Diabetic Complications Consortium

Application Title: Diabetes and miRNA

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1. Project Accomplishments:

Both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) are associated with long-term complications; the most important in terms of increased mortality in adults are macrovascular complications leading to cardiovascular disease (CVD).[1] The majority of diabetes-associated CVD is due to increased atherosclerosis. Mouse models of diabetes-accelerated atherosclerosis demonstrate that diabetes promotes macrophage accumulation in atherosclerotic lesions,[2-4] consistent with findings from human autopsy material from subjects with either T1DM or T2DM.[5] We have recently demonstrated that in two different mouse models of diabetes-accelerated atherosclerosis, accumulation of macrophages in the blood vessel wall is most likely due to diabetes-induced inflammatory activation of these cells, and that blocking this inflammatory activation completely prevents diabetes-accelerated atherosclerosis, but not atherosclerosis in non-diabetic mice.[4] This suggests that diabetes-accelerated atherosclerosis has, in part, a different etiology than atherosclerosis that develops under non-diabetic conditions. We are therefore very interested in identifying mediators of this diabetes-induced macrophage inflammatory activation, with the hope of finding new drug targets to treat or prevent diabetes-accelerated atherosclerosis and perhaps other complications of diabetes that are also dependent on macrophage activation.

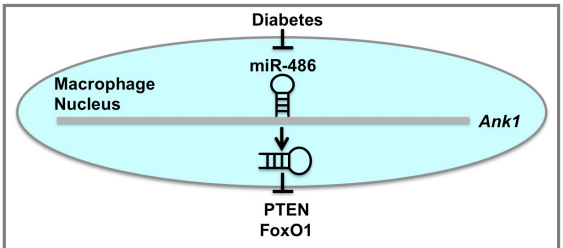

Figure 1. Working hypothesis behind the proposal. Diabetes results in downregulation of miR-486-5p, a microRNA located in the Ankyrin 1 (*Ank1*) locus, in macrophages. Its validated targets are PTEN and FoxO1. MicroRNA-486 normally suppresses PTEN and FoxO1, thereby suppressing inflammatory signaling. Under diabetic conditions, miR-486 is suppressed, the PI3K/Akt pathway is suppressed due to increased PTEN expression, and FoxO1 upregulated, which might contribute to inflammatory activation of macrophages, and diabetes complications, such as atherosclerosis.

This P&F award has allowed us to start to investigate on the role of microRNAs in diabetes. In addition to addressing most of the original aims, the aims of the project were expanded during the funding period. Instead of focusing solely on mir-486 in macrophages, we became very interested in the possibility that miRNAs produced by macrophages might be released into circulation and travel to other target cell types on HDL, where they might mediate important effects.[6] During the funding period, Dr. Kasey Vickers and colleagues showed that miRNAs travel on HDL and mediate important effects on endothelial cells.[7] We therefore initiated a collaboration with Dr. Vickers (Vanderbilt University). We have also started to collect and analyze HDL-associated microRNAs in humans with diabetes.

Our long-term goal is to identify new drug targets to deactivate macrophages, increase the anti-inflammatory effects of HDL in diabetes, and prevent atherosclerosis and other diabetes complications.

2. Specific Aims:

The original application had two specific aims, which were to address the questions:

1. Is miR-486 suppressed by diabetes in monocytes and macrophages and is this associated with hyperglycemia and suppression of Ank1 expression? We hypothesized that miR-486 is suppressed in monocytes and macrophages by diabetes, and that this occurs concomitantly with suppressed expression of the Ankyrin 1 gene, of which miR-486 is a part.

2. Is downregulation of miR-486 in macrophages sufficient for inflammatory activation, mimicking the effect of diabetes? We hypothesize that downregulation of miR-486 in macrophages is sufficient to cause inflammatory activation and to mimic the effect of diabetes, and that these effects are due to increased expression of PTEN and FoxO1.

