# University of Pennsylvania

# Annual Progress Report: 2007 Nonformula Grant

**Reporting Period**

July 1, 2009 – June 30, 2010

**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)*
During this year, the project emphasis was on subject recruitment. 276 subjects completed telephone screening interviews, compared to 97 subjects in the prior year. 30 subjects completed in person screening interviews at our new off-campus recruitment site, City Line Family Medicine in adjacent Montgomery County. 37 subjects completed the informed consent process,

compared to 32 subjects in the prior year. 12 subjects were enrolled, compared to 8 subjects in the prior year. Presently 6 consented subjects are undergoing eligibility determination.

While enrollment has progressed from 8 subjects in the first year to 12 subjects in the second year, our total enrollment remains behind our target of 30 subjects by this point in time. The upper limit of our entry fasting glucose criteria was conservatively set at 149 mg/dl to ensure that few if any subjects would be dropped for inadequate glucose control during the course of the study. In fact, glucose control has been excellent in all subjects, and we presently have submitted a protocol modification to our IRB and CTRC Advisory Committee to raise the upper limit of the entry fasting glucose to 159 mg/dl, which will certainly facilitate enrollment.

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 potential subjects for consent and screening. Importantly, 7 of the 30 subjects interviewed at City Line Family Medicine enrolled in the study, our most successful recruitment strategy success rate. Attempts at targeted recruitment of patients seen at primary care practices of HUP and Presbyterian Medical Center identified through an IRB approved database (PICARD) search, and of patients seen at Penn Endocrinology at Pennsylvania Hospital have not been very successful. We are working to establish a second site for conducting in-person interviews at primary care and diabetes clinics of the VA Medical Center located next door to Penn in Philadelphia. We will continue to explore and implement new strategies to increase our enrollment rate to > 2 subjects per month in the coming year.

Of the 20 enrolled subjects, 5 are women, 10 are African American. Mean ± SE age is 58 ± 2 years, BMI 34 ± 1 kg/m$^2$, fasting glucose 126 ± 3 mg/dl, 2-hour oral glucose tolerance 228 ± 9 mg/dl, and HbA1c 6.3 ± 0.1 %. Importantly, we have maintained a 50/50 split of African American and Caucasian enrollees that should enable the performance of subgroup analyses with respect to racial differences to examine for potential interaction with baseline fasting glucose levels. Such analyses are extremely important since African Americans share a disproportionate burden of type 2 diabetes in Pennsylvania. The baseline oral glucose tolerance test data are given in Fig 1.

*Aim 2: Determine if GLP-1 receptor agonists stimulate human ß cell growth (Stoffers)*
We focused on translation of our findings in murine models to human islets, thereby completing Subaim A of the proposal. Given the association of tacrolimus (Tac) with post-transplant diabetes in humans, we sought to determine how calcineurin inhibition impacts human ß-cell survival and replication. Human islets obtained from the Clinical Core were treated with Tac or vehicle for 48 hours. Tac increased human β-cell apoptosis by 3.1-fold (Fig. 2) with a similar trend in non-β-cells that was not statistically significant (not shown). Ex-4 could partially reverse this effect, improving cell survival by > 2-fold.

We also examined BrdU incorporation as a measure of β-cell replication in human islets treated with vehicle, Tac, and/or Ex-4. Following 24 hours of BrdU labeling, we found rare BrdU-

labeled non-β-cells; however, not one BrdU-labeled β-cell was identified despite counting nearly 100,000 human β-cells among the 3 donors (not shown). These data may reflect the failure of β-cell replication to occur in the *ex vivo* setting and are consistent with the low levels of β-cell proliferation previously reported in adult humans. Taken together, these results demonstrate the deleterious effects of calcineurin inhibition on human β-cell survival.

Akt, also known as protein kinase B (PKB), is a serine–threonine kinase that acts as a major effector of the Irs/PI3K/Akt pathway to coordinate growth factor signaling. Genetic mouse models support the regulation of β-cell replication, survival and function by this pathway. Tac treatment of INS-1 cells reduced Akt phosphorylation, whereas total Akt was unaffected (not shown). Thus, calcineurin inhibition may affect β-cell survival and replication in part through regulation of the PI3K/Akt pathway.

