Diabetic Complications Consortium

Application Title:
The Role of Oxidative Stress in Diabetes Induced Progenitor Cell Deficits

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Project Accomplishments:

As outlined in our previously filed extension, our original projected start date was deferred to 08/01/2015 due to delays in funding release (by Stanford) and unanticipated logistical difficulties in cross-country animal transport, followed by 12-weeks of subsequent animal quarantine. As these animals were recently cleared for release, our experiments have made significant forward progress over the past few months. As such, we are excited to report on the experimental outcomes within the aims of this project. We intend to file an amended and updated final report at year-end.

Broadly, we have successfully established a glyoxalase-1 knockdown (*Glo1* KD) colony within our animal facility at Stanford University independent of the parent colony maintained by the Brownlee group at Albert Einstein. Utilizing an additional transgenic animal model from our collaborators, we also proposed establishing a new *Glo1*-overexpressing mouse colony in restricted germ-layer compartments under pharmacologically inducible conditions. We have begun the breeding of these *Glo1* overexpression mice using commercially available cre- animals, allowing for creation of 2 strains – a global overexpression and a mesenchymal lineage restricted overexpression animal.

Single cell FACS sorting for progenitor cells within the bone marrow (BM) and adipose compartments of wild-type and *Glo1* knockdown animals have demonstrated progenitor cell population depletion similar to derangements found in end-stage diabetic animals, however, notably without accompanying impairment of glucose tolerance. Cells collected from each animal are currently under processing for single cell transcriptional analysis (SCTA) as previously described by our group, to elucidate progenitor cell functionality changes in healthy and diseased states.[1,2] Progress toward individual specific aims with accompanying data will be presented in further detail below.

Specific Aims:

Specific Aim 1:

To identify the role of methylglyoxal-derived reactive oxygen species generation on diabetic progenitor cell subpopulation defects.

Results:

Homozygous *Glo1* knockdown males were successfully mated with heterozygous *Glo1* knockdown females to generate additional homozygous male offspring. Heterozygous females were utilized for breeding purposes to prevent complete loss of methylglyoxal metabolism in-utero and reduce subsequent increases in spontaneous intrauterine fetal demise. C57BL/6 WT males were fed a 20% high-fat diet for a minimum of 12 weeks to generate a type-2 diabetes phenotype, as previously described.

In a 2-hour glucose challenge (2mg glucose/ g body-weight), *Glo1* KD animals, similarly to WT negative controls, demonstrated intact glucose tolerance when compared to diabetic-high fat diet (HFD) positive control animals (Table 1). Their serum glucose levels determined by conventional point-of-care testing did not exceed clinical standards of 200 mg/dL at fasting prior to or after glucose challenge. We emphasize this data to reinforce an important fact that *Glo1* animals do not demonstrate glucose metabolism impairment typically seen in classically diabetic mice.[3] Such a specific condition allows us to better study the impact of methylglyoxal oxidative load with minimal confounding by traditional glucose-related glycation events, which are a known cause of progenitor cell dysfunction in end-stage diabetic pathophysiology.[4]

Strain	24hr Fasting Glucose (mg/dL)	2hr Glucose Challenge (mg/dL)
Wild-Type	148-177	124-155
Glo1 Knockdown	157-186	190-193
High Fat Diet-Diabetic	204-213	**226-368**

Table 1. 2-hour glucose tolerance test results. Animals fasted over a 24 hour period were tested utilizing conventional point-of-care glucometer system to demonstrate degree of glucose metabolism impairment at baseline. 2 hours after injection with 2mg glucose/g body-weight, animals were retested to demonstrate abnormalities in insulin regulation. Animals with a blood glucose levels over 200 mg/dL at the conclusion of the 2-hour glucose challenge are defined as consistent with a type-2 diabetes phenotype.

