM–10:15 AM
Inhibition of Runx1 by the Ro5-3335 benzodiazepine derivative reduces aberrant retinal angiogenesis
Leo A. Kim[1, 2], Jonathan D. Lam[2], Angie Sanchez[2], Dhanesh Amarnani[2], Jonathan Cardona-Velez[2], Joseph Arboleda-Velasquez[2]. [1]Retina Service, Massachusetts Eye and Ear Infirmary, Boston, MA; [2]Schepens Eye Reseach Institute, Boston, MA.
Purpose: Runx1, the runt-related transcription factor 1, has been implicated in endothelial cell function. We recently identified Runx1 expression to be enhanced in endothelial cells isolated from fibrovascular membranes of patients with proliferative diabetic retinopathy. Ro5-3335 is a well characterized inhibitor of Runx1 function in models of leukemia. We hypothesize that inhibition of Runx1 function, with the Ro5-3335 inhibitor, may ameliorate pathologic retinal neovascularization.
Methods: The oxygen-induced retinopathy model of retinal angiogenesis was employed for this study. Wild-type C57BL/6J P7 mice were exposed to 75% O_2 for 5 days and returned to room air (P12). Mice were treated with 75 µM Ro5-3335 inhibitor (n = 7) or DMSO vehicle (n = 7) via intravitreal injections in the left eye at P13 and P15 before sample collection at P17. Retina were dissected and stained for Isolectin B4, CD31, and Runx1 followed by quantification of the vaso-obliterative and neovascular regions.
Results: Untreated eyes stained for Runx1 and Isolectin B4 demonstrate selective staining of neovascular tufts for Runx1. There was significant reduction in neovascular tuft area in the inhibitor treated group (5.2% ± 2.2 whole retina) compared to vehicle (10.4% ± 3.0 whole retina, $p=0.003$). The vaso-obliterative region of the inhibitor treated group (9.9% ± 4.7 whole retina) was comparable to the vehicle treated group (14.2% ± 5.9 whole retina, $p=0.16$).
Conclusions: The localization of Runx1 staining to neovascular tufts suggests it is a novel and specific regulator of endothelial cell function in aberrant retinal angiogenesis. Intravitreal injection of Ro5-3335 was associated with a significant decrease in neovascular tuft area compared to vehicle treated pups. This was not associated with a significant shift in the avascular region suggesting independent regulation between normal and abnormal vascularization of the retina. Inhibition of Runx1 function with Ro5-3335 is efficacious in reducing pathologic retinal neovascularization.



Quantification of vaso-obliteration and neovascularization at P17 in a mouse model of oxygen induced retinopathy. Animals were injected with vehicle (DMSO) control (Top Row) or with 75 µM Ro5-3335 (Bottom Row). Retina were stained for CD31 (First Column), and the area of vaso-obliteration (Second Column) and neovascularization (Third Column) quantified.

Commercial Relationships: Leo A. Kim, U.S. Patent Application No. 62/422,523 (P); **Jonathan D. Lam**, None; **Angie Sanchez**, None; **Dhanesh Amarnani**, None; **Jonathan Cardona-Velez**, None; **Joseph Arboleda-Velasquez**, U.S. Patent Application No. 62/422,523 (P)
Support: NIH K12-EY16335, R21-EY027061, P30-E7003790, and E. Matilda Ziegler Foundation for the Blind

Program Number: 4030 **Poster Board Number:** B0003
Presentation Time: 8:30 AM–10:15 AM
Administration of a gap junction coupler reduces retinal vascular cell loss associated with diabetic retinopathy
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Purpose: In diabetic retinopathy (DR), cell-cell coupling appears to be compromised, leading to retinal vascular cell death and the development of retinal vascular lesions. The aim of this study is to evaluate whether administration of a gap junction coupler may be protective against retinal vascular cell death in the retinas of diabetic rats.
Methods: To determine the optimal concentration of danegaptide (DG), a Cx43 gap junction coupler, two concentrations (200 nM or 1000 nM) were tested via intravitreal injections in streptozotocin (STZ)-induced diabetic rats. Diabetes was induced via STZ for 6 weeks and at the end of the study, eyes were enucleated, and retinas subjected to retinal trypsin digest (RTD) for isolation of capillary networks and stained with Hematoxylin and Periodic Acid Schiff (PAS) to analyze the number of acellular capillaries (AC) and pericyte loss (PL). Based on this pilot study, 1000 nM DG was found to be optimal and therefore injected intravitreally in the follow-up study to determine the effects of improved cell coupling on retinal vascular cell death. Additionally, DG was administered systemically via osmotic pumps. The number of rats used in this study was at least 6 per group. Overall, rats were divided into 6 groups: wild type (WT) control rats, STZ-induced diabetic rats, diabetic rats intravitreally injected with DG, diabetic rats intravitreally injected with water, diabetic rats systemically delivered with DG, and diabetic rats systemically delivered with water. Diabetes was induced for 15 weeks and at the end of the study all animals were sacrificed and their retinas were isolated and subjected to RTD for analyses of AC and PL.
Results: The retinal vasculature showed a significant decrease in the development of AC in diabetic rats treated either via intravitreal injections or systemically with DG (134±28% of control, $p<0.01$ and 133±37% of control, $p<0.001$, respectively) compared to those of diabetic rats (296±26% of control, $p<0.001$). Likewise, retinal capillaries of diabetic rats treated intravitreally or systemically with DG exhibited a significant decrease in the number of PL (112±62% of control, $p<0.01$ and 103±65% of control, $p<0.005$, respectively) compared to those of diabetic rats (335±40%, $p<0.005$).
Conclusions: Findings from this study indicate that improved cell-cell coupling may be protective against retinal vascular cell death associated with DR.
Commercial Relationships: Dongjoon Kim, Zealand Pharma A/S (F); **Dayeun Lee**, Zealand Pharma A/S (F); **Ulrik Mouritzen**, Zealand Pharma A/S (E); **Bjarne D. Larsen**, Zealand Pharma A/S (E); **Sayon Roy**, Zealand Pharma A/S (F)
Support: Zealand Pharma A/S

Program Number: 4031 **Poster Board Number:** B0004
Presentation Time: 8:30 AM–10:15 AM
A novel regulator of cytosolic oxidative stress in the development of Diabetic retinopathy
Manish Mishra, Renu A. Kowluru. Ophthalmology, Kresge Eye Institute, Detroit, MI.
Purpose: In diabetes, increased retinal activity of Rac1, a small molecular weight GTPase, increases NADPH-oxidase 2 mediated cytosolic reactive oxygen species (ROS) that damages retinal mitochondria and accelerates capillary cell death. Activity of Rac1, in turn, is stimulated by p66Shc, an adapter protein that promotes oxidative stress. Regulation of *p66Shc* is controlled by histone acetylation, which facilitates the binding of p53 transcription factor at its promoter. In diabetes, expression of p66Shc and p53 are increased in the retina, activity of Sirt1, a histone deacetylase, is decreased, and induction of Sirt1 prevents increase in cellular ROS. Our aim is to examine the role of p66Shc in Rac1 activation and its regulation in the development of diabetic retinopathy.
Methods: To investigate the interrelationship between p66Shc and the Rac1, human retinal endothelial cells, incubated in high glucose for 24-96 hours were employed and tested for *p66Shc* expression and Rac1 activity using qPCR and G-LISA colorimetric assay respectively. Cellular localization of p66Shc and Rac1 was determined by immunofluorescence imaging. Role of p53 and the effect of histone acetylation on its binding at *p66Shc* promoter was determined in the cells overexpressing *Sirt1* using chromatin immunoprecipitation assay.
Results: High glucose increased *p66Shc* expression as early as 24 hours of its exposure, and it continued with the increased duration of glucose exposure. *P66Shc* expression and Rac1 activation presented a strong correlation, and this was confirmed by cytosolic co-localization of Rac1 and p66Shc. The binding of p53 at *p66Shc* promoter was increased by ~40%, which was prevented in the cells overexpressing *Sirt1*. In the same *Sirt1* overexpressing cells, glucose-induced Rac1 activation was also ameliorated.
Conclusions: In diabetes, p66Shc plays an important role in retinal Rac1 activation, and Sirt1 regulates p66Shc via modulating p53 binding at *p66Shc* promoter. Thus, understanding the relationship between Rac1, p66Shc and Sirt1 will provide a novel mechanism of regulating early oxidative response to maintain cellular homeostasis and prevent the development of diabetic retinopathy.
Commercial Relationships: Manish Mishra, None; **Renu A. Kowluru**, None
Support: EY014370, EY017313, EY022230

