Diabetic Complications Consortium

Application Title: iPS therapy of erectile dysfunction in a rat model of diabetes and obesity

Principal Investigator: Nestor Gonzalez-Cadavid

1. Project Accomplishments:

In the first eight months of work, we have found that:

1) Adult stem cells (muscle derived stem cells or **MDSC),** isolated from young Zucker **(OZ)** rats not yet exhibiting manifestations of type 2 diabetes **(T2D)** or obesity, and implanted into the penile corpora cavernosa of older OZ rats with long-term T2D and morbid obesity, improved erectile dysfunction **(ED)**, specifically corporal veno-occlusive dysfunction **(CVOD)**, and counteracted the underlying penile corpora cavernosal smooth muscle cell **(SMC)/**collagen decrease and fat infiltration, and upregulated nNOS. However, the MDSC isolated from these long term T2D rats acquired an inflammatory/profibrotic/oxidative/dyslipidemic transcriptional phenotype and failed to repair the corporal tissue, thus implying that they are imprinted by the T2D hyperglycemic/dyslipidemic milieu with a noxious phenotype associated with an impaired tissue repair capacity.

2) Induced pluripotent stem cells **(iPS)** obtained from mouse fibroblasts generated with four plasmids implanted into the corpora cavernosa of immunosupressed rats subjected to bilateral cavernosal nerve resection **(BCNR)** as a model of ED subsequent to radical prostatectomy, improved CVOD, the corporal SM/endothelium tissue, and the expression of neural markers, but the induction of myostatin expression may constitute a side effect that should be targeted by an anti-myostatin expression.

3) The OZ-derived MDSC and the iPS incubated by themselves or in dual cultures with penile stem cells **(PSC)** suggest that: 1) hyperglycemia alone did not cause in vitro in general any significant change in differentiation markers expression of the MDSC, exposed or not to T2D, or in the iPS; 2) there was no cross-talk with PSC; 3) however, the T2D serum induced a considerable fat infiltration and interference with MDSC, but not iPS proliferation and differentiation.

4) As a side work the project contributed to finalize a study of MDSC on another mouse model of T2D used for studying the effects of stem cell therapy for critical limb ischemia, where myostatin overexpression by the MDSC may limit their effectiveness.

These results have provided the framework to compare, as the project moves along, the in vivo effects of iPS implanted onto long-term T2D OZ rats in the presence or absence of glycemic control, the in vitro confirmation of the putative resistance of iPS to the T2D milieu, and the determination of what dyslipidemic factors are responsible, by themselves or requiring concurrent hyperglycemia, for damaging the MDSC exposed long-term to T2D

2. Specific Aims:

This project was awarded on 10/15/14, but because of delays with the IACUC approval and the administrative entry into the LABioMed system, it could only start on 03/01/15.

Therefore, this progress report represents only 8 months of work. Because of this situation and some technical difficulties, we are requesting hereby a one year no-cost extension :

Sub-aim a. *To determine whether intracorporal implantation of OZ rats with iPS ameliorates ED and the underlying histopathology of the penile corpora cavernosa induced by T2D and obesity*.

Results. The grant contributed to investigate this sub-aim in two different ways. In the first approach, since the iPS were assumed to be more resistant to the deleterious effects of T2D than the MDSC that we had used in other models of ED, a basic experiment was conducted with these cells to use them as reference for the iPS. This aimed to define the effects of MDSC implanted in the corpora cavernosa of the male obese Zucker fa/fa **(OZ)** rat model with ED, to determine their effects on erectile function and the underlying corporal histopathology. We also studied whether the stem cell tissue repair capacity on the host animals was impaired by the long-term impact of the T2D environment in the donor animals. This work was completed and led to paper 1 that has been currently resubmitted to satisfy some reviewers concerns.

Specifically, in relation to this project, we studied whether: a) MDSC from T2D OZ rats at an early stage of diabetes and no overweight **(ED-SC),** counteract corporal veno-occlusive dysfunction **(CVOD)** and corporal SMC loss/lipofibrosis when implanted in the OZ rats at a late stage of diabetes and severe obesity; b) MDSC from these late diabetes OZ rats **(LD-SC)** differ from ED-SC in their gene transcriptional phenotype and repair capacity. ED-SC and LD-SC were compared by DNA microarray assays, and ED-SC were incubated in vitro under hyperglycemic conditions **(ED-HG-SC).** These three MDSC types were iinjected into the corpora cavernosa of late diabetes OZ rats **(OZ/ED, OZ/LD, and OZ/ED-HG** rats respectively). Untreated OZ **(OZ/UT)** and non-diabetic Lean Zucker **(LZ/UT)** rats were controls. Two months later, rats were subjected to cavernosometry and the penile shaft and corporal tissues were used for histopathology and transcriptional assays. The implanted ED-SC and ED-HG-SC, improved CVOD, counteracted the corporal SMC/collagen decrease and fat infiltration in long-term T2D rats, and upregulated nNOS. LD-SC acquired an inflammatory/profibrotic/oxidative/ dyslipidemic transcriptional phenotype and failed to repair the corporal tissue. This implies MDSC are imprinted by the hyperglycemic/dyslipidemic milieu with a noxious phenotype associated with an impaired tissue repair capacity. T2D-impacted stem cells may lack tissue repair efficacy as autografts, and should either be reprogrammed in vitro, or substituted by stem cells from allogeneic non-diabetic sources.