Primary bone marrow and inguinal fat-pad isolates were harvested, dissociated, and stained in the usual fashion from all three groups in preparation for FACS analysis. Bone marrow multipotent progenitor cell populations were defined as Lin-/CD45-/Sca1+/Kit+. Adipose-derived multipotent progenitor cells were defined as CD45-/CD31-/CD34+. Both populations were clearly delineated on FACS sorting using stringent gating schemes constructed based on unstained sample profiles.

FACS analysis of the BM and adipose progenitor populations both demonstrated a depletion of progenitor cells in *Glo1* KD, and HFD-DM2 positive-controls compared to WT negative-controls. In the adipose tissue of non-diabetic WT C57BL/6 mice, progenitor cells represented 3.25% of the total CD45- population. In diabetic C57BL/6 mice, the quantity of these progenitor cells demonstrated significant decline to 1.14%. *Glo1* knockdown animals maintain a quantity of progenitor cells that lies between these two extremes of positive- and negative-control at 2.74% (Figure 1).

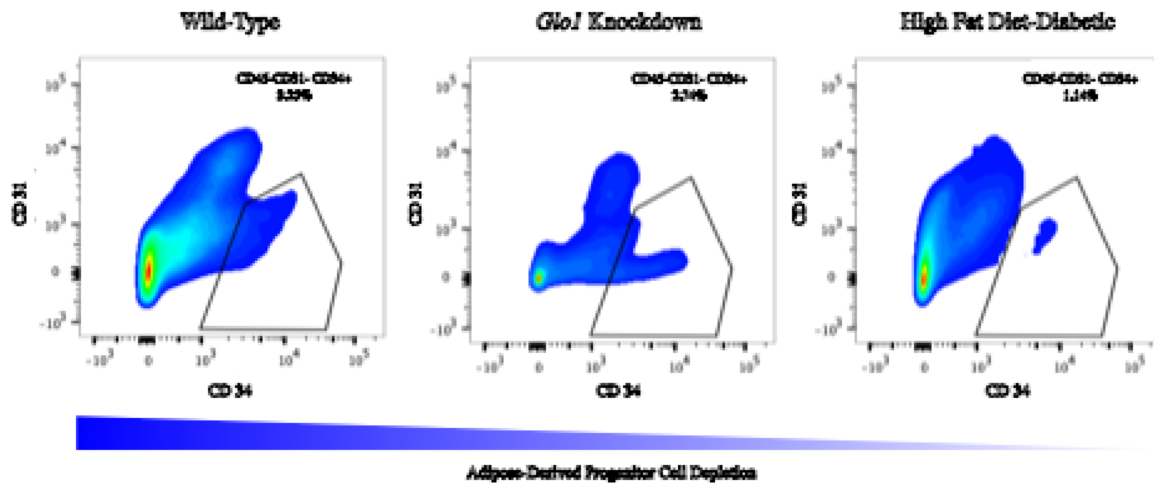

Figure 1. Adipose-derived progenitor cell population depletion. Adipose-derived progenitor cells were defined as CD45-/CD31-/CD34+ cells within the stromal vascular fraction to exclude contaminating hematopoietic and endothelial cells. Flow cytometry gating schemes for adipose-derived progenitor cells demonstrate a depletion of this population as degree of diabetes increases. Glo1 knockdown animals, despite lack of diabetic phenotype, demonstrate population depletion secondary to inappropriate catabolism of methylglyoxal oxidative load. This data supports our hypothesis that methylglyoxal is a potent glycating agent responsible for derangements in progenitor cell function at end-stage diabetes.

Hematopoietic stem cells (HSC) show a similar pattern of decline between groups where WT HSCs accounted for 0.53% of the Lin- population while diabetic C57Bl./6 animals accounted for only 0.30%. Glo1 KD animals demonstrated a quantity between these extremes at 0.36% (Figure 2). Degree of depletion correlates well with the individual animal's severity of diabetes, supporting previously reported findings.[5] Such findings are especially encouraging as they suggest *Glo1* perturbations resulting in dysfunctional methylglyoxal catabolism are significant enough to drive population-level progenitor cell derangements. The data support our hypothesis that methylglyoxal, although representing a small percentage of the overall oxidative load within a diabetic animal, remains an extremely potent glycation agent capable of exerting significant negative impact on progenitor cells despite low concentrations.