Program Number: 4032 **Poster Board Number:** B0005
Presentation Time: 8:30 AM–10:15 AM
Role of activated monocytes in lysyl oxidase-mediated retinal vascular cell stiffening and inflammation associated with diabetes
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Purpose: Leukocyte adhesion to ICAM-1-expressing retinal vascular endothelial cells (EC) is a critical early step in diabetic retinopathy (DR). The adherent (activated) leukocytes release various factors to further amplify inflammation. Since we have recently shown that high glucose-induced vascular stiffening promotes leukocyte-EC adhesion, here we tested the hypothesis that adherent leukocytes also contribute to progressive stiffening of retinal vessels in diabetes, thereby amplifying retinal vascular inflammation associated with DR.
Methods: Human U937 monocytes were activated with high glucose and PMA (200nM). Monocyte activation was confirmed by staining with DCF, a reactive oxygen species (ROS) indicator. Conditioned medium (CM) from activated monocytes was then added to human

retinal ECs (HRECs), followed by qPCR analysis of ICAM-1, lysyl oxidase (LOX), and ROCK2 mRNA, key pro-inflammatory and matrix/EC stiffening mediators, respectively. We next used Amplex Red reagent to determine whether activated monocytes themselves undergo LOX upregulation. Finally, these biochemical measurements were correlated with atomic force microscopy (AFM) measurement and immunofluorescent staining of HREC stiffness and collagen IV matrix deposition, respectively.
Results: High glucose and PMA treatment caused significant activation of U937 monocytes, as judged by a >10-fold increase in ROS levels. Importantly, HRECs treated with monocyte CM exhibited a marked increase in ICAM-1, LOX and ROCK2 mRNA expression. Our AFM measurements confirmed that activated monocytes cause a significant increase in EC stiffness, which correlates with increased endothelial LOX and ROCK2 mRNA. Interestingly, the activated monocytes themselves exhibited a two-fold increase in LOX levels. Finally, our preliminary studies indicate that activated monocytes cause an increase in collagen IV matrix deposition by HRECs. Studies are underway to better understand the molecular mechanisms by which activated monocytes contribute to LOX-dependent retinal vascular stiffening and inflammation.
Conclusions: Our findings indicate a potentially crucial role of activated monocytes in vascular stiffening-dependent retinal inflammation associated with DR and provide rationale to further examine this pathway for the identification of novel anti-inflammatory targets for improved DR therapies in the future.
Commercial Relationships: Andrea P. Cabrera; **Xiao Yang**, None; **Kaustabh Ghosh**, None

Program Number: 4033 **Poster Board Number:** B0006
Presentation Time: 8:30 AM–10:15 AM
Adipose Stem Cell Therapy for Early Retinal Complications of Diabetes in the Ins2Akita Mouse
William Evans, Ramesh Periasamy, RAJI RAJESH LENIN, Rajashekhar Gangaraju. University of Tennessee Health Science Center, Memphis, TN.
Purpose: Diabetic retinopathy (DR) is the most common vascular complication in patients with long-standing diabetes, and is the leading cause of blindness in working-age adults. Early stage DR has been shown to involve neurovascular defects, including the breakdown of retinal barrier integrity driven by ischemia, inflammation and apoptosis. In this study, we hypothesized that CD140b enriched adipose stem cells (ASC) or the paracrine factors released by these stem cells could therapeutically rescue early stage DR features.
Methods: Heterozygous male Ins2Akita mice were intravitreally injected with CD140b+ ASC (1000cells/eye), conditioned media (CM, 20x, 1mL/eye) or saline at 24 weeks of age. Age matched C57BL/6J mice with saline injections served as controls. The mice were analyzed after one and 2-weeks post-treatment by Electroretinography, OCT and OKN. The RayBio® Membrane-Based Antibody Array assessed differences in human cytokines released by CD140b+ASC CM compared with CD140b- ASC CM.
Results: Ins2Akita diabetic mice that received saline injection demonstrated significantly decreased b-wave amplitude, decreased visual acuity and increased need for contrast sensitivity compared to non-diabetic mice. On the other hand, diabetic mice that received ASC or CM injection demonstrated robust increase in b-wave amplitude; visual acuity and a decrease need for contrast sensitivity compared to saline treated Ins2Akita diabetic mice (n=7, $p<0.05$). OCT demonstrated preserved retinal architecture following ASC and CM treatment. Of the 80 proteins tested, 47 proteins remained unchanged (>0.5-<1.5 fold), 9 proteins downregulated (<0.5 fold) and 24 proteins upregulated (>1.5 fold) in CD140b+ASC compared to CD140b-ASC.
Conclusions: First observations of ASC and CM treatment are highly favorable in the treatment against the early retinal complications of diabetes in the Ins2Akita model. Possible beneficial proteins from ASC-CM that are implicated in the observed paracrine effects of ASC in the retina were identified. Future molecular and histological analysis studies are needed to confirm the beneficial effects of the stem cells *in vivo*. Long term studies will look into identifying complications of stem cell treatment, including potential rejection and need for reinjection.
Commercial Relationships: William Evans, None; **Ramesh Periasamy**, None; **RAJI RAJESH LENIN**, None; **Rajashekhar Gangaraju**, Cell Care Therapeutics, Inc (I), Cell Care Therapeutics, Inc (P)
Support: NH Grant EY023427 & RPB Grant

Program Number: 4034 **Poster Board Number:** B0007
Presentation Time: 8:30 AM–10:15 AM
Overexpression of ELOVL4 stabilizes tight junctions and prevents VEGF-induced vascular permeability in diabetic retina and retinal cells
Nermin Kady[1], Xuwen Liu[2], Todd Lydic[1], Segey Seregin[3], Andrea Amalfitano[4], Vince A. Chiodo[5], Sanford L. Boye[5], William Hauswirth[5], Maria B. Grant[6], David Antonetti[2], Julia V. Busik[1]. [1]Physiology, Michigan State university, East Lansing, MI; [2]Ophthalmology and Visual Sciences, University of Michigan -Kellogg eye center, Ann arbor, MI; [3]University of Michigan, Ann arbor, MI; [4]Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI; [5]University of Florida, Gainesville, FL; [6]Opthalmology, University of Indiana, Bloomington, IN.
Purpose: We have previously demonstrated that the expression level of elongation of very long chain fatty acids –like 4(ELOVL4) is significantly reduced in the diabetic retina leading to decreased levels of very long chain (VLC ≥26:2) fatty acids. VLC fatty acids produced by ELOVL4 incorporate into VLC ceramides that are known to play an important role in skin permeability barrier maintenance. We hypothesized that, similar to skin, VLC ceramides could play a beneficial role in maintenance of the blood-retinal barrier and reduction of ELOVL4 in diabetes could lead to increased retinal vascular permeability through the decrease in VLC ceramides.
Methods: Human ELOVL4 was overexpressed in bovine retinal endothelial cells (BRECs) by an E1- and E3-deleted adenoviral vector under the control of CMV promoter. Permeability was determined in confluent monolayers by RITC dextran. Tight junction proteins were assayed by Western blot and immunostaining. Ceramide colocalization with tight junction complex was determined by immunogold electron microscopy. Tight junction isolates were analyzed by tandem mass spectrometry. Streptozotocin (STZ) mice received intravitreal injection of hELOVL4 packaged in adeno-associated virus type 2 containing four capsid Y-F mutations (AAV2 mut quad) under the control of a CMV-chicken β-actin (smCBA) promoter or empty AAV2 mut quad as a negative control. Retinal vascular permeability was assessed by measuring extravasation of FITC-albumin after 8 weeks of diabetes.
Results: Overexpression of hELOVL4 in BRECs significantly decreased basal permeability by 46% (n=3, $P<0.01$), as well as VEGF and IL-1β-induced permeability by 47.7% and 36.2%; respectively (n= 3-6, $p<0.001$, $P<0.01$; respectively). Importantly, hELOVL4 overexpression prevented VEGF-induced decrease in occludin expression and border staining of tight junction proteins; ZO-1 and claudin-5. Ceramides were found to colocalize with tight junction complexes. Lipidome analysis of tight junction isolates

revealed the presence of VLC ceramides. Intravitreal delivery of hELOVL4-AAV2 in STZ mice reduced diabetes-induced increase in vascular permeability by 75% after 8 weeks of diabetes (n= 8-12 per group, *p<0.01*).
Conclusions: Normalization of retinal ELOVL4 expression could prevent blood-retina barrier dysregulation in DR through increase in VLC ceramides and stabilization of tight junctions.
Commercial Relationships: Nermin Kady, None; **Xuwen Liu**, None; **Todd Lydic**, None; **Segey Seregin**, None; **Andrea Amalfitano**, None; **Vince A. Chiodo**, None; **Sanford L. Boye**, None; **William Hauswirth**, Bionic Sight (I), AGTC (I); **Maria B. Grant**, None; **David Antonetti**, None; **Julia V. Busik**, None
Support: RO1EY016077 NIH/NEI, R01EY025383 NIH/NEI, AgBioResearch grant MICL02163, NIH EY012021 and Jules and Doris Stein Professorship,NIH P30EY021721 (WWH), Research to Prevent Blindness (Univ. of Florida)

