AMDCC Annual Report (2011)

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Project Title: Dyslipidemia, Lipoic Acid and Diabetic Vascular Complications in Humanized Mice
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Abstract: While diabetes mellitus can lead to serious damage to many organs, cardiovascular diseases are the major cause of death and morbidity in diabetic patients. Overall, patients with diabetes have a three to five fold increased risk of coronary artery diseases compared to non-diabetics. Our goal is to use mouse genetics for identifying genetic risk factors for the vascular complications of diabetes and for unraveling underlying mechanisms. Although a significant increase in atherosclerosis by diabetes has been demonstrated in atherogenic mouse models, none of these mouse models faithfully replicates the types of dyslipidemia associated with diabetes in humans. We postulate that this failure is due to differences in the relative levels of plasma low density lipoprotein (LDL) and plasma high density lipoprotein (HDL) that are controlled by genetic differences between the two species and genetic polymorphisms in humans. Thus our first hypothesis is that humanizing genes that are involved in lipoprotein metabolism in mice so that they develop a more human-like diabetic dyslipidemia will cause them to replicate better the cardiovascular problems of human diabetic patients. We will test this hypothesis in Specific Aim 1 by inducing diabetes in mice with humanized apoE of the three isoforms (E2, E3, and E4) and humanized LDL receptor (LDLR), with or without overexpression of human apoB. We predict that this will lead to diabetic dyslipidemia and accelerated atherosclerosis in an apoE isoform dependent manner. Our second hypothesis is that since diabetes is generally acknowledged to induce oxidative stress, genetically determined differences in the levels of endogenous anti-oxidants affect the development of cardiovascular complications,. To test this hypothesis, we propose in Specific Aim 2 to develop a new mouse model with a genetically controlled reduction in the production of the endogenous antioxidant lipoic acid (LA). We will modify the LA synthase (Lias) gene in such a way that the stability of Lias mRNA will be drastically reduced in a tissue specific fashion. Our hypothesis predicts that reduced production of LA will increase the oxidative stress already present in diabetic mice and enhance their development of vascular complications. In Specific Aim 3, we propose to combine human-like diabetic dyslipidemia with genetically reduced antioxidant capacity due to LA deficiency to test our overall thesis that interactions between genetic polymorphic differences affecting lipid profiles and genetic differences affecting endogenous antioxidant levels determine the degree to which diabetes enhances cardiovascular disease.

Program Accomplishments:

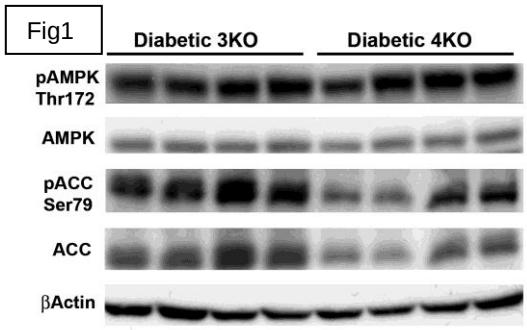
Hypothesis

1. Mice humanized lipoprotein metabolism system will develop a more human-like diabetic dyslipidemia and cardiovascular problems.
2. Genetically determined differences in the levels of endogenous antioxidants affect the development of cardiovascular complications of diabetes.

Progress toward stated aims

1-1. Hepatic origin of diabetic dyslipidemia in LDLR-/- mice expressing human apoE3 or apoE4. We reported previously that the LDLR-/- males expressing human apoE4 isoform (4KO) develop exaggerated dyslipidemia than those expressing human apoE3 isoform (3KO) when they were made diabetic with STZ injection at 2 months of age. Diabetes enhanced atherosclerosis in 4KO mice but not in 3KO mice. However, although increased in amount, plasma VLDL and LDL did not differ in lipid/protein composition, glycated proteins, in the amount of LDL oxidation or in the lipolytic capacity between the two diabetic groups. Therefore, we shifted our attention to one of the most striking difference that the triglyceride content of livers of diabetic 4KO mice was two fold higher than those in the livers of 3KO mice and of non-diabetic controls.