We previously observed that calcineurin regulates Irs2 in a rodent beta cell line and in primary islets. Insulin receptor substrate-2 (Irs2) is a critical adaptor in the PI3K/Akt pathway. Further, Irs2 is a cAMP-responsive element binding protein (CREB) target gene and a target of the calcineurin-dependent CREB co-activator TORC2 in hepatocytes. We observe a reduction in Irs2 mRNA and protein levels in human islets (Fig. 3). Importantly, Ex-4 normalized the expression of IRS2 in Tac-treated human islets. Finally, to determine whether calcineurin could regulate Irs2 via its well known effector NFAT, chromatin immunoprecipitation (ChIP) assays were performed that demonstrated endogenous NFATc1 occupancy of both regions in Min6 cells, which was abrogated by Tac (not shown). Thus, we identify Irs2 as a target of calcineurin, likely mediated by direct occupancy by NFAT.

The species-specific effect on islet cell survival could be due to differences in the response of human and rodent islets to reductions in Irs2 expression. Alternatively, calcineurin inhibition may lead to Irs2-independent effects that differ between humans and rodents. Human and rodent islets, despite similarities in insulin release to secretagogues, vary in terms of histological composition, basal levels of replication, glucose transporter gene expression, and D-cyclin/cyclin-dependent kinase gene expression. *The species-specific differences of our findings further reinforce the need to translate findings from rodent models to human islets.*

*Aim 3: Determine whether systemic Bone Morphogenetic Protein (BMP) regulates rodent and human ß cell growth (Kushner)*

Our currently funded program centers around the hypothesis that Bone Morphogenic Protein (BMP) signals regulate adult ß-cell growth. BMPs are attractive candidate mitogenic signals in adult ß-cells which are particularly strategic targets for ß-cell research, as recombinant human BMP2 is now FDA approved with indications for spinal fusion and tibial fractures. Thus, our aim is to determine whether BMPs regulate rodent and human ß-cell growth in three Aims, progress on two of which will be described.

i: BMP treatment of a mouse model of type 2 diabetes. Dietary fat-induced obesity is associated with insulin resistance, ß-cell dysfunction, and increased ß-cell turnover. Male c57Bl6 mice (Jackson labs) were fed a high fat diet starting at 8 weeks of age and control mice were fed a normal diet. Blood glucose levels were tested weekly. Each month thereafter, a cohort of mice were sequentially labeled with CldU and then IdU in the drinking water for 2 weeks each,

followed by sacrifice. ß-cell proliferation and serial replication behavior were measured by CldU and IdU incorporation.

Mice tolerated 40% high dietary fat treatment. Average body weight was unchanged in each group after several weeks of treatment. Similarly, average blood glucose values were unchanged. Glucose tolerance tests were unchanged in all groups (not shown). Based on the lack of diabetic response, we repeated our studies with 60% dietary fat. These studies reveal greater diabetic response, with greater body weight and moderate glucose intolerance (not shown).

Thymidine analogue labeling. After CldU and then IdU analogue labeling, pancreata were harvested, processed and sectioned for immunohistochemical analysis. Proliferation within ß-cells was measured with insulin and BrdU antisera. These studies reveal that 40% dietary fat is insufficient to increase ß-cell turnover, but 60% dietary fat induces ß-cell turnover by 25 weeks (Fig. 4). Taken together, these studies reveal that 60% dietary fat is required to induce a diabetic state and increased ß-cell turnover. We now plan BMP studies with 60% fat induced diabetes.

<u>iii: BMP treatment of human islets.</u> The main goal is to determine if BMPs augment human ß-cell function or growth. 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.

<u>An innovative high-throughput image processing system for quantifying ß-cells.</u> We use Volocity Acquisition and an automated X-Y stage to image >30,000 ß-cells/ 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. Our algorithm uses successive filters and image overlap and allows for high-speed quantification and simultaneous detection of other ß-cell antigens. This 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 Dr Stoffers' group, in her manuscript recently submitted to the *Journal of Biological Chemistry*.