Program Number: 4035 **Poster Board Number:** B0008
Presentation Time: 8:30 AM–10:15 AM
The miRNA451a/ATF2 signal pathway regulates mitochondrial function of RPE in diabetic conditions
yan shao[1, 2], Lijie Dong[2], Yusuke Takahashi[1], Xun Liu[2], Qian Chen[1], Xiaorong Li[1], Jian-Xing (Jay) Ma[1, 2]. [1]physiology, OUHSC, Oklahoma city, OK; [2]Tianjin medical university eye hospitial, Tianjin, China.
Purpose: Mitochondrial dysfunction plays a pathogenic role in diabetic retinopathy. The purpose of this study was to explore the role of miRNA451a (miR-451a) in mitochondrial dysfunction induced by diabetes.
Methods: Vitreous and epiretinal membrane samples were collected from 14 patients with proliferative diabetic retinopathy (PDR). The membranes were used for immunolabeling of ki-67 protein and RPE65 protein to identify retinal pigment epithelial cells. The vitreous samples were divided into two groups according to Ki-67 expression. Vitreous miRNA profiles were analyzed using miRNA-specific qPCR microarray. The expression of miR-451a was measured by RT-PCR in DB/DB mice. The expression of miR-451a, was quantified by q-PCR in ARPE-19 cells under diabetic conditions. The potential downstream targets of the miRNA were predicted and confirmed by dual luciferase assay, qRT-PCR and Western blotting. Mitochondrial function changes were measured by Seahorse after transfection of miR-451a mimics and inhibitor into ARPE19 cells.
Results: In proliferative vitreoretinopathy membrane, RPE cells account for 2.57%±3.19%(1.61%-11.11%). Proliferative index (PI) = 2.57%±3.19%(1.61%-11.11%). Some RPE cells were Ki-67-positvie cells. MiRNA-specific qPCR microarray showed that the level of miR-451a was substantially higher from the eye with Ki-67-positive membrane than the eye with Ki-67-negative membrane. The expression of miR-451 in the retina was increased by 4-fold in 6-month-old DB/DB mice, compared to age-matched non-diabetic controls (P=0.03). In ARPE19 cells, high glucose up-regulated miR-451a expression. HNE down-regulated miR-451a expression. Bioinformatic analysis and luciferase assay identified activating transcription factor 2 (ATF2) as a potential target of miR-451a. RT-PCR and Western blotting revealed that overexpression of miR-451a down-regulated the expression of ATF2 (P<0.05). The mitochondrial function was enhanced by miR-451a mimics, while suppressed by inhibitor. The basal oxygen consumption rate (OCR) and maximal OCR in miR-451a overexpression group were significantly higher than control group (P=0.014, 0.015). Under high glucose condition, overexpression of miR-451a enhanced the mitochondrial function.
Conclusions: MiR-451a/ATF2 plays a role in regulation of the mitochondrial function in the RPE, which open new perspectives for the development of effective therapies of PDR.
Commercial Relationships: yan shao; **Lijie Dong**, None; **Yusuke Takahashi**, None; **Xun Liu**, None; **Qian Chen**, None; **Xiaorong Li**, None; **Jian-Xing (Jay) Ma**, None
Support: Natural science foundation of Tianjin, China 15JCQNJ11400

Program Number: 4036 **Poster Board Number:** B0009
Presentation Time: 8:30 AM–10:15 AM
Novel Mechanism which Promotes Diabetic Complications in Renal and Ocular Systems
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Purpose: To determine the roles of TGFB1 and TGFB2 receptors, as well as Tranforming Growth Factor Beta-Induced (TGFBI or BIGH3), in the deaths of renal and ocular cells in culture.
Methods: BIGH3 was introduced in to cell medium of cultured RPTEC, REC, and HRPs. Macrophages and macrophage derived TGF-B1/TGFB2 receptor blockers were also added at various concentrations. Cell viability was determined via TUNEL assay per manufacturers instructions.
Renal cells (RPTEC) and Human Retinal Pericytes (HRP's) were purchased from Clonetics/Lonza (Walkersville, MD). Retinal Endothelial Cells (REC) were purchased from ATCC (Manassas, VA). Monocyte-derived Macrophage (MΦ) were prepared in accordance with established methodology (Wintergerst et al., 1998). Polyclonal anti-BIG-H3 was generated in house.
Results: Our study here indicates that macrophages (MΦ) and MΦ-derived TGF-β1 promote BIGH3 Mediated Apoptosis (BMA), thus advancing nephrology and diabetic retinopathy diseases. In addition, we show that in diabetic conditions BMA targets three different cell types. In the retina BMA targets retinal pericytes (HRP) and retinal endothelial cells (RhREC), and in the kidney BMA targets renal proximal tubule epithelial cells (RPTEC). The BMA mechanistic pathway that kills these cells involves MΦ-derived TGF-β1, BIGH3 protein, BIGH3 C-terminal cleavage, C-terminal-derived integrin ligand peptides, and specific integrins expressed on renal, endothelial, and pericyte cell surfaces. After BIGH3 is secreted into the cells' milieu, a crucial step in the BMA mechanism is cleavage of BIGH3's C-terminus which can be accomplished by the peptidases plasmin, matrix metalloproteinase 9, and serine protease high-temperature requirement A1. BIGH3 C-terminal cleavage generates integrin-ligand peptides that manipulate targeted integrin signaling pathways to evoke BMA. We also found that cells in a pre-diabetic environment synthesize BIGH3 protein, indicating BIGH3 is a biomarker signifying a pre-diabetic state that left untreated will likely progress to complications including diabetic retinopathy and nephrology.
Conclusions: Identification of the MΦ source of BIGH3 in diabetic conditions and the sequence of the integrin-ligand peptides derived from BIGH3, as well as the integrins involved in BMA, is expected to offer novel therapeutic targets for interventions to block development and progression of diabetic complications.
Commercial Relationships: Andrew T. Tsin; **Brandi S. Betts-Obregon**, None; **Robert Mortiz**, None; **Richard LeBaron**, None
Support: This study was supported by the National Institute on Minority Health and Health Disparities (G12MD007591) from the National Institutes of Health as well as SALSI grant to RGL.

Program Number: 4037 **Poster Board Number:** B0010
Presentation Time: 8:30 AM–10:15 AM
Endoplasmic reticulum stress at the intersection of inflammation and hyperglycemia in mediating tight junction alterations in diabetic retinopathy
RAJI RAJESH LENIN, Rajashekhar Gangaraju. Department of Ophthalmology, University of Tennessee, Memphis, TN.
Purpose: Endoplasmic Reticulum (ER) stress plays a major role in several metabolic disorders including diabetes. We hypothesized that ER stress pathways interact with inflammatory signaling leading to retinal barrier dysfunction in diabetic retinopathy (DR). Using a well characterized chronic proinflammatory tie2-TNF mouse model and retinal endothelial cells we assessed the connection between ER stress and hyperglycemic stress in mediating vascular permeability.
Methods: Tie2-TNF and age matched wildtype mice were made diabetic with intraperitoneal injections of Streptozotocin and evaluated for retinal vascular permeability by fluorescein labeled-BSA leakage assay. Human retinal microvascular endothelial cells (HREC) were treated with 10 ng/mL TNF-α and 30 mmol/L glucose for 3 hours with and without ER stress inhibitor (10μM TUDCA). Gene and protein expression of ER stress markers were analyzed by Taqman assay and Western blot analysis respectively. Alterations in endothelial junction proteins were evaluated by western blot.
Results: Frozen sections of retina from tie2-TNF transgenic mice demonstrated increased vascular leakage compared to wildtype mice which was further exacerbated with diabetes. Interestingly, whole mount retinal preparation from diabetic tie2-TNF mice demonstrated hyper permeable vessels with strong extravasation of FITC-BSA and extravasation of red blood cells. Retinal extracts from 6-month old tie2-TNF mice exhibited increased mRNA levels of ER stress markers (GRP78, PERK, IRE1α and CHOP) compared to age matched wild type mice (n=3 each, $p<0.05$). In HREC cells, ER stress was significantly upregulated with TNF and high glucose combination as evidenced by increased gene expression of GRP-78, PERK, IRE-1α, ATF-6, XBP-1, CHOP and increased protein expression of GRP78 and a decreased ZO-1 expression ($p<0.05$ compared to control). On the other hand, TUDCA treated cells reversed ER stress markers.
Conclusions: ER stress is a key mediator of vascular damage in the setting of increased inflammatory burden in retinal endothelial cells. Understanding the underlying ER stress mechanisms may shed new insights into novel therapeutic targets for DR.
Commercial Relationships: RAJI RAJESH LENIN, None; **Rajashekhar Gangaraju**, None
Support: EY023427

