University of Pennsylvania

Annual Progress Report: 2007 Nonformula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Nonformula Grant Overview

The University of Pennsylvania received $3,889,058 in nonformula funds for the grant award period June 1, 2008 through May 31, 2012. Accomplishments for the reporting period are described below.

Research Project: Project Title and Purpose

Penn Center of Excellence for Regenerative Medicine - The purpose of this project is to establish a Penn Center of Excellence for Regenerative Medicine at the University of Pennsylvania with collaborative partners across eastern Pennsylvania. A programmatic effort to find new treatments for diabetes will be funded involving basic, translational and clinical investigation at three institutions. There will also be an innovative program to develop a pipeline of new scientists and clinicians in the Commonwealth which will involve outreach in the Philadelphia public schools, Haverford College and Lincoln University.

Anticipated Duration of Project

6/1/2008 - 5/31/2012

Project Overview

The research Specific Aims involve:
A Research Program to develop regenerative therapies for diabetes including clinical, translational and basic studies performed by a collaborative team of investigators at three institutions within the Commonwealth. The project simultaneously advances multiple approaches to encourage islet cell growth and regeneration. Specific aims are:

Aim 1: Determine if GLP-1 sustains or increases functional ß-cell mass in humans in vivo.
Aim 2: Determine if GLP-1 receptor agonists stimulate human ß cell growth.
Aim 3: Determine whether systemic Bone Morphogenetic Protein (BMP) regulates rodent and human ß cell growth.
Aim 4: Determine whether adenoviral over-expression of GDF3 can be exploited to stimulate human ß cell proliferation.
Aim 5: Characterize endothelially derived secreted factors for their potential to improve outcome in human islet transplantation.

Principal Investigator

John Gearhart, PhD
Professor, Department of Cell and Developmental Biology
Director, Penn Institute for Regenerative Medicine
University of Pennsylvania
1151 BRB II
421 Curie Blvd.
Philadelphia, PA 19104-6058
(215) 746-3458

Other Participating Researchers

Jonathan Epstein, MD, Martin Carroll, MD, Klaus Kaestner, PhD, Daniel Kessler, PhD, Edward Morrisey, PhD, Ali Nagi, MD, Michael Rickels, MD, Doris Stoffers, MD, PhD, Robert Gallop, PhD, Kenneth Zaret, PhD, and Jamie Shuda, M Ed - employed by University of Pennsylvania
John Chikwem, PhD - employed by Lincoln University
Stephen Emerson, MD, PhD - employed by Haverford College
Jake Kushner, MD - employed by Children's Hospital of Philadelphia

Expected Research Outcomes and Benefits

The Penn Center of Excellence for Regenerative Medicine will produce both short term and long term benefits. The Center will allow outstanding scientists, educators and clinicians to leverage the power and potential of stem cells and regeneration to develop new therapies. The program stresses collaboration and will promote new research and translational projects with an explicit mandate to obtain durable alternative funding to provide sustained progress in the Commonwealth. The Center will specifically promote promising new approaches to the treatment of diabetes by funding a clinical trial to test a new medication's ability to increase the production of insulin in patients. At the same time, the development of other medications and treatments to promote insulin production from regenerating pancreas cells will be pursued in the laboratory. A world-class group of clinicians and scientists has joined together to attack this vital problem and will synergize to develop new therapies. Cutting edge work being done at the University of Pennsylvania in the area of islet cell transplantation will make it possible for human insulin-producing cells to be provided to the collaborating group of scientists, thereby providing a unique and valuable resource to regeneration scientists to test translational therapies.

