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January 11, 2017

Dear ICOC members,

Thank you very much for your thoughtful consideration of our proposal for funding (**DISC2-09559,** *Thin Film Encapsulation Devices for Human Stem Cell derived Insulin Producing Cells)*. We are heartened by the favorable review that the propopsal has received but wanted to further clarify some of the key questions that came up during review. Below is a point by point response based on concerns raised and we hope that this further strengthens our proposal for funding.

1. There was a concern that the proposal provided little experimental details on differentiation of hESCs to insulin-producing cells (SCIPCs).

We apologize for this but due to the length of the proposal it was difficult to include all the necessary information. However, since submission of the proposal, the Hebrok laboratory has further significantly improved and optimized the differentiation conditions for our hESC-derived insulin-producing cells (SCIPCs). The key change we have made is illustrated in **figure 1**. During embryonic development, beta cells delaminate from the pancreatic epithelium and then reaggregated during islet formation. We have recapitulated this process in our new differentiation protocol and end up with an enriched fraction of immature beta cells directly after sorting. Over 7 days of additional culture, these immature beta cells mature into functional beta cells that we call enhanced Beta cell clusters (eBC). The eBCs are highly enriched for beta cells (~85-90% depending on the exact experiment) and express C-peptide at a high level. Furthermore, when compared to unsorted cells before reaggregation, the beta cells in eBCs show high level expression of critical beta cell factors.

2. The reviewers wanted more data providing evidence that SCIPCs reverse diabetes after transplantation in diabetic immunodeficient mice. It was not clear in the proposal whether the cells can reverse diabetes right after implantation or if they require an 'adaptation period' (before making the recipient mice diabetic by STZ treatment).

We have put stem cell derived beta cells into immune compromised mice (**Fig. 2**). Importantly, these cells function as early as **3 days after implantation**. Furthermore, the enhanced beta cells reduce glucose load in glucose tolerance tests and prevent formation of diabetes upon treatment with streptozotocin. Thus, our preliminary data indicate that the optimization of the stem cell to beta cell differentiation protocol allows for the generation of functional human beta cells that can be tested in transplantation studies.

3. Reviewers questioned whether encapsulating the insulin-secreting cell products in the devices would allow physiological glucose-stimulated insulin secretion. They also wanted to see data providing evidence that cells maintain their functionality in addition to their viability during long-term implantation in the devices.

We were not able to include all of the data that would address this concern in the original submission. We have encapsulated SCIPCs into our device and transplanted them in between the caudate lobe and left hepatic lobe in NSG mice. The engraftment and function of encapsulated SCIPCs was studied by measuring human c-peptide in mouse serum following intraperitoneal glucose stimulation. We demonstrated that after 6 months, encapsulated cells in the device are responsive to glucose with GSI of 6 (**Fig. 3a**). Bioluminescence quantification also confirmed the viability of hSC-βC encapsulated NID grafts (**Fig. 3b**). Finally, ICED-in liver tissue grafts were explanted and immunostained to confirm phenotype of transplanted cells. We find mono-hormonal cells expressing human c-peptide inside our device (**Fig. 3c**). Collectively, these studies provide strong evidence of the device supporting the viability and function of SCIPCs in vivo for 6 months.

4. The rationale for protecting the cells from nutrient deprivation by supplementation with amino acids and from the deleterious effects of cytotoxic cytokines (especially since the cytokines cannot diffuse through the film) is not provided.

It is well established that a large percentage of transplanted islet die independent of immune-mediated rejection due to ischemic and inflammatory injuries. We have quantitatively measured the graft loss using luciferase imaging and found that subcutaneous transplant exacerbate graft loss when compared to kidney capsule transplant (**Fig. 4A and B**). Similar phenomenon is observed when transplanting SCIPCs (**Fig. 4C and D**), although higher proportion of the SCIPC grafts survived after transplant when compared to mature mouse islets. To determine if the difference in survival observed between mouse islets and SCIPCs is due to developmental maturity of the cells, we labeled more mature insulin-producing cells in the SCIPCs by knocking a GFP reporter gene in the insulin locus. Before transplant approximately 40% of the SCIPCs are GFP+ (actively transcribing insulin) and only an average of 12% are GFP+ when the grafts are retrieved 7 days after transplant demonstrating preferential loss of more mature insulin-producing cells (**Fig. 4E**). Moreover, graft loss is exacerbated by encapsulation presumably due to additional barrier to oxygen and nutrient supply to the grafts (**Figure 4E**). These results demsontrate that optimized encapsulation device must include features that promotes graft survival.

