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（様式 10）
（海外特別研究員事業）

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海外特別研究員最終報告書

独立行政法人日本学術振興会 理事長　殿

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（氏名は必ず自署すること）

海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1. 用務地（派遣先国名）用務地：ジョスリン糖尿病センター（国名：　アメリカ合衆国　）

2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。
全ゲノムスクリーンを用いた糖尿病病態における膵β細胞保護機構の解明に関する研究

3. 派遣期間：~~令和~~ 平成 31 年 4 月 1 日 ～ 令和 2 年 9 月 30 日

4. 受入機関名及び部局名
ハーバード医科大学　ジョスリン糖尿病センター

5. 所期の目的の遂行状況及び成果…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**
（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）
（注）「6. 研究発表」以降については様式 10－別紙 1～4 に記入の上、併せて提出すること。

5．所期の目的の追行状況及び成果

（研究・調査実施状況及びその成果の発表・関係学会への参加状況等）

Genome-scale CRISPR screen for beta cell protective mutations identifies the T1D GWAS candidate gene Rnls

As I wrote in the initial application for this fellowship, we have detected 11 target genes as candidates for protection against *in vivo* autoimmune killing using a genome-scale CRISPR screening. Then, we generated 11 mutant NIT-1 cell lines using the same gRNAs identified in the initial screen and started validation experiments both *in vitro* and *in vivo*. Some of the cell lines showed robust protective effect against both *in vivo* autoimmunity and *in vitro* ER stress (figure 4 and 5 in the initial application), and among these we decided to prioritize *Rnls* for validation because *Rnls* has been discovered as the candidate gene for a region in the human genome associated both with the overall risk of T1D[1] and with the age of diabetes onset by GWAS[2].

Rnls deletion protects beta cells against autoimmune killing

We also generated a control cell line by transducing NIT-1 cells with a non-targeting (NT) gRNA to rule out a possible off-target effect by lentiviral transduction. NIT-1 cells were also engineered to carry a luciferase reporter for longitudinal non-invasive imaging of beta cells after transplantation. Using these cell lines, we repeated validation experiments in the following different systems:

1) *Rnls*^{mut} cells and control NIT-1 cells were co-transplanted on opposing flanks of immuno-deficient NOD.*scid* mice, who were also injected with splenocytes from diabetic NOD mice (**Figure 1A**).

2) *Rnls*^{mut} cells and control NIT-1 cells were co-transplanted on opposing flanks of overtly diabetic NOD mice (**Figure 1B**).

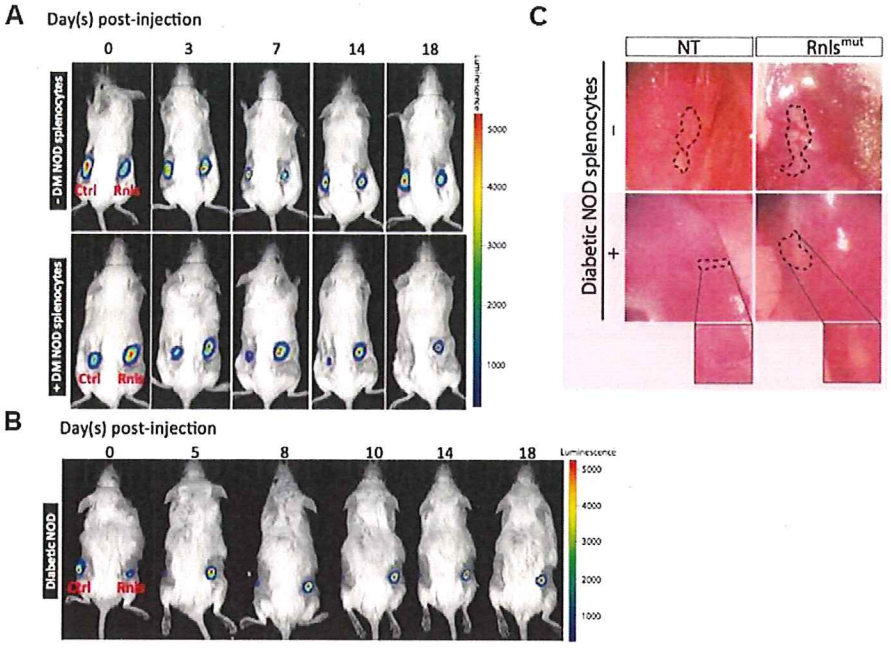
In both experiments, control NIT-1 cells were killed by autoimmunity within one to two weeks after transplantation, while *Rnls*^{mut} NIT-1 cells persisted for up to 2 months in the same recipient mice.

We next tested if the disruption of *Rnls* would similarly protect primary mouse beta cells. We isolated pancreatic islets from NOD.*scid* mice that are devoid of autoimmune infiltrates in the pancreas. Then, we transduced dispersed islet cells with lentivirus encoding rat insulin promoter (RIP)-driven Cas9 endonuclease and either the *Rnls*-targeting (*Rnls*^{mut}) gRNA or a non-targeting (NT) control gRNA and transplanted these islets cells each under one kidney capsule of the same NOD.*scid* mice. After two weeks, we injected graft recipients with splenocytes from diabetic NOD mice to induce autoimmune beta cell killing. As anticipated, autoimmunity decreased the size and insulin

expression in control grafts, while *Rnls*^{mut} islets survived autoimmunity and maintained insulin expression (**Figure 1C**).

Together these results clearly show that targeting *Rnls* in beta cells was protective in pathophysiologically relevant models of autoimmune diabetes.