Summary of Research Completed

Aim 1: Determine if GLP-1 sustains or increases functional ß-cell mass in humans in vivo (Rickels).
This aim involves the conduct of a randomized clinical trial where patients with a fasting glucose 110 – 159 mg/dl off diabetes therapy receive either exenatide (GLP-1 analog), sitagliptin (DPP4 inhibitor that raises levels of endogenous GLP-1) or glimepiride (sulfonylurea as active comparator) for 6 months and undergo determination of β-cell secretory capacity before and after 6 months of treatment. The sample size required to detect a difference of 1 SD in AIR_{max}

between the groups receiving exenatide or sitagliptin and the group receiving glimepiride with > 80% power at $\alpha = 0.05$ (two-tailed) is 20 subjects per group or 60 in total. In the present reporting period, the clinical trial protocol and all supporting documents underwent successful annual review and re-approved by Penn's Office of Human Research, Institutional Review Board (IRB), and Clinical & Translational Research Center (CTRC) Advisory Committee.

During this year, the project made continued progress on subject recruitment:

- 200 subjects completed telephone screening interviews this year, for a study total of 573;
- 40 subjects completed in person screening interviews at our off-campus recruitment site this year, City Line Family Medicine in adjacent Montgomery County, for a study total of 80;
- 31 subjects completed the informed consent process this year, for a study total of 100 subjects;
- 15 subjects enrolled in the trial this year, compared to 12 subjects in the prior year, and 8 subjects in the year before that, for a study total of 35;
- Currently 1 consented subject is undergoing eligibility determination.

Enrollment primarily comes from continued recruitment at the Hospital of the University of Pennsylvania (HUP) that includes targeted recruitment of patients seen at the Penn Rodebaugh Diabetes Center, patients who have participated in the Penn Diabetes Heart Study, and patients responding to flyers and brochures distributed throughout the hospital and physician practices, and from City Line Family Medicine where our research nurse practitioner spends one half day per month meeting with potentially interested subjects for consent and screening. Importantly, 9 of the 40 subjects interviewed at City Line Family Medicine enrolled in the study, our most productive recruitment strategy success rate. This year we attempted to establish another recruitment site at our neighboring VA hospital, but unfortunately the regulatory burden of involving the VA proved impossible. We have recently begun advertisements in newsletters/emails that distribute to both HUP and Penn University employees, as well as posted an ad on craigslist, that to date have been very encouraging additional recruitment strategies.

While enrollment has continued to progress from 8 subjects in the first year to 12 subjects in the second year to 15 subjects in the third year, our total enrollment of 35 subjects remains 25 subjects short of 60. Nonetheless, recruitment was hindered in the last year by the replacement of the study coordinator, and by a nationwide shortage of arginine solution that is used for the baseline and final glucose-potentiated arginine tests that determine the primary outcome measure. We were first notified of the arginine shortage from our supplier (Pharmacia & Upjohn, Kalamazoo, MI) about 6 months ago, and since then had been anticipating a renewed supply on a month by month basis, during which time we had intentionally slowed recruitment to ensure that our supply would support conduct of the final study procedures in each enrolled subject. The most recent information from the company is not to expect stock until the end of September.

Therefore, we have identified an alternate supplier of arginine solution to serve as a bridge until there is resumed manufacture from Pharmacia & Upjohn. The alternate supplier is Franck's Compounding Laboratory & Pharmacy, and we are presently awaiting IRB approval of a protocol amendment to switch suppliers so our recruitment can be accelerated again. With our new study coordinator well in place, and an alternate source of arginine solution expected by the

end of this month, we are confident that we can successfully enroll the remaining 25 subjects in the last year of this project.

Of the 35 enrolled subjects, 11 are women and 15 are African American which should enable the performance of subgroup analyses with respect to gender and racial differences that examine for a potential interaction with baseline fasting glucose levels as suggested by Reviewers 3 and 6 during our Interim Performance Review. Such analyses are extremely important given that African Americans share a disproportionate burden of type 2 diabetes in the State of Pennsylvania.