Using in vitro simulation, we have determined that nutrient deprivation and hypoxia individually can kill about 50% of the and the combination of hypoxia and nutrient deprivation led to killing of nearly all the islets (Gaetano et al, manuscript in preparation, data not shown). SCIPCs are able to better tolerate individual insult of nutrient deprivation and hypoxia with less than 20% cell death, but the combination of the two synergistically kills over 90% SCIPCs (data not shown). Preservation of graft mass thus requires approaches to mitigate impacts of hypoxia and nutrient deprivation.

We decided to control hypoxia damage to the SCIPC grafts by intentional exposure to reduced oxygen tension to induce cell intrinsic adaptation to hypoxia. SCIPICs cultured in 5% instead of 21% pO2 survived better after transplant in the subcutaneous space, but the protective effect was lost in 7 days. Similarly, supplementing the grafts with amino acids was able to improve graft survival in the first week after transplant. Combination of preconditioning in 5% pO2 and amino acid supplementation achieved more durable effect so that >70% of the grafts maintained their initial mass (Gaetano et al, manuscript in preparation, data not shown). Together, these finding provide ther rationale for incorporating this strategy with encapsulation to promote SCIPC survival.

5. No data are provided to support the need of delivering immunosuppressive drugs locally in the device. Some of these drugs may compromise revascularization so they may be deleterious to function of device.

Ischemic islets produce copious amount of inflammatory mediators including Cxcl1 and Cxcl10 that recruit inflammatory cells. The cells produce a wider array of proinflammatory cytokines including IFNg, IL-1, and TNFa that are directly toxic to beta cells. Clinical islet transplant experiences find that inclusion of short-term treatment with anti-TNF and anti-IL-1 and T cell depletion significantly improve rates of insulin independence (PMID: 22723582). It is possible that by preconditioning the SCIPCs grafts with reduced pO2 and supplementation of amino acid, the magnitude of the inflammatory responses of the grafts will be reduced to the point that anti-inflammatory strategies not longer offers benefits, but we feel that it is prudent to not under estimate the potency and the sensitivity of the immune system. Likely in the fully immune competent host, some short-term immune protections would be needed.

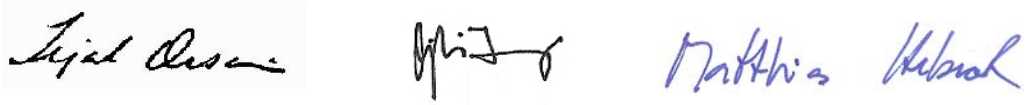
The reviewer is right that rapamycin may impact neovascularization. Using oral gavage, we found that 1mg/Kg rapamycin did not impair revascularization of syngeneic islet grafts in B6 mice (data not shown). It is possible that local delivery of rapamycin will have more potent inhibitory effect. We will monitor vascular growth with or without rapamycin and use alternative agents that specifically target inflammatory agents such as chemokine decoy receptors or antibodies to IL-1 and TNFa.

6. How the device will be modified to get the "best configuration to achieve stable sustained release of amino acids" and what "best" means in terms of measurable outcomes is not specified.

We define graft success using the following composite criteria:
- Maintenance of >80% graft mass in immune competent host without long-term immunosuppression
- Demonstration of stable or increased glucose induced c-peptide production
- Histological evidence of mature islet-like composition

We believe that our approach will have broad implications in the delivery of stem cell derived products for the treatment of Type I Diabetes. The development of alternative encapsulation devices that are both immunoprotective and support cell viability/function long term are essential to moving the field forward.