<u>Research Progress, additional project: Thymidine labeling method development.</u> We have written a manuscript detailing the methods required to perform our thymidine analogue labeling. This manuscript was submitted to *JOVE* on June 29, 2010.

<u>Research Progress, additional project: Gene expression studies.</u> 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 submitted to *Endocrinology* on June 10, 2010.

*Aim 4: Determine whether adenoviral overexpression of GDF3 can stimulate human beta cell proliferation (Kaestner)*

Since the last progress report, we have made good progress on the revised Aim focused on the role of SOCS2 and CISH in beta-cell proliferation. We constructed two adenoviral vectors for shRNA mediated suppression of SOCS2 and CISH expression in ß-cells. We tested the efficacy

of this strategy by transfecting siRNAs into cultured beta-cells and determining CISH mRNA levels after stimulation of cells with prolactin. As shown in Figure 5, the siRNA strategy reduces CISH mRNA levels by about 50%. In addition, we tested the effect of CISH suppression on the accumulation of phosphoSTAT5, the downstream mediator of prolactin signaling in the ß-cell. As predicted, suppression of CISH led to a two-fold increase in p-STAT5 levels in cultured beta-cells treated with prolactin (not shown). We are thus now in a position to move forward with the adenoviral suppression of CISH and SOCS2 in human ß-cells.

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 using mice with targeted mutations in either gene. The SOCS2 null mouse is viable and we have already obtained these mice for the assessment of ß-cell proliferation in pregnancy. CISH null mice, on the other hand, are embryonic lethal. In order to assess the role of CISH in the regenerative response of the beta-cell *in vivo*, we have now constructed a targeting vector for a conditional (loxP) null allele of the gene (see Figure 6). We have transfected the construct into mouse embryonic stem cells, obtained correctly targeted clones, and germ-line transmission of the mutant allele. These mice are currently being bred to Pdx1CreERT2 mice for conditional ablation of the CISH gene in mature ß-cells. We will then assess ß-cell proliferation in the pregnancy paradigm and other regenerative models.

*Aim 5. Characterize endothelially derived secreted factors for their potential to improve outcome in human islet transplantation. (Zaret)*

In the current period, we focused on translating what we learned from our embryonic mouse studies to human islets. Since we discovered that the netrin signaling pathway dynamically governs the specification of pancreatic beta cell progenitors during mouse development, we applied the information to isolated human islets obtained from the Clinical Core. We first asked if Netrin-1 and Netrin-4 are expressed in the human islets, as they are dynamically expressed in early beta cell progenitor development. As seen in Figure 7, both Netrins are expressed sporadically in the human islet (green cells, with red insulin staining as positive control). Since Netrins were originally discovered to be involved in neural pathfinding, we co-stained the islets with the neural glial marker A2B5 (pink). Islet Netrin expression does not coincide with the neural stain; thus Netrins are expressed in other cell types. To obtain these data, and those further below, we spent much of this year working out conditions for immunostaining, including proper antigen retrieval, nonspecific epitope blocking, and specific competitor inhibition (not shown). In further studies, we investigated the expression of DCC, Neogenin, and Unc5b receptors for Netrins in human islets. We previously found that each of these is expressed in embryonic pancreatic development and, notably, we obtained genetic evidence that DCC normally antagonizes the specification of beta cell progenitors. Strikingly, we found that DCC is expressed on many insulin-positive cells in human islets (see arrows indicating yellow staining, indicating co-labeling of DCC and insulin). By contrast, Neogenin is rarely if at all co-expressed in insulin cells (Figure 8) and Unc5b is not expressed in human islets (not shown). These exciting new results set the stage for further studies on the functionality of the DCC receptor in human islets, and the possibility that they may govern beta cell function as they do for progenitors in development.

*Core A: Clinical Core (Naji)*

The human islet isolation core remains committed to providing highly purified and functional

human islets to this program. During the current funding period, the core continued to provide advice on experimental design as well as abundant human islets from multiple individual donors for *ex vivo* studies as well as for transplantation into NSG recipient mice.