We performed an interim analysis of baseline data collected through December 2010 that was included as preliminary data for an U01 application to NIDDK submitted in March 2011 in response to RFA-DK-10-013 entitled "Optimizing Recovery and Preservation of Endogenous Insulin Secretion in Individuals with Pre-diabetes or Recent Onset of Type 2 Diabetes." By the end of 2010, we had enrolled 30 subjects with baseline demographics 29% female, 36% African American, age 58 ± 9 years, BMI 32 ± 6 kg/m^2, FPG 126 ± 13 mg/dl, and HbA1c 6.4 ± 0.4% (data are mean ± SD). The median age was 57.5 years, identical to median age at diagnosis of T2D in the US. The baseline oral glucose tolerance test results are given in Figure 1, indicating that both fasting and oral glucose tolerance is at or just above diagnostic thresholds for the diagnosis of T2D, and so demonstrates that we have been successfully recruiting a cohort with early T2D.

By the end of 2010, 19 subjects had completed the study and had samples from baseline and final glucose-potentiated arginine tests assayed for insulin. The glucose-potentiated arginine test involves assessment of glucose dependent insulin responses to injection of the non-glucose secretagogue under fasting and 230 mg/dl and 340 mg/dl hyperglycemic clamp conditions and provides measures of β-cell secretory capacity, β-cell sensitivity to glucose, and insulin sensitivity. The primary endpoint for this study is β-cell secretory capacity, which has been shown to be impaired in established T2D, while a secondary endpoint is β-cell sensitivity to glucose.

To understand the relative contributions of β-cell secretory capacity and β-cell sensitivity to glucose to the impaired insulin secretion early in the course of T2D, we compared the baseline glucose-potentiated arginine test data from these 19 subjects to data from 16 normal weight nondiabetic subjects obtained in our laboratory as normal controls. Importantly, the glucose infusion rate ($M_{0\text{-}50\ min}$) required to achieve the 230 mg/dl glucose clamp was lower in early T2D than normal subjects (5.7 ± 0.2 vs. 9.1 ± 0.4 mg·kg^{-1}·min^{-1}; $p < 0.0001$), again demonstrating their impaired glucose tolerance, and this despite a trend toward greater second-phase insulin levels ($I_{40\text{-}44\ min}$: 29.2 ± 3.9 vs. 20.8 ± 2.5 µU/ml; $p = 0.091$). Consequently, the clamp-derived measure of insulin sensitivity (M/I) was reduced over 50% in T2D compared to normal (0.24 ± 0.02 vs. 0.53 ± 0.06 mg·kg^{-1}·min^{-1}/µU/ml; $p < 0.0001$), clearly demonstrating markedly impaired tissue sensitivity to insulin action present early in the course of T2D.

β-cell secretory capacity is determined as the acute insulin response to arginine injected during the 340 mg/dl hyperglycemic clamp, and was not different in early T2D compared to normal (180 ± 23 vs. 150 ± 18 µU/ml)(Figure 2, left), although the appropriate control group would be

obese nondiabetic subjects with expected supranormal secretory capacity. However, as shown in Figure 2 (right), in early T2D this reserve capacity for insulin release requires higher plasma glucose levels than in normal subjects, such that the PG_{50}, the plasma glucose level at which a half-maximal insulin response is achieved, is right-shifted (201 ± 14 vs. 155 ± 10 mg/dl; p = 0.013), indicating an impairment in β-cell sensitivity to glucose early in the course of T2D, and as a secondary endpoint the present study we will be able to determine if a GLP-1 based treatment strategy may improve this β-cell defect compared to an effect of sulfonylurea therapy.

Aim 2: Determine if GLP-1 receptor agonists stimulate human ß cell growth (Stoffers)
During the current funding period we focused our efforts on bringing our discovery of the effect of calcineurin inhibition on human beta cell survival and the ability of the GLP1 receptor agonist Exnedin-4 to reverse the toxicity to publication. This work was recently published in the *Journal of Biological Chemistry* (Calcineurin signaling regulates human islet {beta}-cell survival. Soleimanpour SA, Crutchlow MF, Ferrari AM, Raum JC, Groff DN, Rankin MM, Liu C, De León DD, Naji A, Kushner JA, Stoffers DA. J Biol Chem. 2010 Dec 17;285(51):40050-9).