Program Number: 4038 **Poster Board Number:** B0011
Presentation Time: 8:30 AM–10:15 AM
Role of Cathepsin-D in alteration of Endothelium-Pericyte interaction in Diabetic Retinopathy
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Purpose: Previously, we have demonstrated the effect of Cathepsin D (CD) on the mechanical disruption of retinal endothelial cell junctions and increased vaso-permeability, and increased levels of cathepsin D in retinas of diabetic mice. Here we have further examined the effect of CD on the endothelial-pericyte interaction, and the effect of Dipeptidyl peptidase-4 (DPP-4) inhibitor on CD in the endothelial-pericyte interaction. The DPP-4 inhibitor has been shown to alter the function of the IGF2 receptor, which is the presumed binding site for CD on endothelial cells.
Methods: Human retinal endothelial cells (HREC) and human retinal pericytes (HRP) were co-cultured and treated overnight with 25ug/ml of recombinant pro-CD along with 250nM DPP-4 inhibitor. Western blots were performed to check the levels of PDGFR-β, N-Cad, PKC-α and phosphor-PKC-α. Real-time PCR was used to measure angiopoietin-2 (Ang-2) levels in the treated co-cultured cells. GFP-HRP cells were co-cultured with HRECs to check the binding efficiency in treated conditions.
Results: Co-cultured cells treated with pro-CD showed a significant decrease in the expression of PDGFR- β, a tyrosine kinase receptor required for pericyte cell survival and N-Cad, the key adherens junction protein between endothelium and pericytes, and also increase in the vessel destabilizing agent, Ang-2. The effect was reversed in the cells treated with DPP-4 inhibitor along with pro-CD. With pro-CD treatment, there was a significant increase in the downstream signaling protein PKC-α, which disrupts tight junction structure and function, and this was significantly reduced with DPP-4 inhibitor treatment. We observed significantly increased binding of endothelial cells and pericytes when cells were treated with the DPP-4 inhibitor and pro-CD.
Conclusions: The cathepsin D decreases N-cad and PDGFR- β in the endothelium-pericyte alteration in the blood-retinal barrier, and the drug DPP-4 inhibitor can significantly diminish this effect. Thus, the DPP-4 inhibitor may be used as a potential adjuvant therapeutic strategy for treating diabetic macular edema.
Commercial Relationships: Finny Monickaraj, None; **Paul McGuire**, None; **Arup Das**, None
Support: VA Merit Award

Program Number: 4039 **Poster Board Number:** B0012
Presentation Time: 8:30 AM–10:15 AM
ACTIVATION OF THE SIRT1-LXR SIGNALLING PATHWAY IN RETINAL PIGMENTED EPITHELIAL CELLS PROMOTES CHOLESTEROL METBOLISM
Kiana Wood[1], Sandra Hammer[1], Elahe Crockett[1], Maria B. Grant[2], Julia V. Busik[1]. [1]Michigan State University, East Lansing, MI; [2]Indiana University, Indianapolis, IN.
Purpose: Diabetic Retinopathy (DR) is a sight threatening disease with few treatment options. Dyslipidemia has shown to play a significant role in the progression of DR. Liver X Receptors (LXRα/β) are known cholesterol metabolism regulators that also play a role in preventing pro-inflammatory genes upregulation. Retinal Pigmented Epithelial Cells (RPEs) are important in retinal cholesterol uptake and elimination. Additionally, RPE are one of the retinal cells heavily affected by DR. Knockdown of LXR in animals leads to increased retinal cholesterol levels when compared to controls. SIRT1 has recently been shown to activate LXR in non-retinal studies but the role of the SIRT1-LXR signaling axis in DR has not yet been studied.
Methods: Bovine retinal pigmented cells (BRPE) were treated with TNFα (10ng/ml), and the role of SIRT1-LXR signaling axis was examined using SIRT1 activator SIRT1720 (1μM) and LXR activator DMHCA (1μg/ml). LXRα/β, the ATP binding cassette transporters (ABCA1 and ABCG1), and cholesterol metabolizing enzymes (CYP27A1, CYP46A1, and CYP11A1) were analyzed using qRT-PCR. SIRT1-directed siRNA was used to inhibit SIRT1 expression levels.
Results: LXRα, ABCA1 and ABCG1 mRNA levels decreased when BRPEs were treated with TNFα for 24hrs ($p<0.01$, n=6). The cholesterol metabolizing enzymes (CYP27A1, CYP46A1, and CYP11A1) were decreased when treated with TNFα ($p<0.01$, n=6).

Furthermore, treatment with SIRT1 activator, SRT1720, for 24hrs prevented TNFα-induced ABCA1 and ABCG1 downregulation ($p<0.001$, n=9). LXR activator, DMHCA, prevented TNFα-induced ABCA1 and ABCG1 downregulation ($p<0.01$, n=3). Additionally, SIRT1 inhibition it decreased cholesterol metabolizing enzyme CYP11A1 and ABCA1 ($p<0.05$, n=3) while having no statistical effect on LXRα expression.
Conclusions: Inflammatory cytokine stimulation caused a decrease in retinal cholesterol metabolizing enzymes and transporter proteins. Activation of the SIRT1-LXR signaling axis prevented TNFα-induced downregulation of cholesterol metabolism in retinal cells. Taken together this work suggest that activation of the SIRT1-LXR pathway can help restore normal cholesterol metabolism in diabetic retina and prevent dyslipidemia-induced retinal pathology.
Commercial Relationships: Kiana Wood, None; **Sandra Hammer**, None; **Elahe Crockett**, None; **Maria B. Grant**, None; **Julia V. Busik**, None
Support: NH Grant EY025383

Program Number: 4040 **Poster Board Number:** B0013
Presentation Time: 8:30 AM–10:15 AM
Role of high glucose-induced lysyl oxidase overexpression on Ras activity in rat retinal endothelial cells
Brian Chirn[1], Dongjoon Kim[1], Philip C. Trackman[2], Sayon Roy[1]. [1]Departments of Medicine and Ophthalmology, Boston University School of Medicine, Boston, MA; [2]Department of Molecular and Cell Biology, Boston University Henry M. Goldman School of Dental Medicine, Boston, MA.
Purpose: In diabetic retinopathy, cells in the retina undergo significant apoptosis resulting in the development of acellular capillaries and pericyte ghosts. In this study, we determined whether high glucose (HG)-induced lysyl oxidase (LOX) overexpression plays a role in contributing to increased apoptosis by lowering Ras activity, which is known to promote cell survival via the Ras-Raf–ERK pathway.
Methods: Rat retinal endothelial cells were grown in normal (N; 5 mM) or HG (30 mM) medium for 7 days, and in parallel, cells grown in HG were transfected with LOX siRNA, or scrambled siRNA as control. Total protein isolated from the cells was assessed for Ras protein activation using an affinity purification assay that utilizes the Ras Binding Domain. Subsequently, activated Ras derived through pull-down assay was evaluated by Western blot (WB) analysis using a Ras specific antibody. To determine if siRNA mediated LOX inhibition altered Ras activity and thereby promoted apoptosis under HG condition, differential dye staining assay was performed.
Results: WB analysis showed significant LOX upregulation in cells grown in HG medium compared to those grown in N medium (132±11% of control, $P<0.05$). Ras pulldown assay and WB analysis revealed significant decreased Ras activity in cells grown in HG medium compared to those grown in N medium (59±18% of control, $P<0.005$). When HG-induced LOX overexpression was reduced by approximately 40% via LOX siRNA, Ras activity increased by approximately 20%, compared to those grown in HG medium, and those grown in HG medium transfected with scrambled siRNA. As expected, a significant increase in the number of apoptotic cells was observed under HG medium compared to those grown in N medium (210±37% of control). Interestingly, the number of apoptotic cells was significantly decreased when cells grown in HG exhibited increased Ras activity via LOX siRNA transfection (154±48% of control, $P<0.05$).
Conclusions: Findings from this study indicate that HG-induced LOX overexpression promotes apoptosis, at least in part, by inhibiting Ras signaling. Therefore, reducing LOX overexpression may protect retinal vascular cells from undergoing HG-induced apoptosis associated with the pathogenesis of diabetic retinopathy.
Commercial Relationships: Brian Chirn; **Dongjoon Kim**, None; **Philip C. Trackman**, None; **Sayon Roy**, None
Support: NEI, NIH grant EY025528