Compared to the livers of non-diabetic controls, diabetic livers expressed increased levels of genes for FoxO1 and Srebp1c, regulators of lipogenesis and VLDL secretion. Both tended to be higher in diabetic 4KO than in 3KO, but the differences did not reach significance. Expression of genes for Chrebp, Pparα, and Cpt1 in contrast did not change significantly by diabetes, but tended to be lower in 4KO. Importantly, expression of Fasn (fatty acid synthase) reduced to 20% in diabetic 3KO livers compared to non-diabetic control, but no reduction of Fasn was found in 4KO livers. In addition, western blots (Fig.1) showed that, while the levels of AMPK (AMP-activated protein kinase) or its phosphorylated form (pAMPK) did not differ, pACC/ACC (acetyl CoA carboxylase) ratios were significantly reduced in the diabetic 4KO livers. Although the pattern of changes is not completely consistent with the current knowledge of the lipogenic gene/protein regulations, overall the data indicate that a relative increase in fatty acid synthesis and a reduction in fatty acid oxidation underlie the increased lipid store in the livers of diabetic 4KO mice compared to 3KO mice. Indirect calorimetry analysis also showed significantly higher respiratory exchange ratios (VCO_2/VO_2) in the diabetic 4KO mice than 3KO mice during the light cycle, confirming a lower reliance on lipid as an energy source at a whole body level in these mice.

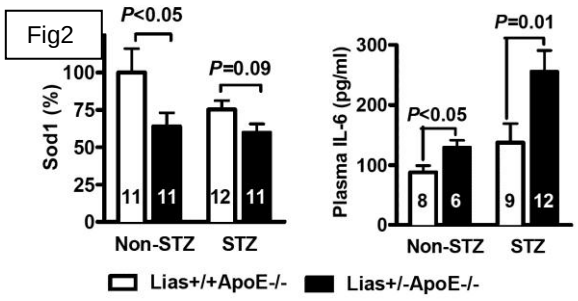


To address glucose and lipid metabolism in more detail, we isolated primary hepatocytes from non-diabetic 3KO and 4KO mice and cultured them for 3 days in medium with high (25mM) or low (5 mM) glucose. Mirroring the increased TG store in the diabetic livers, 4KO cells accumulated twice as much lipids compared to 3KO cells. Culturing in the high glucose medium increased FFA uptake, glucose uptake and de novo lipogenesis by the cells but there were no ApoE-genotype effects. Glucose oxidation was reduced compared to the cells cultured in the low glucose medium, but to similar extents in 3KO and 4KO cells. In a marked contrast, while lipid oxidation in the 3KO cells did not change by the glucose content in the medium, it reduced to 40% in the 4KO cells cultured in the high glucose medium. Together, we conclude that a reduced fatty acid oxidation in the liver leads to an increased lipid store, which in turn leads to two-fold increases of VLDL secretion and plasma lipoproteins in the diabetic 4KO mice. A manuscript describing this work is almost ready for submission to a journal.

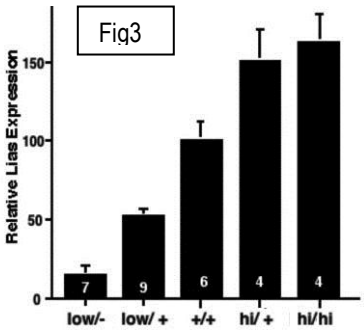
It is not clear how apoE4 protein causes these alterations, except that LDLR is not necessary for these effects. This issue has to be addressed in our future experiments. Nevertheless, we note that LDLR-/- mice are an established model for a diet-induced obesity and insulin resistance. We have also shown than mice expressing apoE4 are more susceptible to diet-induced impairments of glucose tolerance than those expressing apoE3. Thus 4KO mice have a propensity to develop insulin resistance. The apoE4 associated impaired lipid metabolism in the context of insulin deficiency induced by STZ-treatment in these mice extends beyond the scope of type 1 diabetes, making this an excellent model in which to sturdy macrovascular complications of diabetes.