The species-specific differences of our findings further reinforced the need to translate findings from rodent models to human islets. Given the species-specific effects of calcineurin inhibition on cell survival, we examined expression of Akt isoforms in both rodent and human islets. Although all three Akt isoforms are expressed in both rodent and human islets (Figure 3), studies in genetic mouse models show distinct effects of each isoform on the regulation of glucose tolerance. Comparison of the relative mRNA expression of these isoforms between rodent and human islets showed a significant reduction in the relative levels of Akt2 in human islets.

To examine the effect of calcineurin inhibition on human beta cell survival *in vivo* in the transplant model, we transplanted 3,000 human IEQ under the kidney capsule of NOD/SCID/IL2G KO mice (also known as NSG or NOG). Stable engraftment was verified by human specific serum C-peptide concentrations determined by ELISA on day 72 post-transplant. The transplanted mice had subcutaneous vehicle or tacrolimus pellets (1.0 mg/kg/d) placed (n=3 Vehicle group, n=4 Tacrolimus group). Random blood glucose measurements were performed at baseline and following pellet implantation over 14 days. Human and mouse specific serum C-peptide concentrations were measured at baseline and on day 14 post-pellet implantation.

As shown in Figure 4 (upper panel), tacrolimus rapidly caused the development of hyperglycemia. C-peptide levels secreted by both the host (mouse) and the transplanted islets (human) were similarly and significantly reduced (Figure 4, lower panel). Next we submitted the harvested graft-containing kidneys for embedding and sectioning, followed by TUNEL staining. Unfortunately, despite the increased numbers of human islets transplanted under the kidney capsules of these mice compared to previous experiments, we were not able to count sufficient beta cells to obtain a meaningful cell death rate.

Aim 3: Determine whether systemic Bone Morphogenetic Protein (BMP) regulates rodent and human ß cell growth (Kushner)
During the current funding period we focused on Aim 3. In this aim we hope to test if BMPs promote ß-cell growth in cultured human islets. This approach involves quantification of ß-cell turnover of cultured islets, which is currently very difficult to do in an objective manner.

Consequently, we have developed a high-throughput model to measure ß-cell turnover of islets in culture. Our system uses Volocity Acquisition and an automated X-Y stage to image >30,000 ß-cells per hour, which vastly exceeds our capacity to count ß-cells by hand. As a result, we have adopted the PerkinElmer Volocity Analysis platform to quantify ß-cells *in situ* in order to more accurately and efficiently analyze ß-cell turnover. We wrote a ~30 step Volocity quantification algorithm for high-throughput image analysis of ß-cells. Our algorithm uses successive filters and image overlap, and allows for high-speed quantification of ß-cells and simultaneous detection of other ß-cell antigens. This image processing protocol provides unprecedented capacity to quantify ß-cell turnover, allowing us to quantify huge data sets in a single day. Our approach will allow high-resolution quantification of ß-cell turnover of islets in situ or in culture. This technology was used in collaborative studies with the Stoffers group (Aim 2), and was published as part of her manuscript in the *Journal of Biological Chemistry.* With the support of this grant, we also wrote and submitted a manuscript detailing the methods required to perform our thymidine analogue labeling. This manuscript was published in *JOVE* in 2010. Finally, we have carried out a detailed analysis of gene expression changes in islets in response to regenerative stimuli, in response to aging, or both. This manuscript was published in *Islets* in 2010.

Aim 4: Determine whether SOCS2 and CISH regulate human beta cell growth (Kaestner)
Since the last progress report, we have transduced human cadaveric pancreatic islets with two lentiviral vectors for shRNA mediated suppression of either SOCS2 and CISH expression in beta-cells. The transduced islets were then transplanted under the kidney capsule of streptozotocin-treated NOD-SCID mice. After surgery, the mice were given BrdU in the drinking water for continuous labeling of replicating cells. Four weeks later, the mice were sacrificed, the kidneys harvested and processed for immunofluorescence staining for insulin and BrdU. Control mice were treated with a lentivirus encoding a scrambled shRNA. For both SOCS2 and CISH suppression, we observed multiple BrdU-positive beta-cells, which were not seen in the control animals. Quantification of the percentage of prolfierating beta-cells is in progress.