Program Number: 4041 **Poster Board Number:** B0014
Presentation Time: 8:30 AM–10:15 AM
Effect of a single injection of anti-VEGF agent on retinal edema in non-obese diabetic mice
Tamar Leibovitch[1, 4], moshe ben hemo[1, 4], Myles Brookman[1], Orit Barinfeld[1, 4], Orkun Muhsinoglu[2], Shalom Michowiz[2], Nitza Goldenberg-Cohen[3, 4]. [1]Tel Aviv University, Tel Aviv, Israel; [2]Department of Neurosurgery, Rabin Medical Center – Beilinson Hospital, PetachTikva, Israel; [3]Department of Ophthalmology, Bnai Zion Medical Center of Israel, Haifa, Israel; [4]Krieger Eye Research Laboratory Felsenstein Medical Research Center, Beilinson Hospital, PetachTikva, Israel.
Purpose: The aims of the study were to characterize the retinal changes in a mouse model of diabetic retinopathy and to evaluate the response of the retinal edema to a single injection of anti-VEGF-A agent.
Methods: 96 transgenic NOD mice were monitored. Diabetes type 1 (blood glucose levels>250mg/dl) developed spontaneously in 30 (DMT1-NOD group) and was chemically induced in 28 (STZ-NOD group); the remainder served as controls. Mice were examined after the appearance of uncontrolled high glucose levels (>500mg/dl). Retinal blood flow and leakage were evaluated with fluorescein angiography (FA); vascular perfusion, with retinal flat mounts following infusion of India ink or fluorescent gelatin; gliosis and vasculopathy, with Vimentin and GFAP immunostaining; retinal thickness with light microscopy; and expression of genes involved in ischemia (*SOD-1, HO-1*), angiogenesis (VEGF-A, VEGFR-1, -2), gliosis (*GFAP, Vimentin*), and diabetes (*RAGE, IGF-1, EPO*), with RT-PCR. To test the effect of anti-VEGF treatment, the right eyes of STZ-NOD mice were evaluated histologically one week after injection of bevacizumab, ranibizumab, or saline or no treatment.
Results: In the first part of the study, the diabetic mice showed no neovascularization (NVE) on FA. Flat mount show microaneurysms and attenuated retinal vessels, and apparent tufts of NVE in a single retina. Immunostaining revealed reactive gliosis and prominent Muller cells. Mean retinal thickness was significantly higher in the DMT1-NOD mice than the nondiabetic mice. Of all genes evaluated, only *GFAP* decreased significantly (0.5-fold, $p=0.04$). Anti-VEGF treatment led to complete suppression of gliosis with retinal thinning. Bevacizumab (retinal thickness 260±53mm right eye Vs. 241±34mm left eye) appeared to be more effective than ranibizumab (267.5±47mm right eye Vs 223+39mm left eye).
Conclusions: In conclusion, the retinas of DMT1-NOD mice are characterized by vasculopathy and edema, reactive gliosis, and good response to a single injection of anti-VEGF agent. The short survival time of the diabetic mice may have limited the development of NVE. Accordingly, discrepancies were noted between the increase in GFAP histologically and the decrease in GFAP gene expression and between the retinal response to anti-VEGF agents and the lack of change in VEGF gene expression. The reason for the higher effect of bevacizumab than ranibizumab is unclear.
Commercial Relationships: Tamar Leibovitch; **moshe ben hemo**, None; **Myles Brookman**, None; **Orit Barinfeld**, None; **Orkun Muhsinoglu**, None; **Shalom Michowiz**, None; **Nitza Goldenberg-Cohen**, None

Support: D-CURE, foundation, ministry of health & Krieger Eye Research Laboratory Felsenstein Medical Research Center, Petach Tikva, Israel.

Program Number: 4042 **Poster Board Number:** B0015
Presentation Time: 8:30 AM–10:15 AM
Toll-like receptor-mediated up-regulation of xanthine oxidoreductase (XOD) contributes to oxidative stress and inflammation in the diabetic retina
Prerana Malla[1], Menaka Thounaojam[1], Diana Gutsaeva[1], Amany Tawfik[2], Manuela Bartoli[1]. [1]Ophthalmology, Augusta University, Augusta, GA; [2]Oral Biology, Dental College of Georgia, Augusta, GA.
Purpose: Increased oxidative/nitrative stress and chronic inflammatory responses have been implicated in the pathogenesis of diabetic retinopathy (DR). Xanthine oxidoreductase (XOD) is an enzyme involved in purine metabolism which generates superoxide anions and, as catabolic by-product, uric acid (UA). Recently we have shown that XOD is activated in the diabetic retina leading to increased production of UA. The mechanisms by which hyperglycemia stimulates XOD expression and activity as well as its pathogenic consequences in DR are poorly understood. Here, we have investigated the effects of in vivo blockade of XOD in the diabetic retina and the contribution of different toll-like receptors (TLRs) to hyperglycemia-induced up-regulation of XOD in human retinal endothelial cells (HuREC).
Methods: Male streptozotocin-induced diabetic rats (STZ-rats) were kept diabetic for 8 weeks. Some of the STZ-rats received a dose (55 mg/Kg) of the XOD inhibitor allopurinol (ALL) administered orally every other day. At the time of sacrifice, the retinas were excised and analyzed for oxidative/nitrative stress parameters [4-hydroxynonenal (4-HNE) and nitrotyrosine (NY)] and expression of inflammatory markers such as ICAM-1, IL-1beta and IL-6. TLR4, 2 and 7/8 were silenced in HuREC by transfection with specific siRNAs. XOD expression at the mRNA and protein level was measured by qPCR and Western blotting analyses, respectively. XOD activity was assessed by spectrophotometric enzymatic analysis measuring UA production.
Results: Treatments with ALL resulted in significant decreases of 4-HNE and NY and in blunted expression of inflammatory markers, such as IL-1beta, IL-6 and ICAM-1, in the diabetic rat retina. XOD expression and activity in HuREC exposed to glycidic stress (HG = 25 mM D-glucose for 48 hours) were significantly up-regulated in comparison to cells cultured in normal glucose levels (NG = 5 mM D-glucose) or with the osmotic control (LG = 25 mM L-glucose).Transfection of the cells with siRNA specific for TLR4, 2 or 7/8 significantly reduced HG-induced up-regulation of XOD expression and activity and showed highest inhibitory effects by TLR7/8 signaling.
Conclusions: The data obtained suggest that TLR-mediated up-regulation of XOD plays a central role in mediating pro-oxidative/nitrative and pro-inflammatory challenges in the diabetic retina.
Commercial Relationships: Prerana Malla, None; **Menaka Thounaojam**, None; **Diana Gutsaeva**, None; **Amany Tawfik**, None; **Manuela Bartoli**, None
Support: EY022416