1-2. Akita mice expressing human apoE4 and human LDLR develop atherosclerosis without severe hypercholesterolemia. We are excited with the atherosclerotic plaques developing in the aortic root of the 44hAkita mice that express human apoE4 and human LDLR. We have evaluated a total of five mice at 5 months of age to date, and all except one had small plaques consisting of several foam cells and/or infiltration of lipids in the medial layers. In one, the plaques were developed and had distinctive fibrous caps. Because plasma cholesterol levels are low (ca 120 mg/dl) and because the plaques of even apoE-null male mice on C57BL/6 background are small and not well developed at 5 months of age, it is necessary to age these mice further for full evaluation of the plaque development. Unfortunately, we continued to have a breeding problem as well as a reduced lifespan of this strain of mice. We have sent breeders to the Jackson laboratory with a hope to overcome the breeding problem with their expertise, but so far they have not been able to supply any experimental mice or young breeders. We have commenced crosses to generate Akita mice that are homozygous for apoE4 but this will take three generations of crossing to make more experimental animals.

2-1. Atherosclerosis in Lias+/-Apoe-/- mice made diabetic by a STZ treatment. We reported last year that a 50% reduction of endogenous LA production and diabetes appears to have independent additive effects in exaggerating atherosclerosis. Lias+/-Apoe-/- male mice have higher atherosclerosis than Lias+/+Apoe-/- males, and this is associated with lower expression of Sod1 and Sod2. Gene expression of Sod1 and Sod2 in aorta was reduced in diabetic mice compared with non-diabetic mice suggesting that STZ treatment increased oxidative stress. However, they did not differ significantly by the Lias genotype. Possibly, an enhanced oxidative stress by diabetes blurs the genotype effect. In contrast, MCP-1 gene expression in the aorta was increased by diabetes as well as by the reduction of Lias, indicating that increased monocyte recruitment is an additive effect. In addition, plasma IL-6 level was significantly increased, suggesting that enhanced inflammation contributes to formation of larger lesion in diabetic Lias+/-apoE(-/-) mice. Although atherosclerotic plaques in the diabetic Lias+/-Apoe-/- males were approximately 20% larger in size than those in the diabetic Lias+/+Apoe-/- male, there were no notable differences in the complexity of lesions under light microscope.

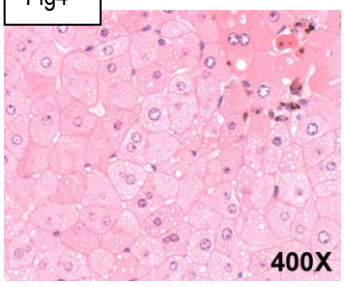


2-2. Mice with low and high Lias gene expression. We have imported from JAX Lias-H (high) and Lias-L (low) mice and have established the colonies at UNC. We first determined the expression levels of the Lias gene in the liver using RT-PCR, which demonstrated that the steady state levels of Lias transcripts in mice with a Lias-L allele was about 60% while those in mice with a Lias-H allele was about 150% normal. As shown in the Fig3 on the right, we are able to generate a series of mice varying in the Lias gene transcript from about 15% to 200%.



Initial characterization of animas showed that both male and female Lias-L/L mice tended to die suddenly at about 6 month of age. Increased incidents of death of Lias-L/L mice at similar age were also observed at JAX, and Dr. Leiter has informed us that necropsy showed that liver failure is the potential cause of death of these mice. Histologically, sizes of nuclei are not uniform: some are condensed, pyknotic or completely lost. Areas of necrosis were also evident (top right in the Fig4). Initial study of Lias-L/L mice surviving at 6 month of age (n=6) showed that they have lower body weight (29.5±1.5g) than Lias-L/+ (33.8±1.0g, n=36) and WT mice (32.4±1.8g, n=19). Although the difference did not reach statically significance due in part because of a small sample size, the large standard deviation suggests that we need to monitor the weight gain up to 6 month more carefully. Plasma glucose levels were incrementally, although slightly, reduced in L/+ (159±7 mg/dl) and L/L (149±6 mg/dl) mice compared to wild type mice (168±8 mg/dl). Plasma cholesterol, triglycerides and free fatty acid, and lipid peroxidation levels indicated by 4-xydroxynonenal were not significantly different in the three groups. Oral glucose tolerance test (OGTT) was normal in the L/L mice. Plasma pyruvate level had an incremental increase as the expression of Lias decreases, with the levels in the L/L mice (0.69±0.24 nmole) approximately twice those in WT mice (0.29±0.14 nmole). Plasma lactate levels were not different. Blood pressure was also not different (99±3.8 mmHg in L/L, 106±5.3 mmHg in L/+, and 101+2.8 mmHg in WT mice). To elucidate the mechanism underlying the premature death of the Lias-L/L homozygotes have isolated fibroblasts from the L/L mice and will be examining their oxidative stress status and metabolic changes.