In order to be able to assess the loss-of-function phenotypes for CISH and SOCS2 for beta-cell proliferation *in vivo*, we are also employing mice with targeted mutations in either gene. For the derivation of a conditional allelee for SOCS2 we have obtained a targeting vector constructed by the EUCOMM consortium and will use this to transform mouse embryonic stem cells for targeted gene replacement. Because CISH null mice are embryonic lethal, we have constructed a conditional allele for the gene, as shown in Figure 5. In order to assess the role of CISH in the regenerative response of the beta-cell *in vivo*, we have constructed a targeting vector for a conditional (loxP) null allele of the gene and successfully performed gene targeting in ES cell lines from C57BL6 mice. These mice have been bred to Pdx1CreERT2 mice for conditional ablataion of the CISH gene in mature beta-cells.

Aim 5. Characterize endothelially derived secreted factors for their potential to improve outcome in human islet transplantation (Zaret)
We had originally proposed to screen, in year 4 of the grant, for new molecules that play a role in endocrine and beta cell development, and we are completing the study now. We had discovered that netrin-4 promotes the early differentiation of pancreatic beta cell progenitors in mouse

development, and further found an inhibitory role for DCC, a netrin receptor. However, despite that DCC can bind netrin-4, there is dispute in the literature about whether DCC is the biologically relevant receptor for netrin-4. New data in the field demonstrate that neogenin can serve as a receptor for netrin-4. In last year's report, we showed that neogenin is not expressed in the adult islet. However, we hypothesized that neogenin may be important in embryonic development of the beta cells, and therefore in the past year investigated the expression and function of neogenin, and its cross-regulation with DCC.

We found that early mouse embryos, at embryonic day of gestation 8.5 (E8.5), do not express neogenin (data not shown), but by E9.5, neogenin is induced in the pancreatic endodermal epithelium and in the local mesenchyme (Figure 6). However, the expression of neogenin is remarkably brief, in that by E10.5, neogenin expression is absent in the Pdx1+ pancreatic bud and nearby embryonic domains (Figure 6). Note positive green signals at E10.5 are due to autoflourescent blood cells. These data are consistent with the possibility that initially, at E9-9.5, neogenin functions positively to respond to the netrin-4 cue. We know that the positive effect of netrin signaling is brief, and ends by E10.5, whereupon the DCC netrin receptor functions to inhibit endocrine and beta cell development. We therefore examined neogenin expression in DCC homozygous null embryos, which, from E10.5 onwards, exhibit larger pancreas buds, then larger pancreases and more beta cells. Remarkably, we observed a huge induction of neogenin expression in the DCC -/- embryos at E10.5 (Figure 6). This unexpected result has led to a new hypothesis for the dynamic effects of netrin signaling in early pancreas development: Netrin-4 initially and briefly functions positively, via neogenin, and then its effects are suppressed by DCC, by repressing neogenin expression. Thus, DCC suppresses the extent of pancreas and beta cell development. In the absence of DCC, neogenin increases and positively stimulates beta cell formation. In the coming year, we will test these hypotheses.

Core A: Clinical Core (Naji). The human islet isolation core remains committed to providing highly purified human islets to this program. During the previous funding period, the core provided critical advice on experimental design to the investigators and human islets for *ex vivo* studies and for transplantation into diabetic NSG recipient mice.

Core B: Xenotransplantation Core (Carroll). Core B continued to work with program investigators and Core A to develop the *in vivo* model for islet transplant. We transplanted greater numbers of human islets than before (3,000 human islets/mouse) and experimented with withholding the streptozotocin induction of diabetes prior to transplant to avoid potential toxic effects to the host.

Education

Student Internships

Between July 2010 and June 2011, we awarded 9 Center of Excellence Research Internships. In addition, another 6 undergrads (some only recently graduated from High School) who did not qualify for the "Center of Excellence Research Internship", but who still had an interest in stem cell biology or regenerative medicine, were awarded "IRM Summer Internships *for* Undergrads".

