1. **Study Title:**
   Immunoengineering of Stem Cell-derived Insulin Producing Cells (SCIPC) to Reduce Immunogenicity and Potential Rejection

2. **Proposing Trainee Investigator:**
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3. **Clinical Mentor/Principal Investigator:**
   Peter Stock, MD
   Professor of Surgery and Chair, Department of Surgery Research Committee

4. **Scientific Mentor/Principal Investigator:**
   Qizhi Tang, PhD
   Professor of Surgery and Director, Transplantation Research Laboratory

5. Study Rationale, Background, and Preliminary Data:

Islet transplantation is a proven effective therapy for patients with type 1 diabetes (T1D). In recent years, remarkable progress has been made with regard to transplantation of cadaveric islets, however, donor shortage limits this therapy to very few patients. Recent improvements in differentiation protocols of human embryonic stem (hESC) cells now allow for reliable production of human Stem-Cell-derived Insulin-Producing Cells (SCIPCs) that mimic the functional properties of human beta cells (1-3). These cells present a possibility for an unlimited supply of cells for the treatment of diabetes. However, one of the remaining problems concerns the autoimmune and alloimmune responses present in T1D patients against transplanted beta cells.

Here, we propose to take advantage of CRISPR/Cas9 mediated gene editing to generate SCIPCs that are minimally alloimmunigenic. In particular, we will eliminate components of the major histocompatibility complexes (MHC) from beta cells. Eliminating MHC I & II from beta cells is an innovative strategy aimed at 'hiding' SCIPC from detection by the immune system and ultimately from destruction. Successful concealing of SCIPCs could revolutionize modern cell therapy approaches and would have direct implications for T1D patients.

6. Hypothesis and Study Objective(s):

We hypothesize that by eliminating specific HLA class I and class II components, we will prevent rejection of SCIPC grafts, thus allowing for their long-term functionality and survival and function in the absence of debilitating immunosuppressive strategies. We will test this hypothesis in both in vitro cultures and in vivo using a humanized mouse model.

Aim 1: To produce SCIPC with minimal genetic modifications to allow long-term survival in allogeneic hosts without immunosuppression.

Aim 2: To obtain the preclinical safety and efficacy data to support future generation of GMP-compliant minimally immunogenic hESCs to enable phase I clinical trial in patients.

7. Study Design and Methodology:

Aim 1: Immunoengineering of hESC: Using CRISPR/Cas9 technology, we will abolish HLA class I expression by deleting the HLA-A, B and C genes. Gene elimination will be accomplished by introducing CRISPR-mediated indel that cause frameshift mutations in protein-coding portion of three HLA genes (HLA-A, B and C genes). We will use flow cytometry to compare HLA-A, B, C expression on cells that have been treated with various gRNAs to determine the efficiency of targeting and thus confirm elimination of HLA-A, B, C expression. Successfully targeted cells can be recovered using FACS, thus streamlining the selection and cloning strategy. Once an HLA-A/B/C^{null} subline is confirmed, we will express a firefly luciferase gene in the HLA-A/B/C^{null} line to enable *in vivo* real-time monitoring of the grafts (proof of principle experiment shown in Fig. 1) (4).

HLA class II may be induced on beta cells under inflamed conditions in the presence of IFNg and TNF and provide a source of alloantigen. An efficient way to prevent expression of these genes is to eliminate the class II transactivator (CIITA) that serves as a master transactivator of HLA class II expression (5). We propose to use the HLA-A/B/C^{null}.Luc line and employ the same CRISPR/Cas9 strategy to eliminate the CIITA gene. We will using a combination of IFNg and TNF to stimulate hESCs to induce HLA-II expression to facilitate detection of successfully targeted cells (6).

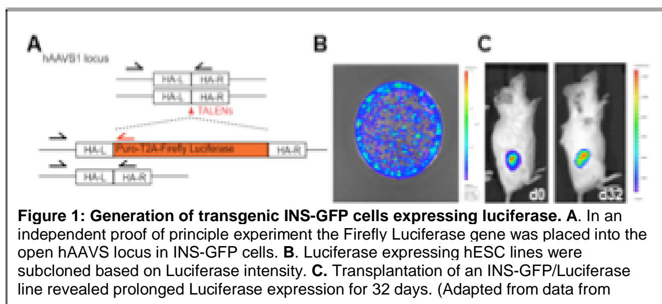

Figure 1: Generation of transgenic INS-GFP cells expressing luciferase. A. In an independent proof of principle experiment the Firefly Luciferase gene was placed into the open hAAVS locus in INS-GFP cells. **B**. Luciferase expressing hESC lines were subcloned based on Luciferase intensity. **C.** Transplantation of an INS-GFP/Luciferase line revealed prolonged Luciferase expression for 32 days. (Adapted from data from

We will perform functional assays, including *in vitro* glucose-stimulated insulin secretion, and *in vivo* function in diabetic NSG mice. Furthermore, we will conduct whole genome sequencing of the edited hESC lines to ensure that CRISPR/Cas9 manipulation has not introduced unwanted off target mutations that might compromise functionality and viability, or introduce tumorigenic potential.