*Core B: Xenotransplantation Core (Carroll)*
Core B continues to work successfully with Core A and with the projects to develop an in vivo model for islet cell transplant. The goals for this past year were to optimize transplant methodologies and to increase our understanding of the variables critical to successful islet transplantation. To achieve these goals: we: (1) further demonstrated technical proficiency of our transplantation technique using various doses NSG-derived islets; (2) evaluated the impact of transplanting higher numbers of human islets (up to 3,000 per mouse as a means to improve consistency among animals and islet donors, and (3) determined that human islet function could be maintained long-term in NSG (as determined by human C-peptide levels) in the absence of streptozocin (STZ) pre-conditioning. Since reduced culture time is associated with better islet survival and function, this approach allowed us to significantly reduce the islet culture period (only 2-3 days) compared to using mice induced only after STZ-induced diabetes. We are also evaluating insulin implants to control glycemia in STZ-induced mice. This will allow us to rapidly transplant human islets after isolation and to use glycemia as an experimental readout. We will continue to work with the projects to begin to test effects of compounds on islet cell growth in the xenotransplant model.

Education
*Student Internships*
In May, the Institute awarded internships to 13 applicants (from Penn and Haverford) to this year's program. Additionally, another 11 undergrads 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".

In addition to interested undergraduate students, the IRM had a large number of high school students who wanted to apply to the internship program. In response, we announced an "IRM Summer Internship for High School Students". Over 90 students applied for 10 internship opportunities.

*Haverford College*: The Center of Excellence's first summer intern, Yusup Shin (Summer 2008), is now beginning his year-long thesis project in Dr. Emerson's laboratory on the role of hematopoietic stem cell self-renewal genes in HSC-thymocyte commitment, i.e. his summer internship successfully stimulated his interest in stem cell biology. He will most likely apply to M.D.-Ph.D, programs with a focus on stem cells and development. Two additional Haverford students are participating in this summer's Center of Excellence Research Internship in the laboratories of Drs. Bhandoola and Naji.

*Project BioEyes/Bridge to ReBIO:* This year, Bridge to ReBIO and Project BioEYES reached more underserved students in Philadelphia and nationally than ever before. Since 2002, Project BioEYES has served 19,445 Philadelphia students and close to 9,000 students in partnering cities such as Baltimore, MD, South Bend, IN, and Williamstown, MA. BioEYES was also replicated in Melbourne, Australia this summer. Along with expanding the curriculum, Project BioEYES

has also launched a new website (www.bioeyes.org) where students and teachers can learn about the program. The outstanding success of these programs is primarily a result of the efforts of Dr. Jamie Shuda, who joined Penn in August 2009 as the Director of Life Sciences for the Institute for Regenerative Medicine. As founder of Project BioEYES and a former K-12 educator, Dr. Shuda oversees and evaluates all outreach efforts and works in collaboration with Penn's Barbara and Edward Netter Center for Community Partnerships to create systemic change in how students are exposed to science in and out of the classroom.

*Bridge to ReBIO* had many successes in its' 2nd year. First, in early October we hosted a "Research Tools 101" workshop for 30 Philadelphia High School students. Not only did this excite the ReBIO students about doing their own research project, but it also provided them an opportunity to understand scientific tools and the impact of the research conducted by IRM faculty. This introductory workshop was followed by the formation of research teams, involving 24 high school students and 12 UPenn, Drexel, and Haverford student and faculty mentors, who worked together to generate independent research projects that were carried out at the high schools or IRM laboratories. On March 9, 2010, the School District of Philadelphia hosted the 31st annual George Washington Carver Science Fair. Overall, 19 of the 24 ReBIO students received awards at the Carver Fair. This includes students from four West Philadelphia High Schools that were represented at the city-wide competition for the very first time. Of these schools in particular, ReBIO mentored students took two first place finishes, one third place, one honorable mention, and one special award given by the Navy for "Exemplary scientific thought and creativity." All ReBIO students who placed at the fair then competed in the Delaware Valley Science Fair in April. Again, these students did tremendously well with 14 of the 19 students placing in their category. To celebrate the hard work and dedication of the students, mentors, and staff, Bridge to ReBIO hosted the 2nd annual Science Celebration on May 4, 2010.