Due to the overwhelming response of high school students who wanted to apply to the internship program, we restricted this year's applications to those students who would be seniors in the Fall of 2011 and awarded another 5 "IRM Summer Internships *for* High School Seniors".

Haverford College: This past year we offered two education/research opportunities supported by the State of PA. As in the past, the great majority of the students taking advantage of these opportunities are now studying and/or researching in biomedical research, at the University of Pennsylvania, MIT, CalTech, Columbia, Stanford, Duke, MSK Institute and other major research institutions. 1) Biol 363 Senior Research Seminar/Tutorial in Stem Cell Biology: This research literature based course enrolled 14 students, and explored classic and recent papers in stem cell purification, receptors, niches, cancer stem cells, tissue engineering, and adult pluripotent stem cells. 2) Biol 412 Senior Research in Stem Cell Biology: Two senior students, Susan Weiner and Yusup Shin, completed research theses with the partial support of this grant. Yusup Shin: *PolyIC induces late-stage apoptosis in hematopoietic cells in a Sca-1 associated manner that is antagonized by Bcl-2 expression.* A manuscript based on Yusup's thesis is now being prepared for submission to Blood. Yusup is now working as a Research Assistant at Memorial Sloan Kettering Institute, and will be applying to M.D.-Ph.D. programs next year. Yusup was the first Center of Excellence Research Intern. Susan Wiener: *TAT-Mediated NF-Ya Protein Transduction to Encourage the Differentiation of Human Embryonic Stem Cells into Hematopoietic Stem Cells*. Susan's research, the first undergraduate research on hESCs and IPSCs at a liberal arts college, is now being extended by one of this year's rising seniors, through her summer research. Susan was also a co-author on a book chapter on TAT-transduction in human hematopoietic stem cells that will be published later in 2011. She will continue it through the year. Susan is currently working as a Research Assistant at the Children's Hospital of Philadelphia, and will be applying to medical school within the next two years.

Lincoln University: The SCBRM course has been developed. The laboratory component of the course has also been developed and resources allocated that will enable students to have a hands on and practical lab experience. Two students are currently participating in a summer internship at the University of Pennsylvania.

Project BioEyes/Bridge to ReBIO: In addition to teaching Project BioEyes in Philadelphia schools and again mentoring high school students for their science fair projects (Bridge to ReBIO), high school students, teachers, and Penn mentors from the Bridge to ReBIO program participated in a round-table discussion about science outreach, as part of the education session at this year's Mid-Atlantic Society for Developmental Biology Meeting, hosted by Penn on June 3rd-5th. Panelists and developmental biologists from around the region discussed the benefits and challenges involved with creating effective science outreach projects, while emphasizing the importance of such outreach work to both the public and to scientists. High school students participating in the conference then stayed to present their Bridge to ReBIO projects at the meeting's poster session.

FIGURES AND TABLES:

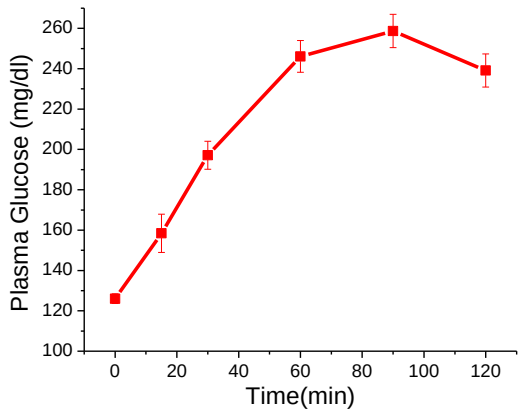

Figure 1. Plasma glucose in response to ingestion of 75 g glucose at t = 0 min in our cohort enrolled as of December 2010 (n = 30) with prediabetes or early T2D.