Fig4



We have found that the Lias-H/H male mice are aggressive and fight with littermates. This has hampered the characterization of the series of mice with increased Lias expression. We are considering to place the mice in individual cages to protect and avoid adverse effect on analysis.

Plans for the year and completion of the project

1. We will expand Akita diabetic mice expressing human apoE4 and human LDLR.
2. Interactions between apoE4 protein and protein components of mitochondrial Complex III and Complex IV have been described in neurons. Mitochondrial dysfunction has been also implicated in FAA oxidation deficiency that proceeds hepatic steatosis. We will assay the degrees of mitochondrial dysfunction in the diabetic 4KO and 3KO mice. Finally, we will complete the experiments that address the potential implication of apoE4-specific predisposition to insulin resistance in diabetic dyslipidemia under the settings of dietary stress.
3. We will complete the manuscript describing the effects of reduced endogenous LA production in diabetic complication in kidneys.
4. We are expanding a colony of Akita mice with Lias-L allele to develop models for diabetic complication. We will complete the initial characterization of mice with varied levels of the Lias gene expression.

1.**Collaborations:**

With other AMDCC PIs : Because of the proximity, collaboration between students in the Oliver Smithies's group have been excellent. For example, they together worked to set up assays of mitochondrial dysfunction using the Seahorse Cellular Bioenergetics Analyzer.

With Jax : Generation of mice with high and low expression of Lias has been completed. We have imported these mice to UNC and initial characterization is on going as summarized above (2.2) Breeding Akita mice with those low expression of Lias has also been completed at JAX and we have received some for breeders. To increase the number of supply of 4hAkita mice expressing human LDLR (h/+) and human apoE4 (4/4) we have sent JAX 4/4 Akita mice and 4/4/ h/+ mice to be bred at JAX. The supply of these mice is crucial for study of atherosclerosis and of other studies by the AMDCC groups - such as neuropathy.

With the MMPCs : None. Generally, setting up collaborations with MMPC has not been successful for our group partly because graduate students and postdoctoral fellows prefer performing experiments themselves. This is also because mutant mice we have been characterizing are limited in supply and we were not able to ship them to MMPCs. We have inquired for the possibility of histological (EM) analysis of kidneys at one of the MMPC, but the time line was faster if we did in house.

With other non-AMDCC PIs : We have been collaborating with Dr. Volker Nickeleit, nephropathologist at UNC, and Dr. Leighton James, nephrologists at the University of Florida for the kidney phenotypes.

Address previous EAC comments:

- Good progress and interesting results for all projects. Thank you.

- These are very exciting results for the 4hAkita mice. The model is a beautiful example for the power of humanizing mice. Yes we believe so. We are gearing up to increase the number of mutant mice for further analyses in our laboratory as well as in JAX.

- From the experiments described (diet in 4KO) it seems that the hypothesis that apoE isoforms affect insulin sensitivity is not correct, but that E4 has a direct effect on plaque formation independent of dyslipidemia. Are there plans to address that? We believe that the apoE-isoform affect insulin sensitivity through its effects on adipocyte differentiation. Diet study in 4KO mice was designed to test the effect of either high lard or high sucrose in diet. As such, although both diet decreased glucose tolerance slightly but significantly, the effect of apoE-isoform was not significant. However, we have not ruled out the potential apoE4-effects on insulin resistance in 4KO mice, because the effects of these two diets are small. Western-type diet that is high in fat, sucrose and cholesterol was not tested because of its profound effects on dyslipidemia. As summarized above in 1-1, our studies have demonstrated that lipid/glucose handling in the diabetic 4KO mice differed from that in diabetic 3KO mice, and this is contributing to the enhanced diabetic dyslipidemia and atherosclerosis in the 4KO mice. We note the possibility that apoE4 affects plaque formation directly through isoform-dependent difference in function of vascular cells such as in macrophage. We have previously shown that there is no significant difference in cholesterol uptake and efflux between 4KO and 3KO macrophage, and we are currently examining the effects of sucrose and lard diets. Interactive effects between apoE-isoform and diabetes on macrophage function have yet to be analyzed.