Results: In order to identify miRNAs affected by diabetes in macrophages, we performed miRNA PCR arrays (SA Bioscience; n=3). Diabetes was induced by streptozotocin (STZ) in LDL receptor-deficient (*Ldlr*$^{-/-}$) mice (one of our models of diabetes-accelerated atherosclerosis[4]). After 4 weeks of diabetes (blood glucose levels ~300-500 mg/dl), peritoneal macrophages were isolated by thioglycollate-elicitation, as described previously.[4] Interestingly, only one miRNA was significantly increased in macrophages from diabetic mice (mmu-miR-1193-5p; 3.4-fold increase; p=0.045), whereas 27 miRNAs were significantly downregulated by diabetes. The most marked downregulation was seen for mmu-miR-486-5p (22.2-fold; p=0.03). MicroRNA-486-5p is conserved in mice and humans,[8] and is part of the ankyrin 1 (*Ank1*) locus in both species. A volcano plot of the array data is shown in Fig. 2A, and data on miR-486-5p is shown in Fig. 2B. The phosphatase PTEN is a known target of miR-486,[9] and we therefore measured the effect of *Pten* mRNA in mouse macrophages after inhibition of miR-486 by anti-miR-486. In preliminary experiments, *Pten* mRNA levels were increased by inhibition of miR-486 (Fig. 2C). Together, these preliminary results suggest that diabetes results in changes in several miRNAs in macrophages in mice, and that diabetes-mediated suppression of miR-486 might contribute to increased levels of PTEN in macrophages. Increased levels of PTEN might lead to inhibition of PI3K/Akt signaling and important downstream events.

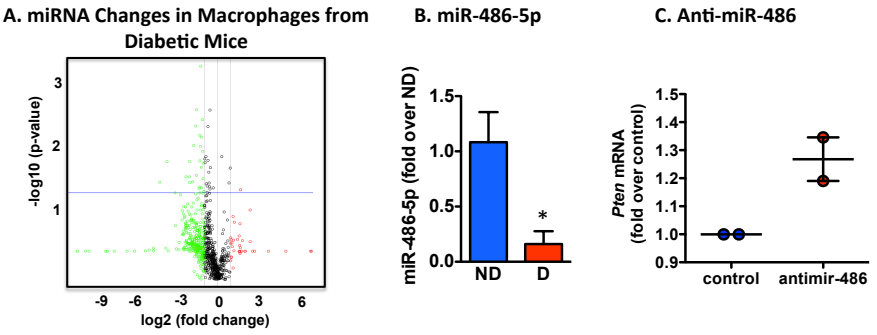

Figure 2. MicroRNA-486 is reduced in macrophages from diabetic mice. A. Peritoneal macrophages from diabetic (D) and non-diabetic (ND) *Ldlr*$^{-/-}$ mice (4 weeks of diabetes) were elicited by thioglycollate. Changes in microRNAs were evaluated by arrays, and plotted in a volcano plot. MicroRNAs above the blue line were considered to be significantly different in macrophages from non-diabetic mice versus diabetic mice. Micro-RNAs in green were reduced by diabetes and micro-RNAs in red were increased by diabetes. B. MicroRNA-486-5p levels were measured in macrophages by arrays. C. Mir-486 was inhibited in macrophages by anti-miR-486. Levels of the target gene *Pten* were measured by real-time PCR. Statistical significance is indicated by * ($p<0.05$ vs. monocytes/macrophages from non-diabetic mice; n=3 in A-B and n=2 in C) and was performed by unpaired two-tailed Student's t-test.

This P&F award has also allowed us to establish an exciting collaboration with Dr. Vickers and to expand our studies to human subjects with diabetes and to study miRNA changes in HDL. HDL was isolated from streptozotocin-diabetic *Ldlr*$^{-/-}$ mice by apoA-I affinity chromatography, and changes in HDL-associated miRNAs were measured by real-time PCR.

The diabetic mice had elevated blood glucose levels as expected (Fig. 3A). The HDL-associated microRNA results are very preliminary, but suggest that several HDL-associated miRNAs are altered by diabetes (Fig. 3B). Interestingly, miR-486 appears to be reduced in HDL from diabetic mice, suggesting perhaps that changes in HDL-associated miRNAs in part reflect changes observed in macrophages. Dr. Vickers is currently analyzing a larger number of samples from our non-diabetic and diabetic mice, using sophisticated methods he has established,[6-7] and results will be generated within the next few weeks.