Program Number: 4043 **Poster Board Number:** B0016
Presentation Time: 8:30 AM–10:15 AM
Hydrogen sulfide as a novel therapeutic for diabetic retinopathy
Claire Allen[1], Jacqueline L. Whatmore[2], Mark E. Wood[3], Matthew Whiteman[2], David O. Bates[1]. [1]Cancer Biology & Stem Cells, The University of Nottingham, Nottingham, United Kingdom; [2]Medical School, University of Exeter, Exeter, United Kingdom; [3]Biosciences, University of Exeter, Exeter, United Kingdom.
Purpose: Diabetic retinopathy (DR) is a major complication of diabetes and a leading cause of blindness worldwide. Current treatments for DR are limited, expensive and non-specific. The anti-inflammatory gaseous transmitter hydrogen sulfide (H_2S) is significantly reduced in type 2 diabetic patients and correlates with microvascular dysfunction. H_2S is thought to contribute to the healthy functioning of the microvasculature by preventing loss of the retinal endothelial glycocalyx and subsequent breakdown of the blood retinal barrier.
Methods: Fundus Fluorescein Angiography (FFA) was performed in isofluorane anaesthetised Norway Brown rats on day 0 and 7 using the Micron IV retinal imaging microscope (Phoenix Research Labs). Animals received an intraocular injection of the slow release H_2S donor sodium 4-methoxyphenyl(morpholino)-phosphinodithioate (NaGYY4137; 1μM) on day 0 (Prevention) or on day 6 (Treatment). On day 1 some animals received a single dose of streptozotocin (50mg/kg, i.p.) to induce diabetes and blood glucose levels measured. Angiograms were imported into ImageJ software and fluorescence was measured in the interstitium and vessel. The ratio of interstitial to vascular fluorescence was adjusted for background and plotted against time and the slope used to determine an estimate of permeability.
Results: NaGYY4137 significantly ($p < 0.05$) reduced retinal permeability in non- and diabetic rats on day 7 when given as a single intraocular preventative dose on day 0. In addition NaGYY4137 significantly reduced ($p < 0.05$) retinal permeability in non-diabetic rats when administered therapeutically (day 6) and stabilised retinal permeability in rats with pre-existing diabetes. This was further supported by fundus images showing increased capillary leakage and microaneurysm formation in the retina of diabetic animals.
Conclusions: NaGYY4137 protected the retinal endothelial permeability barrier from diabetes-associated loss of integrity and reduced the progression of DR. Slow release H_2S donors may therefore be a potential alternative and more specific therapeutic candidate for DR.
Commercial Relationships: Claire Allen, NuVision (P); **Jacqueline L. Whatmore**, None; **Mark E. Wood**, None; **Matthew Whiteman**, None; **David O. Bates**, None

Program Number: 4044 **Poster Board Number:** B0017
Presentation Time: 8:30 AM–10:15 AM
NFAT-dependency of IL-1β-induced diabetes-relevant behaviors in human retinal microvascular endothelial cells and Müller cells
Meredith J. Giblin[1], Megan E. Capozzi[2], Gary W. McCollum[3], John S. Penn[3, 1]. [1]Cell and Developmental Biology, Vanderbilt University, Nashville, TN; [2]Molecular Physiology & Biophysics, Vanderbilt University, Nashville, TN; [3]Department of Ophthalmology and Visual Sciences, Vanderbilt University, Nashville, TN.
Purpose: Early diabetic retinopathy (DR) involves chronic low-grade inflammation, characterized by elevated vitreous cytokines, such as IL-1β. This inflammation promotes many pathologic consequences, including the hallmark vascuar changes for which DR is best known. Nuclear factor of activated T cells (NFAT) is involved in the regulation of inflammatory mediators, extracellular matrix proteins, and adhesion molecules and thus may control multiple pathogenic

steps early in DR. We have examined NFAT's role in IL-1β auto-amplification and explored the NFAT-dependency of endothelial cell responses to IL-1β, including monolayer permeability and expression of targets involved in leukocyte adhesion and basement membrane thickening.
Methods: For IL-1β production, human Müller cells (HMC) were treated with 50pg/mL IL-1β, Inhibitor of NFAT-Calcineurin Association-6 (INCA, 2.5μM) and proper vehicles for 8 hrs before collection for qRT-PCR. For ICAM expression, human retinal microvascular endothelial cells (HRMEC) were treated with 1ng/mL IL-1β and 2.5μM INCA for 2 hrs. For collagen IV expression, HRMEC were treated with 10ng/mL IL-1β and 1μM INCA for 48 hrs. In permeability experiments, HRMEC were pre-treated with vehicle or 2.5μM INCA for 16 hrs before 24 hrs of exposure to 0.5ng/mL IL-1β with/out INCA. Transendothelial electrical resistance values were measured using the $EVom_2$.
Results: IL-1β increased HMC IL-1β expression by 82-fold ($p<0.01$); INCA inhibited this induction by 28% ($p<0.05$). HRMEC treatment with IL-1β caused a 328-fold induction of ICAM expression ($p<0.01$); INCA inhibited this induction by 20% ($p<0.01$). HRMEC treatment with IL-1β caused a 2-fold induction of collagen IV expression ($p<0.01$); INCA inhibited this induction by 50% ($p<0.01$). Treatment with IL-1β decreased HRMEC monolayer resistance by 25% (measured vs resistance at time=0, $p<0.01$), which was partially rescued (44%, $p<0.01$) by INCA treatment.
Conclusions: These data demonstrate the potential of NFAT as a multi-targeted therapy for retinal inflammation secondary to diabetes. NFAT inhibition not only prevented HMC IL-1β auto-amplification, but also inhibited the pathogenic response of HRMEC to IL-1β, including the expression of adhesion proteins, excessive extracellular matrix deposition and increased permeability. Future work will investigate the therapeutic potential of *in vivo* NFAT inhibition.
Commercial Relationships: Meredith J. Giblin, None; **Megan E. Capozzi**, None; **Gary W. McCollum**, None; **John S. Penn**, None
Support: T32EY021453, R01-EY007533, R01-EY023639, and P30-EY008126; Research To Prevent Blindness and The Reeves Foundation.

Program Number: 4045 **Poster Board Number:** B0018
Presentation Time: 8:30 AM–10:15 AM
TLR4 inhibits insulin signaling, leading to increased apoptosis in mouse retina
Youde Jiang, Li Liu, Elizabeth Curtiss, Jena J. Steinle. Anatomy and Cell Biology, Wayne State University, Detroit, MI.
Purpose: We recently reported that Compound 49b, a b-adrenergic receptor agonist, could reduce toll-like receptor 4 (TLR4) levels and TNFalpha in retinal endothelial cells and diabetic mouse retina. Since we also have reported that Compound 49b restored normal insulin signaling in the retina, we hypothesized that TLR4 would inhibit insulin signaling, leading to apoptosis in the retina.
Methods: TLR4 floxed mice (B6 Cg-$Tlr4^{tm1.1karp}$/J) and B6.FVB-Tg (cdh5-cre)7Mlia/J Cre mice purchased from Jackson lab were cross bred to produce endothelial cell-specific TLR4 knockout mice. TLR4 overexpressing mice were acquired from Dr. Fukuchi. Mice from each group were used at 3 months of age for Western blotting or ELISA for insulin receptor, insulin receptor substrate 1 (IRS-1), Akt, and cleaved caspase 3.
Results: Insulin receptor phosphorylation and Akt phosphorylation were increased in the retinal lysates fromTLR4 conditional knockout mice when compared to their floxed littermates. TLR4 overexpression significantly reduced insulin receptor and Akt phosphorylation in mouse retina. TLR4 floxed mice had significantly more phosphorylation of IRS-1Ser307 when compared to the conditional knockout mice. TLR4 overexpression increased IRS-1Ser307 phosphorylation in the mouse retina. Apoptosis was reduced in the retina from TLR4 conditional knockout mice, as well as in the littermates of the TLR4 overexpressing mice, when compared to TLR4 floxed and TLR4 overexpressing mice, respectively.
Conclusions: TLR4 signaling inhibits insulin signaling in the mouse retina, leading to increased apoptosis. Therapies designed to block TLR4 signaling may be protective to the retina.
Commercial Relationships: Youde Jiang, None; **Li Liu**, None; **Elizabeth Curtiss**, None; **Jena J. Steinle**, None
Support: R01EY022330