- Considering all the progress, we think it may be worthwhile for multiple complications (like nephropathy and

hypertension) to combine the LiasL with the 4hAkita. We agree. We are presently combining LiasL with Akita and plan to complete initial characterization by the end of the granting period. Our current problem is the difficulty in breeding 4hAkita mice. Combining mutations at four loci (two homozygous and two heterozygous) is quite a challenge, and requires time and patience.

- Dr. Maeda's models should be tested for neuropathy if it isn't being done already. This is an excellent suggestion considering the well-known association between apoE4 isoform and neuronal diseases including Alzheimer's. As summarized above (1-2) we have been having breeding problems but working hard to produce 4hAkita male mice at both UNC and JAX. Enhancement of diabetic nephropathy and atherosclerosis due to the 50% reduction of LIAS is significant but subtle. We are currently evaluating whether or not Lias-L/L Akita mice in which the Lias transcripts are 25% or less will provide models with further enhancement. This will likely provide better model for assessing other complications such as neuropathy.

- There remains a problem with 4hAkita mice as they do not survive to an age to develop atherosclerotic lesions. In the past year, 3 more mice have survived to 6 months of age adding to the 2 that were studied last year. Do the investigators have a clearer understanding of why the animals are dying? What do necropsy results indicate? It is difficult to catch mice that are dying, and necropsy of a couple of mice we caught shortly after death has not been informative. We hope to generate a larger number of 4hAkita males and monitor them intensively, once we overcome the current breeding problems.

- It is somewhat interesting that the investigators are pursuing a role for 4h in insulin sensitivity. While the studies with Western diet certainly argue for this line of investigation, it does not necessarily follow the STZ experiments as insulin production/release should be eliminated. By contrast, this may be an effect of glucose toxicity. Our diet studies have suggested that mice carrying apoE4 have a predisposition to reduced glucose tolerance and developing insulin resistance compared to those carrying apoE3. Our current working hypothesis is that apoE4 influences energy balance within a cell differently from other apoE isoforms, particularly when cells are metabolically stressed by hyperglycemia. Although both low-dose STZ treatment and Akita mutation are considered to be the models of Type 1 diabetes with impaired insulin production, cellular energy balance which are considered more relevant to metabolic syndrome and Type 2 diabetes could interact significantly in the development and progress of diabetic complications. The possible effect of glucose toxicity to vascular cells, beyond oxidative stress and AGE, is an interesting suggestion and will be considered carefully.

- Have there been attempts to determine the cellular constituents of the lesions? Are they all monocyte-rich foam cell lesions or is there added complexity. More information regarding cellular constituents may give some insight into the direction of future studies (e.g. more inflammation). Lesions in the diabetic 4hAkita males at 4 months of age are limited to clusters of monocyte-derived foam cells in 4 of 5 mice. One mouse had a lesion that progressed to fibrous caps and lipid infiltration into medial layers. The plaque appears to have less lipids but more fibrous compared to the plaques similar in size seen in apoE-deficient mice, probably reflecting the low plasma cholesterol. Since inflammation in the vascular wall increases as plaque develop, comparison of inflammatory changes in the 4hAkita vessels (that have plaques) with 3hAkita or 4+Akita (which do not have lesions) does not make sense. We need to approach this in an in vitro model system.

- LIAS+/- apoE(-/-) have higher atherosclerosis in males, but not in females. Interestingly, this is associated with lower expression of SOD1 and SOD2, likely further enhancing oxidative stress. Also very interesting results when the animals were made diabetic. It would thus be expected that there may be more monocyte recruitment and vascular smooth muscle proliferation. Has complexity of lesions been investigated? We additionally found that gene expression of Sod1 and Sod2 is reduced in the aorta of STZ treated apoE-/- mice compared to non-diabetic

mice. In contrast, Sod1 and Sod2 gene expression was increased in the kidneys of the diabetic mice, suggesting the regulation of these genes in response to diabetes is tissue specific. We did not detect marked differences of lesion complexity under light microscope between diabetic and non-diabetic mice: the plaques are fully developed and complex at the stage we evaluated. Alteration in monocyte recruitment is difficult to evaluate in vivo. Perhaps we need to examine the plaques at earlier age, but this will shorten the time that animals has been diabetic and inflammatory effects of the STZ treatment cannot be excluded.