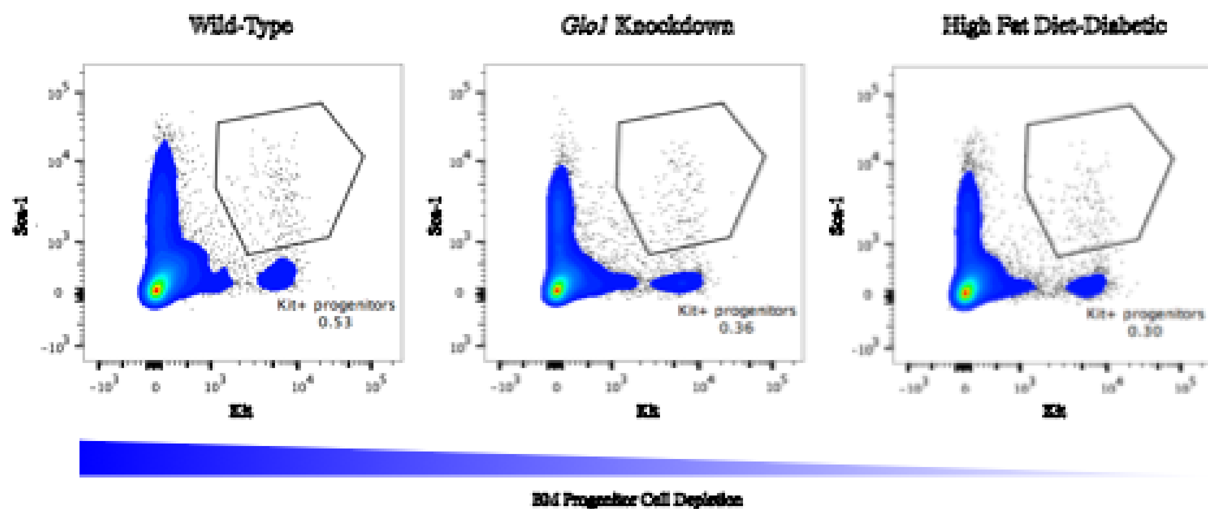

Figure 2. Bone marrow hematopoietic progenitor cell depletion. Hematopoietic progenitor cells were defined as Lin-/Sca-1+/cKit+ cells within the bone marrow fraction to exclude contaminating immune and mesenchymal cells. Flow cytometry gating schemes demonstrate a depletion of this population as degree of diabetes increases analogous to adipose-derived progenitor cell populations. Glo1 knockdown animals, despite lack of diabetic phenotype, demonstrate population depletion secondary to inappropriate catabolism of methylglyoxal oxidative load. This data supports our hypothesis that methylglyoxal is a potent glycating agent responsible for derangements in multiple progenitor cell populations at end-stage diabetes.

Single cells were collected from each animal and stored in -80C in preparation for multiplexed, microfluidic high-throughput RT-PCR via the Fluidigm Biomark system. A 96 element gene-list was constructed based on extensive literature review to assay for progenitor cell function (Table 2).