Aim 2: Determine immunogenicity of immunoengineered SCIPCs: We will deploy a humanized mouse model to test the efficiency of the gene manipulations on reducing alloimmune responses. SCIPC cells are not expected to express costimulatory molecules to sufficiently activate naïve T cells; therefore *in vitro* assays would not have the same sensitivity as *in-vivo* models. Therefore, we plan to develop a modified humanized mouse model based on the human $CD34^{+}$ stem cell transfer approach. Through contracts with JAX lab, we have developed a mouse strain to optimize human immune system reconstitution without GVHD. The immunodeficient NSG mice recipients will have human HLA-A2 and DR4 transgenes to allow selection of human T cell precursors with human HLA restriction resulting in mature T cells in this model that will be allogeneic to the SCIPC grafts. The β2 microglobulin (b2M) is required for MHC class I expression and thus we will use a transgenic line with a HLA-A2 and

human b2M fusion construct to ensure optimal expression of HLA-A2. We will also eliminate endogenous mouse MHC class II expression so that all human $CD4^+$ T cells developed in these mice will be restricted to HLA-DR4. To enhance human NK cell engraftment, we will further introduce a human IL-15 transgene knocked into the mouse IL-15 locus. Altogether, the mouse model we aim to generate for the *in vivo* analyses in this study is $NSG.A2.DR4.AB^o$. hIL-15Tg ($NSG.MHC^h$.15).

We will use a novel *in utero* transplant approach to introduce human CD34+ cells into NSG recipients. With the help of the Mackenzie lab, we will monitor the development of human T, myeloid, NK, and B cells over time along with mouse immune phenotyping to determine the kinetics of human immune system development and the composition. Once the human immune cells reach equilibrium, we will transplant $luciferase^+$ parental HLA expressing SCIPCs in the kidney capsules and determine rejection kinetics using bioluminescence imaging, flow cytometry and multiplex immunofluorescence on histological sections (Figure 2). We expect that graft will be rejected and T and NK cells to be present in the graft at the peak of the rejection response.

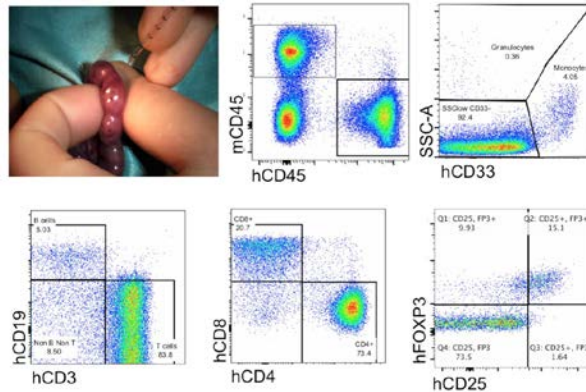

Figure 2. ***In utero*** **transplantation of human CD34+ cells.** Fetuses of embryonic day 14.5 mice were exteriorized for intrahepatic injection of 25,000 to 50,000 human cord blood CD34+ cells. The fetuses were allowed to mature. The presence of human immature cells in the lymphoid organs of the transplanted mice was analyzed at 20 to 24 weeks of age using flow cytometry. Used with permission from Hebrok

Aim 3: Evaluate immune responses to modified SCIPCs: We will use sequential *in vitro* and *in vivo* screens to determine the immunogenicity of the modified SCIPC cells.

A. *In vitro* screen: We will screen the immunogenicity of the $HLA\text{-}A/B/C^{null}$ and HLA^{null} hESC line using an *in vitro* complement-dependent cytotoxicity assay. We have already determined the HLA types of the hESC lines. Anti-HLA-A2, anti-HLA-A24, HLA-B7 and B64 antibodies are available commercially. For HLA-class II, we will use combined TNF and IFNg stimulation and anti-HLA-DR/DP/DQ antibodies are available commercially. In addition to flow cytometry, we will also use alternative complement-medicated cytotoxicity assays to assess HLA ablation.

B. *In vivo* screen: We will transplant the parental or modified SCIPC cells into humanized mice and analyze graft survival using real-time bioluminescence imaging for at least 100 days after transplant or until graft loss. Graft loss may be due to T cell- or NK-mediated rejection or SCIPC death due to ischemia injury. Analyzing the graft using flow cytometry and immunofluorescence will help us to determine the cause of graft loss.