- What is the effect of diet upon LIAS gene expression in both wild type and hets? Are there known ways (other than the genetic methods that are being pursued) of enhancing LIAS gene expression and/or protein activity? Not much is known about the regulation of the Lias gene expression / protein activity at present. A small but significant increase of Lias gene expression in aorta has been reported by the treatment with TZD such as rosiglitazone. We also observed an 80% decrease in the kidneys of diabetic mice and lipoic acid supplementation further reduced this expression. In another experiment we found feeding Western-type high fat diet for 3 months did not affect liver expression of the Lias gene in WT animals. We have not examined the diet effects in heterozygotes. This is a good suggestion, and we will set up either Lias+/- or LiasL/L mice on an western-type diet.

- The goal of this project is to generate humanized lipoprotein mice for studies of atherosclerosis. The PI has made reasonable progress in the last year; she has generated the 4hAkita mouse which develops atherosclerosis independently of hyperlipidemia. She is currently trying to expand this mouse colony with the help of JAX. She has also begun studying diabetic nepropathy lipoic acid synthase knockout mice on an Akita background. She also found that these mice have higher blood pressure, so she plans to study this more in detail. In sum, the investigator is on track to complete goals, and has expanded lipoic acid synthase studies to include nephropathy and hypertension. No changes in direction are suggested. Thank you.

- Below is a list of your AMDCC publications from the website. Should any publications be added or subtracted? Has all of the relevant data from these publications been uploaded to the website? Please work with Dr. Rick McIndoe to ensure that the website and database are up-to-date and complete. Five new publications have been added. We are in the process of uploading the primary data of diabetic Lias+/-, 4KO, and 3KO mice.

1. alpha-Lipoic acid protects diabetic apolipoprotein E-deficient mice from nephropathy. Yi X, Nickeleit V, James LR, Maeda N. J Diabetes Complications. 2010 Aug PMCID: PMC3010318

2. Pparg-P465L mutation worsens hyperglycemia in Ins2-Akita female mice via adipose-specific insulin resistance and storage dysfunction. Pendse AA, Johnson LA, Tsai YS, Maeda N. Diabetes. 2010 Nov;59(11):2890-7. PMCID: PMC2963548

3. Impaired adipogenic response to thiazolidinediones in mice expressing human apolipoprotein E4. Arbones JM, Johnson LA, Altenburg MK, Kim HS, Maeda N. Faseb J 2010 Oct;24(10):3809-18. PMCID: PMC2996914

4. Genetic reduction of endogenous alpha-lipoic acid synthesis modestly increases atherosclerosis in male but not female apolipoprotein E deficient mice. Yi X, Xu L, Kim K, Kim H-S, Maeda N. Atherosclerosis. 2010 Aug;211(2):424-30. PMCID: PMC2914155

5. Macrovascular complications of diabetes in atherosclerosis-prone mice. Johnson LA, Maeda N. Expert Rev. Endocrinol. Metab. 2010. 5(1), 89-98. (invited review)

6. Mice with heterozygous deficiency of lipoic acid synthase have an increased sensitivity to lipopolysaccharide-induced tissue injury.
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10. A de novo deafwaddler mutation of Pmca2 arising in ES cells and hitchhiking with a targeted modification of the Pparg gene.
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11. Senescence-associated phenotypes in Akita diabetic mice are enhanced by absence of bradykinin B2 receptors
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The Journal of clinical investigation, 2006 (116), 1302 - 1309
12. Endogenous production of lipoic acid is essential for mouse development.
Yi X, Maeda N
Molecular and cellular biology, 2005 (25(18)), 8387 - 8392
13. PPARgamma: a critical determinant of body fat distribution in humans and mice.
Tsai YS, Maeda N
Trends in cardiovascular medicine, 2005 (15(3)), 81 - 85
14. Hypertension and abnormal fat distribution but not insulin resistance in mice with P465L PPARgamma.
Tsai YS, Kim HJ, Takahashi N, Kim HS, Hagaman JR, Kim JK, Maeda N
The Journal of clinical investigation, 2004 (114(2)), 240 – 249