Sincerely,



Tejal Desai, Qizhi Tang, and Matthias Hebrok
Scientific Team

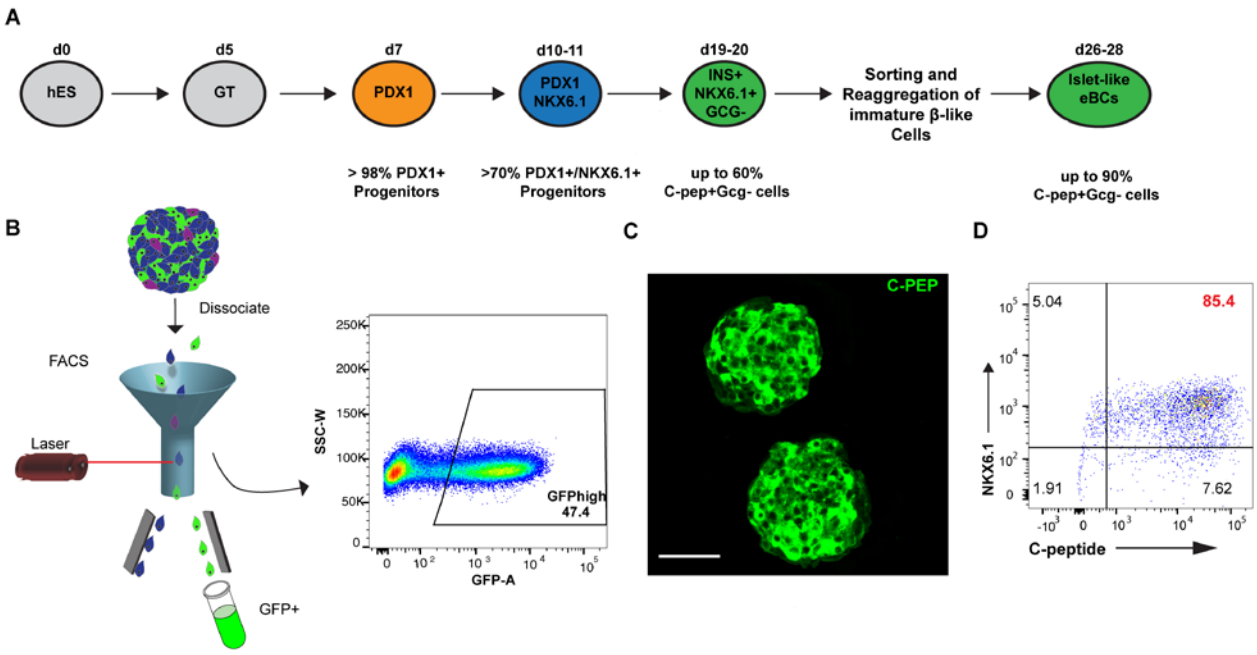


Figure 1: Generation of Islet-like Beta-clusters. (A) Schematic representation of directed differentiation of hESCs to pancreatic β-cells through 26-28 days that recapitulates embryonic development and includes a new strategy of reaggregation of immature β-like-cells to mimic β-cell coalescence into islets. (B) Isolation of immature β-like-cells by FACS sorting of d19-20 Ins-GFP+ cells, and (C) subsequent reaggregation into 100 µm sized clusters (enhanced Beta-clusters-eBCs stained after additional 7 days of culture with C-peptide). Scale bar, 100 µm. (D) Quantitative analysis of d26-27 Beta-clusters by flow cytometry confirmes that majority of the population is bona fide β-cells that are C-peptide+/NKX6.1+.