For the second year in a row *Project BioEYES* has exceeded its own goal of educating over 2,000 Philadelphia students. In Philadelphia alone, approximately 3,469 students were engaged in Project BioEYES outreach program between September 2009 and June 2010. The breakdown of students reached includes: 527 elementary students, 1,335 middle school students, and 1,607 high school students.

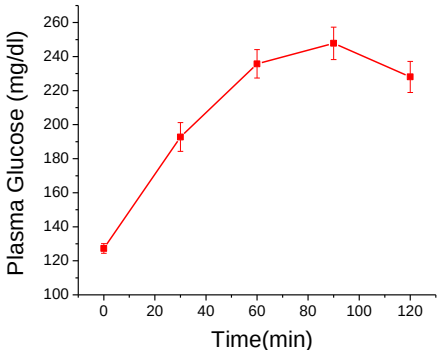



**Figure 1.** Plasma glucose during a 75 g oral glucose tolerance test in enrolled subjects (*n* = 20). Mean fasting and 2-hour glucose levels were at or just above cutoffs for the diagnosis of diabetes established by the World Health Organization, indicating successful recruitment of an "early" type 2 diabetic population.

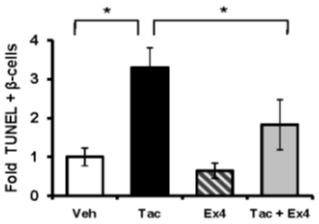



**Figure 2.** Calcineurin inhibition induces human ß-cell apoptosis. Quantitation of human ß-cell TUNEL rates after 48 hours of vehicle, 10ng/mL Tac, 10nM Ex-4 or combination of Tac and Ex-4. Data presented as mean (±SEM) of 3 independent human islet preparations and expressed as fold compared to the vehicle group. A total of 5,000-30,000 human ß-cells were counted per treatment group for each preparation.* p<0.05.

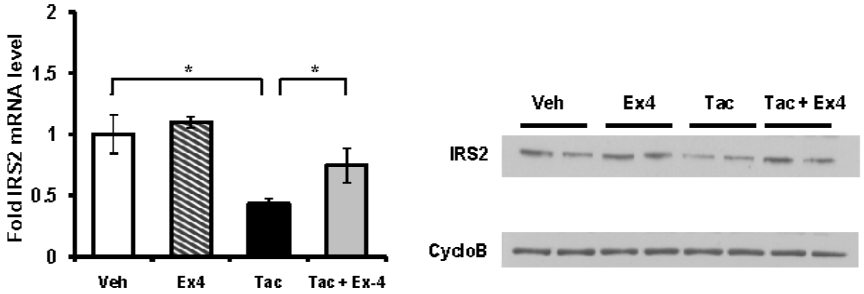
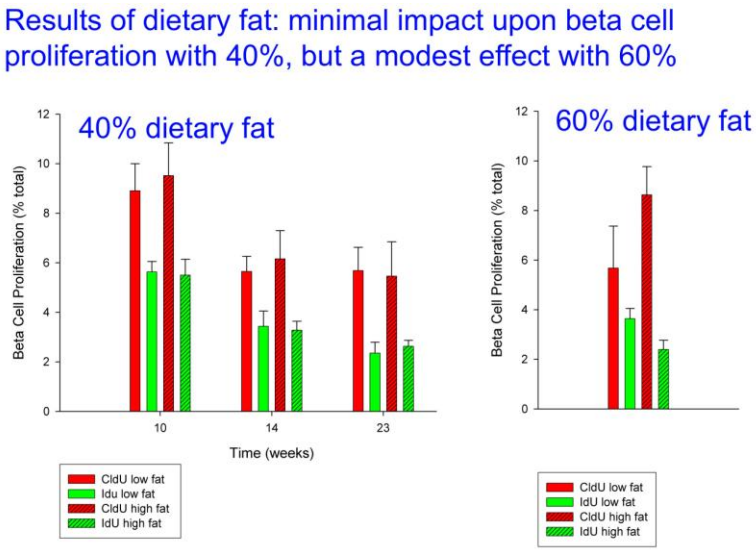