Our second approach was to define the effects of the iPS on erectile function and the underlying histopathology in a non-diabetic rat model of ED (paper 3). Specifically, in relation to this project, we obtained and propagated mouse fibroblasts generated with the mfiPS clone by transfection with a polycistronic plasmid for Oct4, Sox2, Klf4, and c-myc, without transgene genomic insertion (inconsequential myc risks). mfiPS were cultured on mouse embryonic feeder layer (FL) in KO-DMEM. As a preliminary approach we studied whether iPS can counteract neuropraxia-induced CVOD and the underlying corporal SMC and neural histopathology in a rat model of BCNR subjected to immunosuppression. Rats were implanted into the corpora cavernosa with FL/KO-DMEM mfiPS (BCNR-iPS), and compared at 2 months with untreated controls (BCNR-UT). CVOD was determined by cavernosometry. The underlying corporal histopathology was defined by histochemistry, western blot and ad-hoc assays

We found that the mfiPS (- FL/DMEM) cross-talked with PSC, upregulating Oct-4 (stem cells) and the calponin/ACTA2 ratio (SMC/myofibroblasts ratio), but down-regulating nanog

(stem cells), the individual ACTA2 and calponin, and CD31 (endothelium). Erectile function in BCNR-iPS, versus BCNR-UT, was normalized via increasing the papaverine response and reducing the drop rate, presumably by the increase in the corporal calponin/Acta2 ratio, and eNOS (endothelium) and the reduction of collagen (hydroxyproline). Neurogenesis markers, such as NF70, nNOS and PnNOS, were up-regulated. Myostatin, the main inhibitor of striated muscle mass, and also a pro-fibrotic effector that we found in another study that was expressed in the corpora cavernosa, was upregulated by the iPS implantation, as had also been observed with MDSC. Therefore, iPS implanted into the corpora of BCNR rats improve CVOD, the corporal SMC/endothelium, and the expression of neural markers, but the induction of myostatin expression may constitute a counteracting side effect that should be reduced by an anti-myostatin expression.

Budget limitations do not allow to test in vivo findings that were not predicted in the project, i.e., the combination of an antimyostatin approach with the iPS implantation, or the effects of T2D on the donor fibroblasts from the OZ rat that would generate the autologous iPS. However, with the results so far obtained for this sub-aim it will be possible to conduct the iPS treatment of the OZ rat, as planned, but considering in the interpretation of the future results the future directions.

Sub-aim b. *To determine whether concurrent glycemic control in the host may reduce the noxious effects of T2D on the iPS once implanted;*

Results. This experiment has not yet been initiated because it is an integral part of the design originally proposed, and will be performed as planned

Subaim c. *To determine whether issue repair by iPS occurs in part by their cross-talk with endogenous PSC, leading to a differentiation of both types of stem* cells *and/or paracrine modulation of differentiated cells, that may be impaired by hyperglycemia/dyslipidemia.*

Results. The PSC were obtained initially from Sprague Dawley rats, because all the OZ rat penises were used for the experiments above, but if possible we will repeat some key experiments with PSC from OZ rats. Cultures of the iPS, together with the ED-SC, LD-SC, and ED-HG-SC from the OZ rats in the experiments above were tested after 1 and 2 weeks incubations by western blot and immunocytochemistry (tests and assays are still are ongoing), in the following way: 1) effect of high glucose (25 mM, 450 mg/dl)) versus low glucose (5.8 mM, 100 mg/dl) on differentiation of each cell culture in specific media into SMC, adipose cells, myofibroblasts, and neural cells; 2) similar effects and tested in dual culture for paracrine crosstalk (dual culture in DMEM or KO-DMEM) with rat PSC under the influence of ED-SC or iPS, on the differentiation of PSC into various cell types; 3) effect of 0.5 to 5% serum obtained from the early and late T2D OZ rats, added to single cultures of ED-SC and iPS, on morphology, fat infiltration and differentiation.