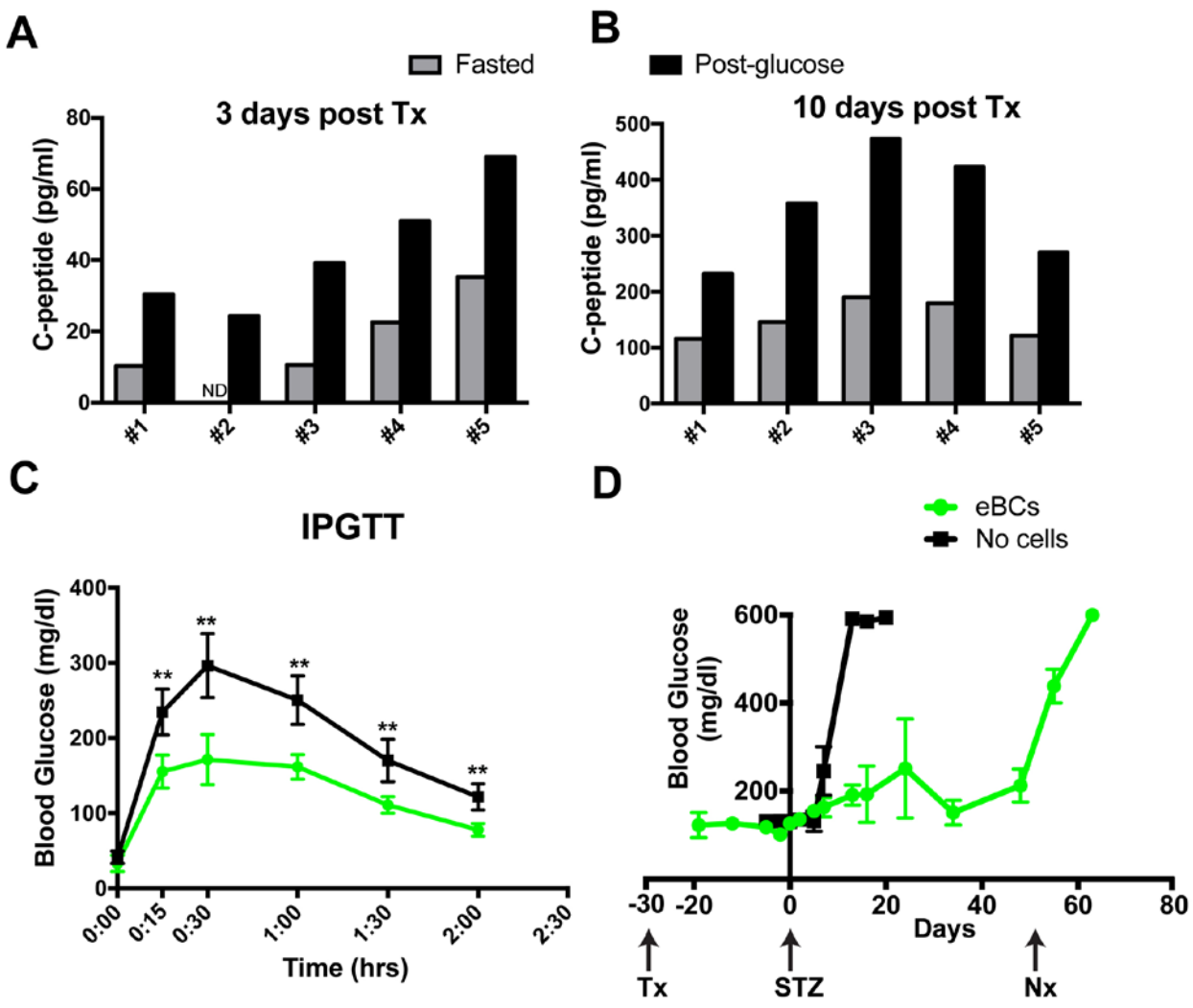


Fig 2. eBCs are functional *in vivo* as early as 3 days after transplant. (A) *In vivo* glucose challenge test at the indicated time points. 700 eBCs (0.7x10^6 cells) were transplanted under the kidney capsule of non-diabetic male NSG mice. Human C-peptide levels in the serum were measured following an overnight fast and 30min after an IP glucose bolus **3 days** after the transplant. The numbers on the X axes indicate individual animals. ND: not detected. (B) 4000 eBCs (4x10^6 cells) were transplanted under the kidney capsule of non-diabetic male NSG mice. Human C-peptide levels following fasting and 60min after an IP glucose bolus were measured **10 days** after the transplant. The numbers on the X axes indicate individual animals. (C) Intra-Peritoneal Glucose Tolerance Test (IPGTT) was performed on NSG mice 30 days after transplantation with 700 eBCs (green, n=4) and control NSG mice that did not receive any cells (no cells) (black, n=5). **P<0.01, ****P<0.0001 determined by two-tailed unpaired t-tests. (D) STZ was used to induce diabetes in control NSG mice (no cells, black line, n=2) or NSG mice transplanted with 700 eBCs (green line, n=2). Random fed glucose measurements were taken during the course of the study. Mice that did not receive eBC-grafts died 22 days after STZ treatment, whereas the mice that received eBC-transplants maintained normoglycemia until their engrafted kidney was removed via survival nephrectomy at day 50.