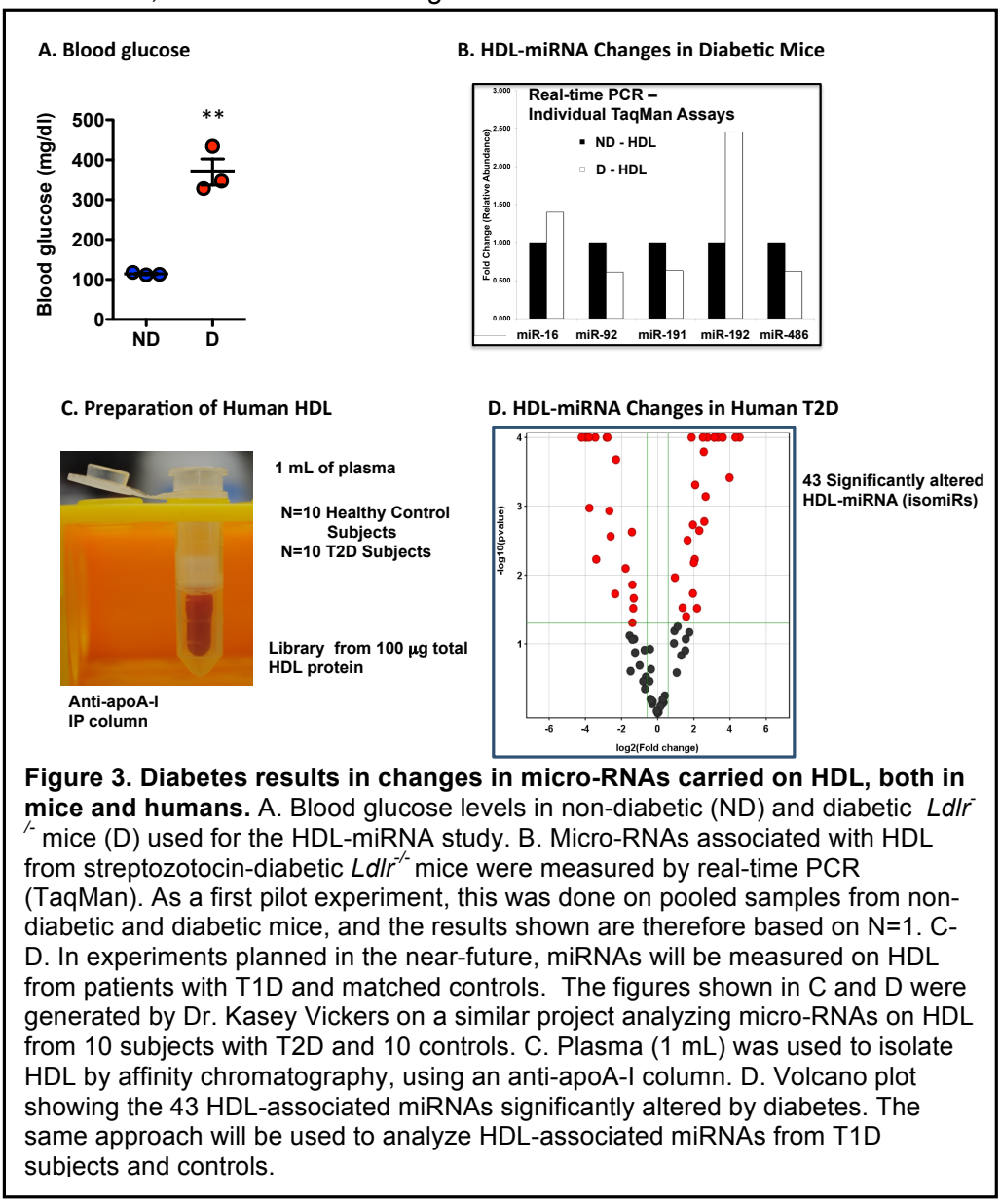

Figure 3. Diabetes results in changes in micro-RNAs carried on HDL, both in mice and humans. A. Blood glucose levels in non-diabetic (ND) and diabetic *Ldlr*$^{-/-}$ mice (D) used for the HDL-miRNA study. B. Micro-RNAs associated with HDL from streptozotocin-diabetic *Ldlr*$^{-/-}$ mice were measured by real-time PCR (TaqMan). As a first pilot experiment, this was done on pooled samples from non-diabetic and diabetic mice, and the results shown are therefore based on N=1. C-D. In experiments planned in the near-future, miRNAs will be measured on HDL from patients with T1D and matched controls. The figures shown in C and D were generated by Dr. Kasey Vickers on a similar project analyzing micro-RNAs on HDL from 10 subjects with T2D and 10 controls. C. Plasma (1 mL) was used to isolate HDL by affinity chromatography, using an anti-apoA-I column. D. Volcano plot showing the 43 HDL-associated miRNAs significantly altered by diabetes. The same approach will be used to analyze HDL-associated miRNAs from T1D subjects and controls.

We will also measure changes in HDL-associated miRNAs in human subjects with T1D and matched controls (N=15). These samples have already been collected. Fig. 3C-D shows the approach we plan to take, based on methods used by Dr. Vickers to analyze HDL-associated miRNAs in subjects with T2D and controls. In that study, significant changes in 43 miRNAs were found (Fig. 3D).

Overall, this P&F award has allowed us to generate exciting preliminary data on changes in microRNAs by diabetes in macrophages and HDL, has allowed the lab to move into a new and translational area of diabetes complications research, and will provide data for larger grant applications and multiple publications within the next few years.

Literature Cited:

1. Emerging Risk Factors Collaboration, Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, Di Angelantonio E, Ingelsson E, Lawlor DA, Selvin E, Stampfer M, Stehouwer CD, Lewington S, Pennells L, Thompson A, Sattar N, White IR, Ray KK, Danesh J. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet. 2010*;375(9733):2215-22.