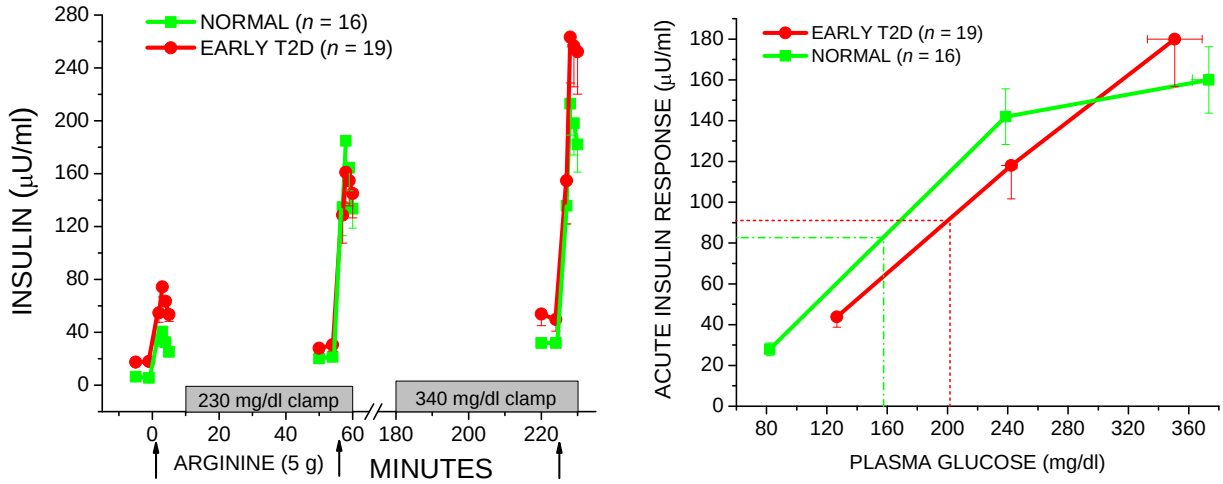

Figure 2. Left, plasma insulin in response to intravenous injection of arginine under fasting, 230 mg/dl and 340 mg/dl hyperglycemic clamp conditions, demonstrating relatively preserved β-cell secretory capacity in early T2D relative to normal weight nondiabetic controls. Right, the acute insulin response to arginine as a function of the prestimulus plasma glucose level, indicating a right-shift in PG_{50}, a measure of β-cell sensitivity to glucose, in early T2D compared to normal (201 ± 14 vs. 155 ± 10 mg/dl; p = 0.013).

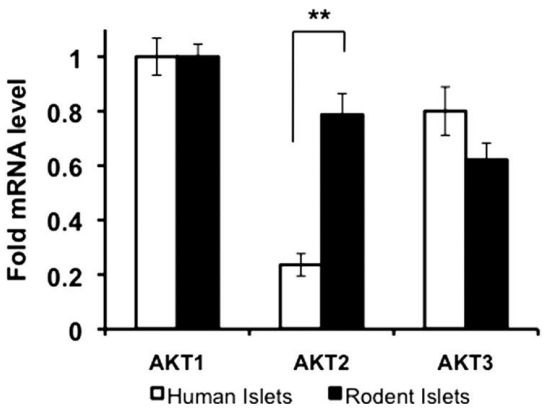

Figure 3. Quantitative PCR analysis of Akt isoform mRNA expression in cultured human and rat islets. Isoform expression is presented relative to Akt1 mRNA levels. Data are presented as mean ± S.E. (*error bars*) of three independent rat islet preparations and four independent human islet preparations (each analyzed in triplicate). *, $p < 0.05$; **, $p < 0.01$.