Program Number: 4046 **Poster Board Number:** B0019
Presentation Time: 8:30 AM–10:15 AM
TLR-4 phosphorylation at tyrosine 674 is necessary for NF-kB and inflammasome activation in the diabetic retina and in retinal endothelial cells exposed to glucidic stress
Manuela Bartoli[1], Menaka Thounaojam[1], Diana Gutsaeva[1], Wan Jin Jahng[2]. [1]Ophthalmology, Augusta University, Augusta, GA; [2]Petroleum Chemisttry, American University, Yola, Nigeria.
Purpose: Up-regulation of Toll-like receptor 4 (TLR4) expression and signaling has been implicated in the pathogenesis of diabetic retinopathy (DR), however the molecular mechanisms of TLR4 activation in the diseased retina are poorly understood and often referring to promiscuous downstream signaling events. We have conducted studies assessing the occurrence and biological significance of TLR4 phosphorylation at tyrosine 674, which is located in the highly biologically active toll-IL-1 resistance (TIR) domain of this molecule.
Methods: Western blotting analysis was conducted to measure phospho-Tyr (674)-TLR4 in diabetic and non-diabetic human *postmortem* retinas and in retinas of streptozotocin-induced diabetic rats (STZ-rats) as compared to their respective normoglycemic controls. The occurrence of TLR4 phosphorylation at Tyr674 was also measured by immunoblotting in human retinal endothelial cells exposed to glucidic stress (HG=25mM D-glucose) or to normal glucose levels (NG=5mM D-glucose) or the osmotic control (LG=25mM L-glucose). HuREC were transfected with TRL4-Tyr-(674)-Ala mutants and exposed to the different glucose conditions for 48 hours. Downstream TLR4-mediated signaling events were measured by assessing NF-kB and p38MAPK phosphorylation /activation and expression of IL-1beta in cells exposed to the different treatments.
Results: TLR4 expression and phosphorylation at Tyr 674 was significantly up-regulated in the diabetic rat retinas as well as in human *postmortem* diabetic retinas as compared to their respective normoglycemic controls. Importantly, HG stimulated TLR4 expression and phosphorylation at Tyr674 and consequent phosphorylation/activation of NF-kB and p38MAPK as well as production of mature IL-1beta. In addition blockade of TLR4 phosphorylation at Tyr674, achieved by transfection of HuREC with Tyr674-Ala mutants, resulted in signficantly decreased phosphorylation/activation of NF-kB and p38MAPK in these cells as well as decreased levels of mature IL-1beta.
Conclusions: Our data suggest that monitoring and study of TLR4 phosphorylation at Tyr 674 in response to hyperglycemia may offer a more specific diagnostic and therapeutic target for the prevention and treatment of DR and, potentially, of other ischemic retinopathies involving TLR4 activation
Commercial Relationships: Manuela Bartoli, None; **Menaka Thounaojam**, None; **Diana Gutsaeva**, None; **Wan Jin Jahng**, None

Support: EY022416

Program Number: 4047 **Poster Board Number:** B0020
Presentation Time: 8:30 AM–10:15 AM
Role of N-methyl-D-aspartate receptor activation in Blood-Retinal barrier dysfunction: Potential role in Diabetic retinopathy
Riyaz Mohamed[1], Ahmed Ibrahim[1, 2], Nehal M. El-Sherbiny[2], Mohamed A. Al-Shabrawey[1], Amany M. Tawfik[1]. [1]Oral Biology department; Vision Discovery Institute, Augusta University, Augusta, GA; [2]Department of Biochemistry and Clinical Biochemistry, Mansoura University, Mansoura, Egypt.
Purpose: Homocysteine (Hcy) is a risk factor for neurodegenerative and cardiovascular diseases and has been reported to be elevated in patients with diabetic retinopathy (DR). Our recent studies showed that Hcy induced retinal ischemia, neovascularization and Blood-Retinal Barrier dysfunction (BRB). The current study is aiming to investigate the role of N-methyl-D-aspartate receptor (NMDAr) in Hcy induced BRB dysfunction.
Methods: We confirmed the expression of NMDAr in Human Retinal Endothelial Cells (HRECs) by real-time polymerase chain reaction (qPCR) in comparison with Retinoblastoma cell line as a positive control. In addition, HRECs treated with or without different concentrations of Hcy (20, 50 and 100mM) were subjected to Evaluation of NMDAr activation in by Western blot (WB) and immunofluorescence (IF). FITC–Dextran flux permeability assay in presence and absence an NMDAr antagonist (MK801, 25μM). Furthermore, mice with increased level of Hcy (*cbs*$^{+/-}$) and different mouse models of diabetes (Streptozotocin (STZ), db/db and Akita) mice were evaluated for NMDAr activation in retinal cryosections by Immunofluorescence (IF).
Results: NMDAr is expressed in HRECs and WB and IF analysis showed that Hcy significantly increased NMDAr expression in HRECs in a dose-dependent manner, increased FITC-dextran leakage in HRECs which was rescued by NMDAr antagonist MK801. Furthermore, NMDAr expression was increased in *cbs*$^{+/-}$ mice retina as well as the diabetic mice retinal sections in comparison to control mice.
Conclusions: Homocysteine disturbs the BRB function via activation of NMDAr receptor in HRECs. Targeting NMDAr may propose new therapeutic avenue for patients with DR.
Commercial Relationships: Riyaz Mohamed, None; **Ahmed Ibrahim**, None; **Nehal M. El-Sherbiny**, None; **Mohamed A. Al-Shabrawey**, None; **Amany M. Tawfik**, None
Support: AHA Grant SDG

Program Number: 4048 **Poster Board Number:** B0021
Presentation Time: 8:30 AM–10:15 AM
Rap1 activation promotes vascular blood-retinal barrier
Carla Jhoana Ramos, Xuwen Liu, Chengmao Lin, David Antonetti. Ophthalmology and Visual Science, University of Michigan, Ann Arbor, MI.
Purpose: Purpose: Activation of Rap1 through the cAMP-dependent guanine nucleotide exchange factor EPAC was reported to enhance barrier function in human umbilical endothelial cells. However, the effect of EPAC-Rap1 signaling on retinal endothelial barrier, tight junctions, and the relationship to vascular endothelial growth factor (VEGF)-induced permeability are unknown. We hypothesize that EPAC-Rap1 activation inhibits VEGF-induced permeability in retinal endothelial cells.
Methods: Methods: Primary, bovine retinal endothelial cells (BREC) were used to model the blood retinal barrier. The EPAC-specific activator cAMP analog 8-pCPT-2-O-Me-cAMP-AM (8CPT) was used. Cell monolayer permeability was measured by electrical resistance using the ECIS-ZΘ system or by measuring 70kDa RITC dextran flux across transwell filters. Rap1 activation was determined using a Rap1 capture assay with GST-RalGDS followed by Western blot. EPAC specific antagonist (ESI-09) or Rap1B siRNA (bovine) were used to inhibit EPAC or silence Rap1 expression in BREC. Confocal microscopy assessed junctional protein organization. Male Sprague-Dawley rats were used to test retinal vascular permeability *in vivo*.
Results: Results: Treatment of BREC monolayers with 8CPT and activation of Rap1 prevented both VEGF or VEGF and tumor necrosis factor (TNF)-induced permeability to 70kDa dextran ($p<0.0001$) and reduction in electrical resistance ($p<0.0001$). 8CPT treatment reversed VEGF-induced permeability to solute and ion flux. EPAC inhibition or Rap1 silencing led to an increase in basal permeability and VEGF had no additional permeability increase. Pre-treatment with 8CPT decreased VEGF signaling in the Erk pathway but Rap1 knockdown revealed no effect on VEGF signaling. Immunofluorescence staining showed disruption of tight junction organization in VEGF treated cells and 8CPT blocked VEGF junctional disruption ($p<0.0001$). Permeability studies *in vivo* showed that VEGF/TNF increased permeability and 8CPT blocked VEGF/TNF permeability.
Conclusions: Conclusions: These data demonstrate that activation of Rap1 through EPAC in retinal endothelial cells promotes barrier properties, inhibits and reverses VEGF and VEGF/TNF-induced permeability, and protects tight junction organization. Collectively, these data suggest activation of Rap1 through cAMP analogs may provide a therapeutic means to restore barrier properties in diseases of increased retinal permeability.
Commercial Relationships: Carla Jhoana Ramos, None; **Xuwen Liu**, None; **Chengmao Lin**, None; **David Antonetti**, None
Support: 5T32EY013934-14

Program Number: 4049 **Poster Board Number:** B0022
Presentation Time: 8:30 AM–10:15 AM
Genetically modified probiotics for oral delivery of Angiotensin-(1-7) confers protection against diabetic complications
Qiuhong Li[1], Zhibing Liang[1], Kang Xu[1], Tao Du[1], Ping Zhu[1], Tuhina Prasad[1], Shenquan Liao[1], Manoj Kulkarni[1], Amrisha Verma[1], Mohan Raizada[2]. [1]Ophthalmology, University of Florida, Gainesville, FL; [2]Functional Genomics & Physiology, University of Florida, Gainesville, FL.
Purpose: Previous studies have established that activation of the members of the vasoprotective axis of the renin-angiotensin system [ACE2 or Angiotensin-(1-7) (Ang-(1-7)] prevents and arrests progression of diabetic retinopathy. The objective of present study was to generate a probiotic containing Ang-(1-7) and test the hypothesis that oral administration of such a probiotic would provide protection against diabetic retinopathy.
Methods: We genetically modified the commensal bacterium Lactobacillus paracasei (LP) to serve as a live vector for the oral delivery of Ang-(1-7) and investigated its therapeutic potential in attenuating diabetes and associated complications. Ang-(1-7) was expressed as a secreted fusion protein with a trans-epithelial carrier to allow uptake into circulation. The vector pTRKH3-ldh-SP-Ang-(1-7) or pTRKH3-ldh-GFP were introduced by electroporation into LP. Two-month old adult diabetic eNOS-/- mice induced by intraperitoneal injection of streptozotocin and Akita mice were orally gavaged every other day with $1x10^9$ CFU of LP secreting Ang-(1-7) (LP-A), LP secreting GFP (LP-GFP), or LP alone for eight and twelve weeks respectively.