2. Renard CB, Kramer F, Johansson F, Lamharzi N, Tannock LR, von Herrath MG, Chait A, Bornfeldt KE. Diabetes and diabetes-associated lipid abnormalities have distinct effects on initiation and progression of atherosclerotic lesions. *J Clin Invest.* 2004;114:659-68.
3. Lamharzi N, Renard CB, Kramer F, Pennathur S, Heinecke JW, Chait A, Bornfeldt KE. Hyperlipidemia in concert with hyperglycemia stimulates the proliferation of macrophages in atherosclerotic lesions: potential role of glucose-oxidized LDL. *Diabetes.* 2004;53:3217-25.
4. Kanter JE, Kramer F, Barnhart S, Averill MM, Vivekanandan-Giri A, Vickery T, Li LO, Becker L, Yuan W, Chait A, Braun KR, Potter-Perigo S, Sanda S, Wight TN, Pennathur S, Serhan CN, Heinecke JW, Coleman RA, Bornfeldt KE. Diabetes promotes an inflammatory macrophage phenotype and atherosclerosis through acyl-CoA synthetase 1. *Proc Natl Acad Sci U S A.* 2012;109:E715-24.
5. Burke AP, Kolodgie FD, Zieske A, Fowler DR, Weber DK, Varghese PJ, Farb A, Virmani R. Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. *Arterioscler Thromb Vasc Biol.* 2004;24:1266-71.
6. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol.* 2011;13(4):423-33.
7. Tabet F, Vickers KC, Cuesta Torres LF, Wiese CB, Shoucri BM, Lambert G, Catherinet C, Prado-Lourenco L, Levin MG, Thacker S, Sethupathy P, Barter PJ, Remaley AT, Rye KA. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat Commun.* 2014;5:3292.
8. Alexander MS, Casar JC, Motohashi N, Myers JA, Eisenberg I, Gonzalez RT, Estrella EA, Kang PB, Kawahara G, Kunkel LM. Regulation of DMD pathology by an ankyrin-encoded miRNA. *Skelet Muscle.* 2011;1:27.
9. Small EM, O'Rourke JR, Moresi V, Sutherland LB, McAnally J, Gerard RD, Richardson JA, Olson EN. Regulation of PI3-kinase/Akt signaling by muscle-enriched microRNA-486. *Proc Natl Acad Sci U S A.* 2010;107(9):4218-23.

3. Publications:

We expect to submit manuscripts describing the results from this project in 2015.