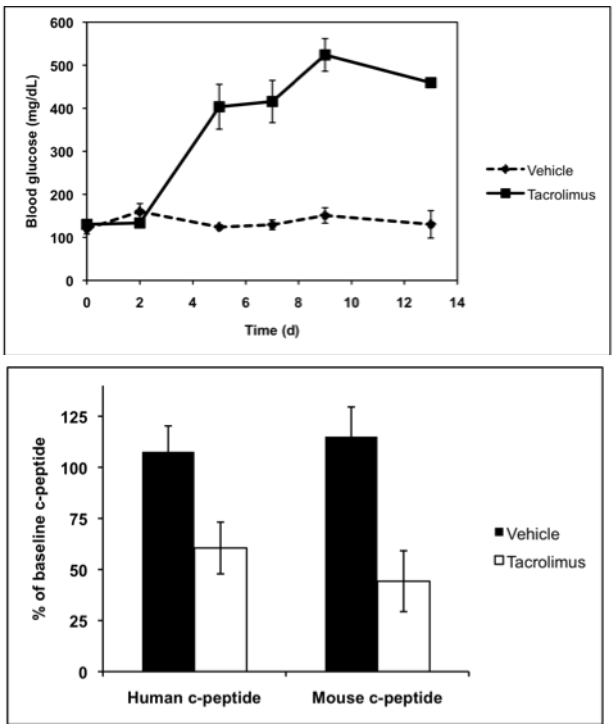

Figure 4. Upper panel, Random blood glucose levels in vehicle and tacrolimus treated NOG mouse recipients of 3,000 human IEQ under the kidney capsule. *Lower panel*, human- and mouse-specific serum C-peptide concentrations measured at baseline and on day 14 post-pellet implantation, determined by ELISA and compared to levels on day 72 post-transplant.

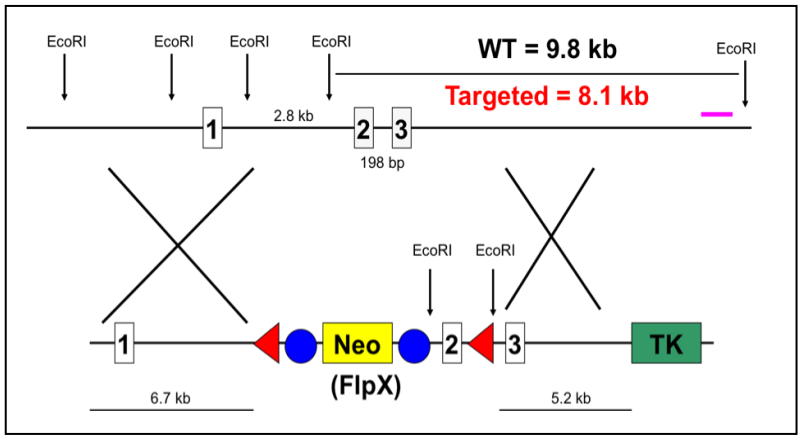

Figure 5. Targeting strategy for the derivation of a CISH^{loxP} allele. The neomycin resistance cassette (Neo, in yellow) has already been removed through expression of Flp-recombinase.

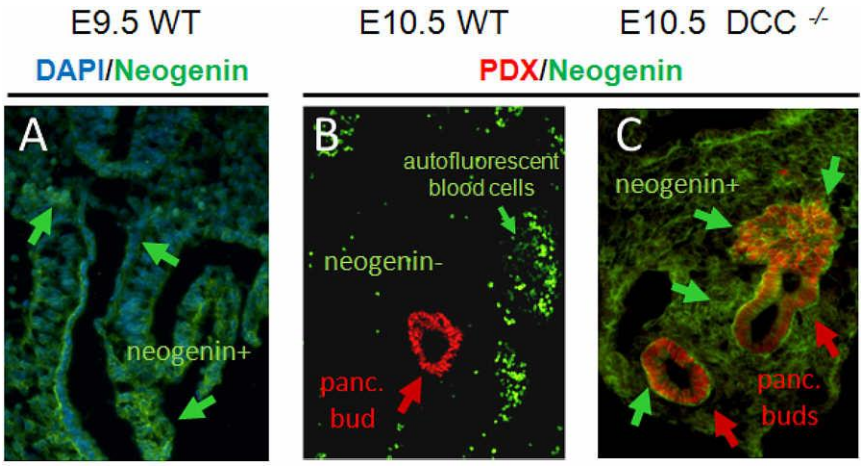

Figure 6. Neogenin expression in the developing pancreas of wild-type and *DCC*$^{-/-}$ mice