Assay ID	Gene Name	Assay ID	Gene Name
Mm00802048 m1	*Ace*	Mm00492322 m1	*Ggt1*
Mm00607939 s1	*Actb*	Mm00439306 m1	*Hg-egf*
Mm00545742 m1	*Adam10*	Mm01135193 m1	*Hgf*
Mm00456503_m1	*Angpt1*	Mm00439560_m1	*Igf1*
Mm00545822 m1	*Angpt2*	Mm00492632 m1	*Igfbp2*
Mm00437762 m1	*B2m*	Mm00516037 m1	*Igfbp5*
Mm00477631 m1	*Bcl2*	Mm00446726 m1	*Il13ra1*
Mm00437783 m1	*Bcl2l1*	Mm01275139 m1	*Il4r*
Mm00599749_m1	*Birc5*	Mm00439653_m1	*Il6r*
Mm01340178 m1	*Bmp2*	Mm00439797 m1	*Itga5*
Mm01609165 g1	*Bst2*	Mm00443980 m1	*Itgb3*
Mm00441242 m1	*Ccl2*	Mm00496902 m1	*Jag1*
mm00441259 g1	*Ccl3*	Mm01222421 m1	*Kdr*
Mm00432359_m1	*Ccnd1*	Mm00445212_m1	*Kit*
Mm00487740 m1	*Cd200*	Mm00516104 m1	*Klf4*
Mm00547485 s1	*Cd248*	Mm00440181 m1	*Lepr*
Mm00507988 m1	*Cd320*	Mm01340842 m1	*Mef2c*
Mm00519283 m1	*Cd34*	Mm00485028 m1	*Mme*
Mm00681165_m1	*Cd44*	Mm00487803_m1	*Myc*
Mm00495005 m1	*Cd47*	Mm00446296 m1	*Ngfr*
Mm00438377 m1	*Cd55*	Mm00440502 m1	*Nos2*
Mm00483149 m1	*Cd59*	Mm00501910 m1	*Nt5e*
Mm01966817 g1	*Cd63*	Mm00448100 g1	*Pcna*
Mm03047340_m1	*Cd68*	Mm01205760_m1	*Pdgfa*
Mm00658576 m1	*Cd74*	Mm00440701 m1	*Pdgfra*
Mm00504869 m1	*Cd81*	Mm00435546 m1	*Pdgfrb*
Mm00440239 g1	*Cd93*	Mm01242584 m1	*Pecam1*
Mm04214669 u1	*Cd99*	Mm01302896 m1	*Pgf*
Mm00486938_m1	*Cdh5*	Mm00440911_m1	*Plaur*
Mm01255578 m1	*Cflar*	Mm01236713 m1	*Plxnc1*
Mm00517812 m1	*Ctnnb1*	Mm00440992 m1	*Procr*
Mm00445552 m1	*Cxcl12*	Mm01132688 m1	*Ptn*
Mm00469712 m1	*Cxcl16*	Mm01292575 m1	*Ptprc*
Mm00494538_m1	*Dpp4*	Mm00445392_m1	*Pvrl1*
Mm00438656 m1	*Edn1*	Mm00436144 m1	*Pvrl2*
Mm00433023 m1	*Egfr*	Mm03928990 g1	*R18s*
Mm00468256 m1	*Eng*	Mm00443243 m1	*Tek*
Mm00658541 m1	*Erbb2*	Mm00446232 m1	*Tgfa*
mm00438853_m1	*F3*	Mm01178820_m1	*Tgfb1*
mm01204974 m1	*Fas*	Mm00436955 m1	*Tgfb2*
Mm00433287 m1	*Ffg2*	Mm00493681 m1	*Thy1*
Mm00438917 m1	*Ffg4*	Mm00441786 m1	*Tie2*
Mm00433291 m1	*Ffg7*	Mm00445273 m1	*Tlr4*
Mm00442795_m1	*Fgf9*	Mm00443258_m1	*Tnf*
Mm01269930 m1	*Fgfr2*	Mm00441875 m1	*Tnfrsf1*
Mm00438980_m1	*Flt1*	Mm01263821_m1	*Tnfrsf25*
Mm00433382 m1	*Fzd4*	Mm01320970 m1	*Vcam1*
Mm01235633 m1	*Gata6*	Mm01281447 m1	*Vegfa*

Table 2. Prospective gene names and assay IDs for single-cell gene expression analyses of adipose and bone-marrow derived progenitor cells. Genes specifically relating to tissue regeneration, cell stemness, survival, and proliferation were chosen, in addition to selected control and surface marker related probes. Genes were cross-verified with differentially expressed genes from previous single cell studies.