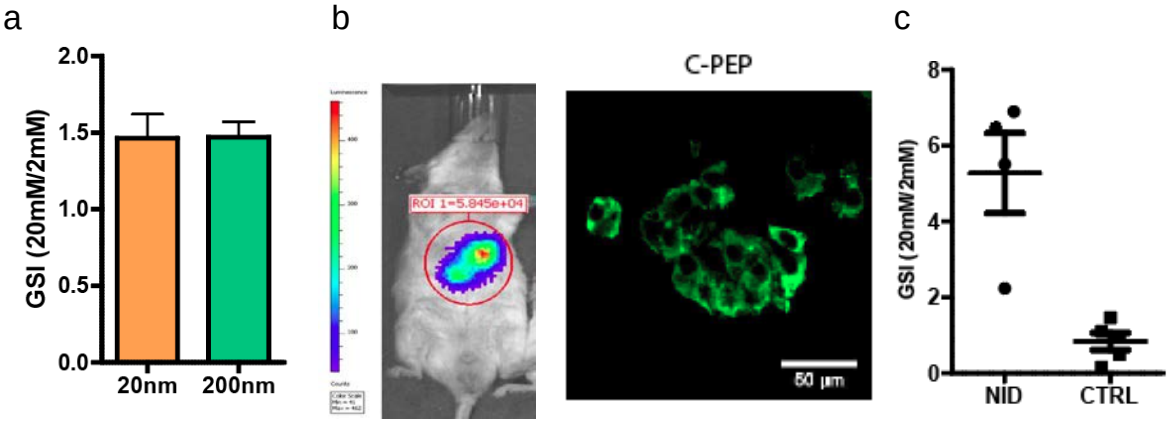


Figure 3. In vitro and In vivo viability of function of SCIPC encapsulated in device. a, GSI of encapsulated SCIPC within device. **b**, in vivo luminescence imaging and immunofluorescence staining of SCIPC in device after 6 month transplantation **c**, GSI of encapsulated SCIPC in vivo after 6 months transplanatation

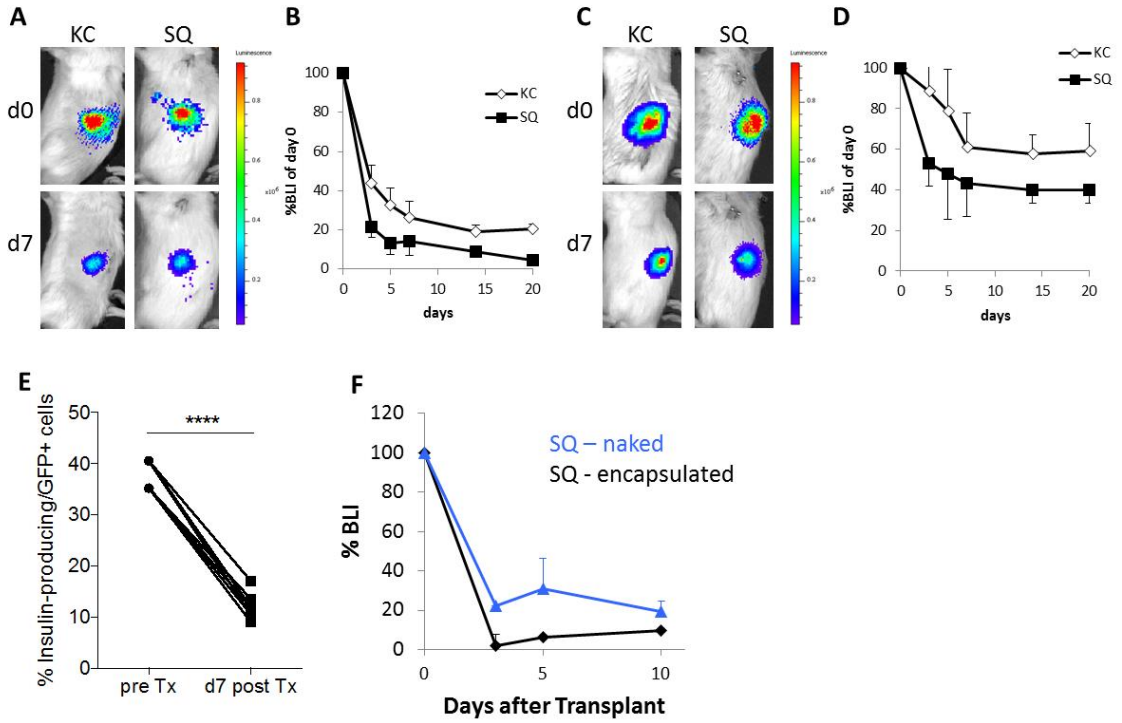


Figure 4. Mature mouse islet and SCIPC-derived grafts are impacted by primary graft failure. (A) Representative pictures of MIP.Luciferace B6 islets transplanted into B6 albino mice under the kidney capsule (KC, n=10) or subcutaneously (SQ, n=3) at day 0 and at day 7. (B) The bioluminescent intensity (BLI), showed as percentage of day 0, was quantified overtime. (C) Representative pictures of SCIPC.LUC transplanted into NSG mice under the kidney capsule (KC, n=8) or subcutaneously (SQ, n=10) at day 0 and at day 7 after transplant. (D) The residual SCIPC grafts were quantified during the duration of the experiment using bioluminescent intensity, showed as percentage of day 0. (E) SCIPCs with a GFP reporter for insulin expression were transplanted under the kidney capsule of NSG mice as shown in C and D. At day 7 post transplant, the grafts were retrieved and % GFP+ human cells were determined using flow cytometry. The results show selective loss of more mature GFP+ cells after transplant. (F). SCIPCs were transplanted SQ with or without encapsulation. Results shod enhanced graft loss when encapsulated.