The preliminary results (see paper 4) show: 1) hyperglycemia alone did not cause in vitro in general any significant change in the in vitro tests so far conducted with MDSC exposed or not to T2D or iPS; 2) there was no evidence in vitro of a cross-talk that would modify the expression or phenotype of the PSC under the influence of the MDSC types or iPS that were implanted in vivo; 3) however, T2D serum added to as low as 0.5% to the ED-SC induced fat infiltration in MDSC and interference with their proliferation and differentiation, whereas the

initial tests with iPS appear to indicate that they are more resistant than the MDSC, but this requires confirmation

Incubations of MDSC and iPS with a soluble cholesterol bioreagent, and saturated noxious lipids, i.e., palmitic acid and palmitate triglyceride albumin conjugates, at concentrations found in the OZ serum, alone or in combination, and either in the absence or presence of hyperglycemia, are ongoing. They hopefully will discern which is the main factor responsible for the deleterious effects of the T2D serum on MDSC, and whether iPS are resistant to this damage.

Additional approach

The grant has also partially helped with a project related to the use of another T2D/obesity animal model, the db/db mouse, on the effect of MDSC and their pharmacological modulation for the treatment of critical limb ischemia **(CLI)** (see paper 2), as a preliminary approach to the use of the same experimental design for the use of iPS. CLI is a condition with high risk of amputation and post-surgical mortality, and no effective medical treatment. Stem cell therapy, mainly with bone marrow mesenchymal, adipose derived, endothelial, hematopoietic, and umbilical cord stem cells, is promising in T2D/CLI mouse and rat models and is currently in clinical trials. Using a T2D mouse model of CLI induced by femoral artery ligation, we tested: a) the repair efficacy of MDSC implanted into the ischemic muscle and its potential stimulation by concurrent intraperitoneal administration of a long acting nitric oxide generator, molsidomine; and b) whether implanted stem cells may partially counteract their own effects by stimulating the expression of myostatin, the main lipofibrotic agent in the muscle and inhibitor of muscle mass. MDSC reduced mortality, but without improving limb preservation, and in the ischemic muscle, increased stem cell number and myofiber central nuclei, reduced fat infiltration, myofibroblast content, and myofiber apoptosis, and increased vascular and neural factors. However, the stimulation of early myofiber repair and neural factors did not lead to increased myogenesis or neurogenesis, and raised collagen deposition, probably due in part to overexpression of myostatin induced by MDSC. Supplementation of MDSC with molsidomine failed to stimulate, or directly counteracted, the beneficial effects of MDSC, with the only exception of reducing myostatin overexpression and avoiding limb loss. Molsidomine given alone was rather ineffective, except for reducing myostatin overexpression. Therefore, like for ED, follistatin administration or other anti-myostatin approaches may be used as a combination treatment to improve the effects of stem cell therapy of CLI in T2D.

3. Publications:

All the articles below, as well as any additional future one, formally acknowledge the partial funding contribution of the DIACOMP grant for some aspects of the work

Submitted papers:

1. Kovanecz I, Vernet D, Masouminia M, Gelfand R, Loni L, Aboagye J, Tsao J, Rajfer J, **Gonzalez-Cadavid NF**. Implanted muscle derived stem cells ameliorate erectile dysfunction in a rat model of type 2 diabetes, but their repair capacity is impaired by their prior exposure to the diabetic milieu. J Sex Med, to resubmit, major revision

2. Tsao J, Kovanecz I, Awadalla N, Gelfand R, Sinha-Hickim I, White R, and Nestor **Gonzalez-Cadavid NF**. Muscle derived stem cells counteract fat infiltration and stimulate the early stages of myofiber repair in diabetic muscle ischemia, in a process associated with overexpression of myostatin. J Transl Med, submitted, under revision

Papers in writing

3. Vernet D, Kovanecz I, Masouminia M, Loni L, Aboagye J, Rajfer J, Izpisua-Belmonte JC, **Gonzalez-Cadavid NF**. Induced pluripotent stem cells ameliorate corporal veno-occlusive dysfunction in a rat model of bilateral cavernosal nerve resection. To be submitted by 12/15
4. Vernet D, Masouminia M, Tsao J, Loni L, Aboagye J, Rajfer J, **Gonzalez-Cadavid NF**. Dyslipidemia, but not hyperglycemia alone, is the main factor in the impairment of the survival and differentiation capacity of skeletal muscle derived stem cells in a diabetic milieu, as compared to iPS. To be submitted by 01/16