**Figure 3**. Tacrolimus decreases IRS2 expression, which is restored by cAMP stimuli, in human islets. (left) Quantitative PCR analysis of IRS2 mRNA following treatment with vehicle, 10ng/mL Tac, and/or 10nM Ex-4 for 6 hrs in 3 independent human islet preparations. Data presented as the mean (±SEM) fold vs. vehicle. (right) IRS2 protein expression in representative Western blot of human islets following same treatment. * p<0.05

**Figure 4**. Beta cell proliferation analysis.



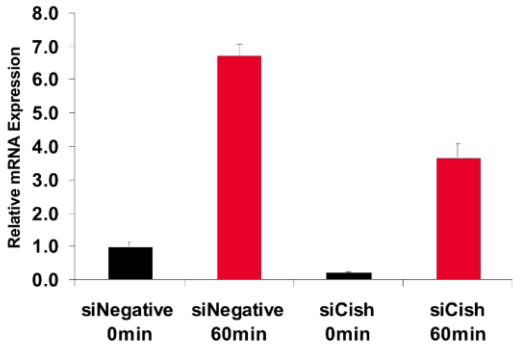



**Figure 5**: siRNA suppression of CISH leads to decreased CISH mRNA levels in both the basal (Black bars) or prolactin-stimulated condition (red bars).

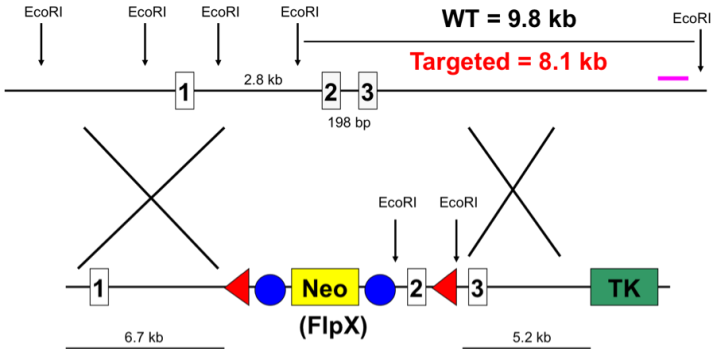



**Figure 6.** Targeting strategy for the derivation of a CISH$^{loxP}$ allele. The neomycin resistance cassette (Neo, in yellow) has already been removed through expression of Flp- recombinase.

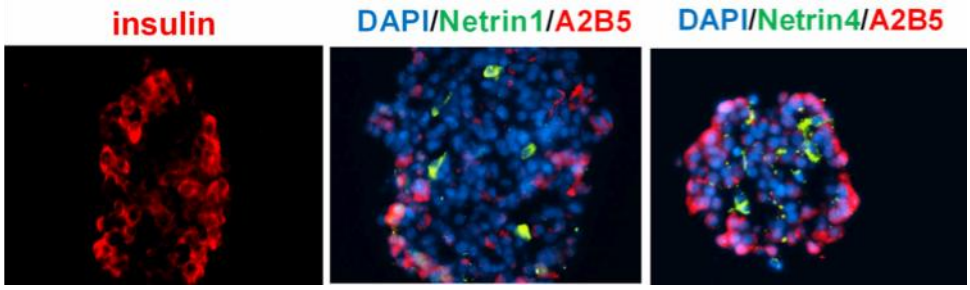



**Figure 7**

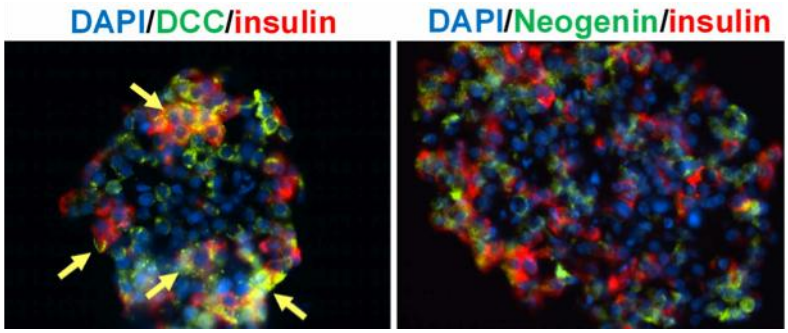



**Figure 8**