Our future goals for this aim involve completing single-cell transcriptional analysis utilizing the data generated from our microfluidic RT-PCR assays. The obtained data will provide valuable insight into key differences in functionality between progenitor cell subpopulations from the different experimental groups. We intend to repeat an additional iteration of this process in wounded animals sustaining ischemic flaps to further determine whether proliferation, recruitment, and activation of these adipose and bone-marrow derived progenitor populations are also impaired in *Glo1* KD animals compared with controls.[6]

Specific Aim 2:

To prevent diabetes-induced defects in progenitor cell populations by *Glo1* overexpression

Results:

To better assess the impact of progenitor cell dysfunction reversal, two commercially available transgenic mouse strains, cre-CAG and cre-Prrx1 were acquired and bred with floxed *Glo1* overexpression strains. The resultant crosses yield one strain with a *Glo1* cartridge downstream of a ubiquitous beta-actin promoter and another strain with a *Glo1* cartridge downstream of a mesenchymal-specific Prrx1 promoter. These two strains represent a global, constitutively overexpressed *Glo1* phenotype and a mesenchymally overexpressed *Glo1* phenotype, respectively. Moving forward, these two models will serve to further address the utility of local and systemic overexpression of *Glo1* in ameliorating or reversing deleterious effects of methylglyoxal on progenitor cell populations.[7,8]

SCTA of bone marrow and adipose progenitor populations from the same workflow and 96-element gene-list outlined in Specific Aim 1 will demonstrate the degree of progenitor cell population depletion and dysfunction in an overexpressed animal. We would expect to see a recovery of both cell numbers and functionality as overexpression of *Glo1* increases. Our central hypothesis posits that amplifications in methylglyoxal catabolism will actively decrease oxidative load and protect progenitor cells.

Specific Aim 3:

To reverse longstanding defects in diabetic progenitor cell populations by controlled *Glo1* overexpression

Results:

Experimental studies to assess stability of a tamoxifen inducible *Glo1* overexpression downstream of a b-actin promoter are in progress. Upon successful crossing and genotyping of these offspring, we will begin initiating a high-fat diet in these animals to recapitulate a type-2 diabetes phenotype.

Publications:
None

Abbreviations:
Glo1 – glyoxalase 1
FACS – fluorescence activated cell sorting
BM – Bone marrow
KD – knockdown
WT – wild-type
HFD – high-fat diet

References:

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2. Januszyk M, Sorkin M, Glotzbach JP, et al. Diabetes irreversibly depletes bone marrow-derived mesenchymal progenitor cell subpopulations. Diabetes 2014;63:3047-56.

3. Giacco F, Du X, D'Agati VD, et al. Knockdown of glyoxalase 1 mimics diabetic nephropathy in nondiabetic mice. Diabetes 2014;63:291-9.

4. Rodrigues M, Wong VW, Rennert RC, Davis CR, Longaker MT, Gurtner GC. Progenitor Cell Dysfunctions Underlie Some Diabetic Complications. The American journal of pathology 2015;185:2607-18.

5. Rennert RC, Sorkin M, Januszyk M, et al. Diabetes impairs the angiogenic potential of adipose-derived stem cells by selectively depleting cellular subpopulations. Stem cell research & therapy 2014;5:79.

6. Suga H, Rennert RC, Rodrigues M, et al. Tracking the elusive fibrocyte: identification and characterization of collagen-producing hematopoietic lineage cells during murine wound healing. Stem cells 2014;32:1347-60.

7. Brouwers O, Niessen PM, Miyata T, et al. Glyoxalase-1 overexpression reduces endothelial dysfunction and attenuates early renal impairment in a rat model of diabetes. Diabetologia 2014;57:224-35.

8. Berner AK, Brouwers O, Pringle R, et al. Protection against methylglyoxal-derived AGEs by regulation of glyoxalase 1 prevents retinal neuroglial and vasodegenerative pathology. Diabetologia 2012;55:845-54.