8. Anticipated Challenges:

Considerations of tumorigenic potential: Transplantation of in vitro derived SCIPCs might lead to grafts with tumorigenic potential in NSG mice. This would likely be due to the presence of residual cells with progenitor capacities that have not yet undergone terminal differentiation. In the unlikely case we were to observe

tumorigenic potential of hESC-derived cells, it is possible to use the CRISPR/Cas9 system to express suicide gene(s) whose function could be induced to trigger apoptosis of tumorigenic cells.

Considerations of recurrent autoimmunity: It is known that beta cell antigen specific autoreactive T cells can contribute to rejection of transplanted beta cells (7). While we expect that ablating HLA expression on beta cells will prevent direct attack of HLA-restricted cytotoxic T cells on SCIPCs (8), it is possible that this strategy may not be effective in fully preventing damages caused by autoreactive T cells that indirectly attack the graft by secreting cytokines or by activating other cells types such as macrophages. We anticipate transgenic expression of A20 may reduce proinflammatory responses by SCIPCs.

9. Expected Outcome and its Impact on Transplantation:

We anticipate that the successful completion of our proposal would have a great impact on cell therapies in general. We believe that the proposed studies are the first steps toward modulating gene expression in hESC-derived beta cells that will eventually result in new strategies to fully protect such cells from auto- and allo-immune assaults. Proof-of-concept results obtained in this study will provide the rationale to invest in GMP processes for producing immunoengineered hESCs to support future clinical trials in patients.

10. Coursework Plan (100 hours per year):

As part of the FAVOR T32 training grant, I will participate in the FAVOR T32 Immersion workshop (20 hours), summer and fall TICR coursework (20 hours), and I will plan to complete the following courses when they are next offered in online format: Designing Clinical Research (EPI 202), Biostatistical Methods for clinical research (BIOSTAT 200), and Introduction to Statistical Computing in Clinical Research (BIOSTAT 212). Additional course work will include the Transplant Seminar Series and the Human Immunology Seminar, and additional pertinent BMS mini-coursework in Spring 2018.

11. Tentative Budget for Project Completion:

Our approximate budget for supplies, animals, reagents, and core services totals $100,000. This project has been funded by a Juvenile Diabetes Research Foundation Grant awarded to the Hebrok and Tang laboratory.

Our budget includes:

<u>Animals:</u>

Mice: Approximately 280 mice will be used to complete the experiments proposed in Aims 1 and 2. These mice will be purchased for experiments and the purchase cost will be $15,000. We expect average housing to be 4 months. Combined mouse purchase and housing costs will be $25,000/first year.

<u>Reagents:</u>

-Histology ($3,000):. The costs include purchase of primary antibodies, conventional or fluorescent secondary antibodies, antibody detection kits, etc.

-Cell Culture and Reagents ($15,000): Significant cell culture reagents will be required to support this proposed study. Costs include media, culture plates, etc.

<u>Other costs:</u>

-BLI recharge: For in vivo monitoring of transplant function. We expect 30 hour usage/month which will cost $750/month at recharge rate of $25/hr. Total of $9,000 recharge is expected.

- Microscopy recharge: Histological analysis of devices and adjacent tissue next to implantation site will incur an average of 20 hr usage/month at recharge rate of $25/hour. Total recharge microscopy and flow cytometry will be about $6,000/year.

References:

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3. **Rezania A, Bruin JE, Arora P, Rubin A, Batushansky I, Asadi A, et al.** Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. Nature biotechnology. 2014;32(11):1121-33.
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5. **Chang, C. H., Guerder, S., Hong, S. C., van Ewijk, W. and Flavell, R. A.** (1996). Mice lacking the MHC class II transactivator (CIITA) show tissue-specific impairment of MHC class II expression. *Immunity* 4, 167–178.
6. **Pujol-Borrell, R., Todd, I., Doshi, M., Bottazzo, G. F., Sutton, R., Gray, D., Adolf, G. R. and Feldmann, M.** (1987). HLA class II induction in human islet cells by interferon-gamma plus tumour necrosis factor or lymphotoxin. *Nature* 326, 304–306.
7. **Pugliese, A., Reijonen, H. K., Nepom, J. and Burke, G. W.** (2011). Recurrence of autoimmunity in pancreas transplant patients: research update. *Diabetes Manag (Lond)* 1, 229–238.
8. **Prange, S., Zucker, P., Jevnikar, A. M. and Singh, B.** (2001). Transplanted MHC class I-deficient nonobese diabetic mouse islets are protected from autoimmune injury in diabetic nonobese recipients. *Transplantation* 71, 982–985.

By signing below, I certify as a proposing mentee that I have read through the T32 FAVOR summary and application, and am aware of my expectations.

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SIGNATURE of Proposing Mentee DATE

By signing below, I certify as a mentor that I have reviewed the proposing mentee's T32 initial proposal, and agree to provide guidance and funding for the study.

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SIGNATURE of Clinical Mentor (CRT) DATE

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SIGNATURE of Scientific Mentor (SRT) DATE