Results: Compared to age-matched untreated diabetic control animals, oral feeding of LP and LP-A significantly improved STZ-induced damage to insulin producing beta cells in pancreas in diabetic eNOS-/- mice. LP-A treatment also enhanced glucose tolerance, improved structure and morphology of islets and kidney, increased insulin expression in both diabetic eNOS-/- mice and Akita mice; and are associated with reduced retinal gliosis, inflammation, apoptotic neuronal cell death and loss of retinal vascular capillaries.
Conclusions: Oral administration of a genetically modified commensal bacterium that can secrete Ang-(1-7) provides protection against diabetes-induced tissue damage and diabetic retinopathy. Thus, LP-based delivery of Ang-(1-7) may hold important therapeutic potential for the treatment of diabetic complication.
Commercial Relationships: Qiuhong Li, None; **Zhibing Liang**, None; **Kang Xu**, None; **Tao Du**, None; **Ping Zhu**, None; **Tuhina Prasad**, None; **Shenquan Liao**, None; **Manoj Kulkarni**, None; **Amrisha Verma**, None; **Mohan Raizada**, None
Support: Supported in part by NIH grants EY021752, EY024564, American Diabetes Association, BrightFocus Foundation (QL) and NIH grant R01 HL102033 (MKR). Core facilities were supported by NEI grant P30 EY02172 and Research to Prevent Blindness to University of Florida.

Program Number: 4050 **Poster Board Number:** B0023
Presentation Time: 8:30 AM–10:15 AM
Hypoxia-Inducible Factor 1-Regulated Angiogenic Cytokines in Prolifeartive Diabetic Retinopathy
Haley Megarity[1], Monika Deshpande[1], Savalan Babapoor-Farrokhran[1], Brooks Puchner[1], Gregg Semenza[2], Silvia Montaner[3], Akrit Sodhi[1]. [1]Ophthalmology, Johns Hopkins, Baltimore, MD; [2]Pediatrics, Institute for Cell Engineering, Baltimore, MD; [3]Pathology, School of Medicine, Baltimore, MD.
Purpose: Recent studies have demonstrated that monthly intravitreal injections with therapies targeting VEGF delays the development of PDR in some, but not all, treated patients. This suggests that additional factor(s) may contribute to the development of retinal neovascularization in diabetic patients. In addition to VEGF, hypoxia inducible factor (HIF)-1 regulates the expression of other angiogenic factors in ischemic retinal disease, and plays a central role in the promotion of retinal neovascularization in PDR. Here we set out to examine the potential contribution of three other HIF-1-regulated angiogenic cytokines, angiopoietin 2 (ANGPT2), erythropoietin (EPO), and angiopoietin-like 4 (ANGPTL4), all previously implicated in the promotion of diabetic eye disease, to the development of PDR.
Methods: HIF-1-regulation of VEGF, AGPT2, EPO, and ANGPTL4 mRNA and protein expression was assessed in vitro by qPCR and ELSIAs in primary and immortalized (MIO-M1) retinal Müller cells in vitro or the OIR model in vivo. The angiogenic potential was assessed using the tubule formation assay. Expression of these factors in vitreous biopsies from patients with active NV due to PDR was determined by ELISA. Complementary IHC studies were performed in a second group of eyes from PDR patients. Mann-Whitney U test and Spearman correlation statistical models were used to compare protein concentrations and to identify factors that independently correlate with PDR.
Results: VEGF, ANGPT2, EPO and ANGPTL4 mRNA levels were elevated in hypoxic Müller cells in vitro and in the OIR model in vivo; inhibition of HIF-1 blocked their expression. However, protein levels of EPO and ANGPT2 were not significantly increased in retinal Müller cells, suggesting that these proteins may be expressed by a different cell type, or their local (intraocular) expression may be increased in diabetic eyes due to increased vascular permeability. Vitreous concentrations of VEGF, ANGPT2, EPO, and ANGPTL4 were all significantly higher in PDR patients compared to controls and correlated with the presence of PDR. Following anti-VEGF therapy, vitreous VEGF levels decreased; however levels of ANGPT2, EPO, and ANGPTL4 remained elevated.
Conclusions: In addition to VEGF, our results suggest that ANGPT2, EPO, and ANGPTL4 – or HIF-1 to target all four – may be potential therapeutic targets for the prevention and/or treatment of PDR.
Commercial Relationships: Haley Megarity, None; **Monika Deshpande**, None; **Savalan Babapoor-Farrokhran**, None; **Brooks Puchner**, None; **Gregg Semenza**, None; **Silvia Montaner**, None; **Akrit Sodhi**, None

Program Number: 4051 **Poster Board Number:** B0024
Presentation Time: 8:30 AM–10:15 AM
Cell Therapy Model of Diabetic Retinopathy Using Vascular Progenitor Cells Derived from Human iPSC
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Purpose: Successful cellular therapy of diabetic vascular complications (DVC) will require novel sources of angiogenic progenitors that can sustain long-term functional recovery in a clinical setting. Since adult stem cell sources such as diabetic endothelial progenitor cells (D-EPC) have proven to be scarce or have impaired migration into the injury sites, human induced pluripotent stem cell (hiPSC) technology may offer unlimited amounts of embryonic vascular progenitor (VP) to repair ischemic diabetic tissues with clinically relevant and efficient protocols for reprogramming and differentiation.
Methods: To test this potential, we generated diabetic iPSC (D-iPSC) from skin fibroblasts of type-I diabetic patient using non-integrative episomal reprogramming system. Short exposure to a GSK3β inhibitor and ascorbic acid dramatically facilitated the reprogramming kinetics. Additionally, we recently developed novel chemical methods [LIF with GSK3β, ERK and tankyrase inhibitors (L3i)] that stably revert hPSC to a mouse ESC-like naïve state. We demonstrated that LIF-3i-reverted hPSC acquired transcriptomic, epigenetic, and signaling signatures of naïve-pluripotency, and significantly improved multi-lineage differentiation efficiencies with reduced lineage bias.
Results: This L3i reversion method was successfully applied to generating naïve D-iPSC to obtain higher quality of therapeutic VP. Primed- (traditional) and naïve-D-iPSC were compared for differentiation potential, vascular functional assays in vitro and in vivo. To investigate the regenerative capacity of iPSC-VP in a DVC-relevant model, we employed a streptozotocin (STZ) injected diabetic athymic nude rat model that exhibited hyperglycemia over 24 weeks and decreased retinal function as measured by electroretinography. Our preliminary results showed that injection of healthy hiPSC-VP into this diabetic rat model validated human cell capacity to migrate and engraft to injured sites. We are currently examining the engraftment of D-iPSC-VP, naïve-D-iPSC-VP, healthy cord blood- and fibroblast-iPSC-VP. Furthermore, intravitreous injection of hiPSC-VP expressing a luciferase transgene allowed detection of human cell migration using noninvasive live animal bioluminescent imaging.

Conclusions: This STZ-induced diabetic rat model provides a translational opportunity to evaluate the use of patient specific iPSC-VP for treatment of DVC.
Commercial Relationships: Tea Soon Park; **Imran A. Bhutto**, None; **Ludovic Zimmerlin**, None; **Il Minn**, JHU (P); **Nensi Ruzgar**, None; **Martin Pomper**, JHU (P); **Mary A. Johnson**, None; **Gerard A. Lutty**, None; **Elias Zambidis**, JHU (P), Thermo Fisher Scientific (R)
Support: NIH/NHLBI grant U01HL099775 (ETZ), NIH/NICHD grant R01HD082098 (ETZ), Maryland Stem Cell Research Fund (MSCRF) grants 2011-MSCRF-II-0008-00 (ETZ), 2007-MSCRF-II-0379-00 (ETZ), 2014-MSCRF-II-118153 (TSP), 2013-MSCRF-III-114936 (LZ), core NIH grants P30 CA006973 and EY01765