Figure 1. In vivo validation of β-cell survival under autoimmunity

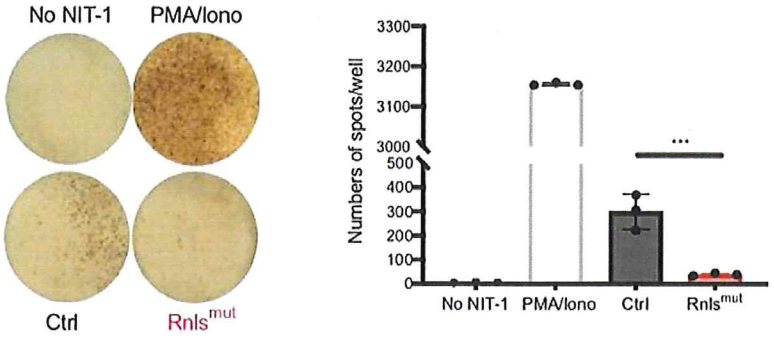


Rnls mutation diminishes immune recognition of beta cells

We proceeded to ask if *Rnls* deficiency had a direct effect on immune recognition. The expression MHC class I and class II molecules on the surface of *Rnls*^{mut} NIT-1 cells was comparable to that of control cells. *Rnls* mutation did not significantly affect the response of beta cell-reactive (BCD2.5 TCR transgenic) CD4^{+} T cells co-cultured with antigen presenting cells and NIT-1 beta cells. However, *Rnls*^{mut} NIT-1 cells elicited a significantly weaker response from polyclonal beta cell-reactive CD8^{+} T cells isolated from diabetic NOD mice (**Figure 2**). We also transplanted *Rnls*^{mut} and control NIT-1 cells into opposite flanks of MHC-mismatched C57BL/6 mice and found that both beta cell grafts were rapidly destroyed by the strong allogenic response of host immune cells, showing that *Rnls* deficiency did not affect allo-rejection.

These data suggest that *Rnls*^{mut} beta cells are not impervious to immune detection or killing but that they are less prone to stimulating autoreactive CD8^{+} T cells.

Figure 2. Immune recognition by CD8+T cells (ELISPOT assay)

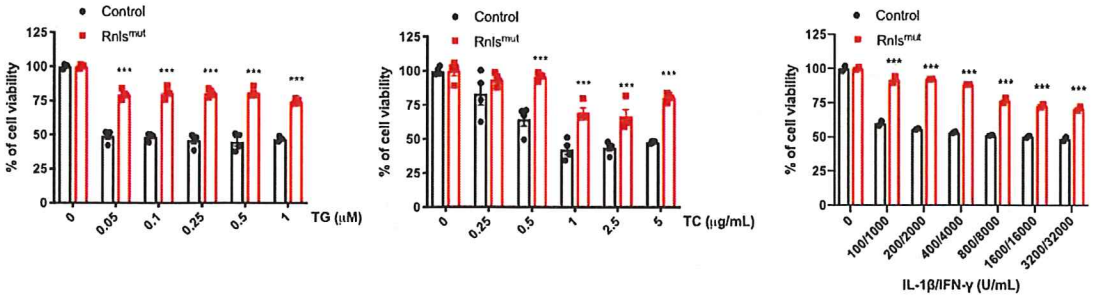


Rnls mutation confers ER stress resistance and modifies the cellular response to ER stress

As I proposed in the initial application, a growing body of evidence supports a role for ER stress in the demise of beta cells in diabetes. The unfolded protein response (UPR) that is triggered by ER stress has been implicated in beta cell apoptosis in both T1D and type 2 diabetes[3-5].

To test the effect of *Rnls* mutation on ER stress response, we challenged NIT-1 cells with the ER stressor thapsigargin (TG) and tunicamycin (TC). Control cells were highly sensitive to TG and TC treatment, while *Rnls*^{mut} cells withstood even a 20-fold greater concentration of TG and TC. Furthermore, *Rnls* mutation made cells resistant to the apoptotic effect of the inflammatory cytokines IL-1β and IFN-γ implicated in beta cell stress and killing in T1D (**Figure 3A**).

Figure 3A. Cell stress assay



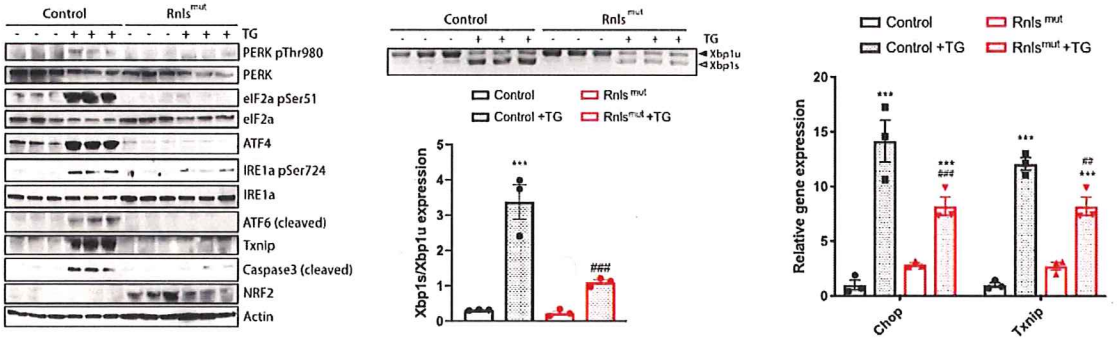
To exclude possible off target effects on ER stress resistance, we also tested ER stress resistance using additional cell lines in which *Rnls* exons 2 to 4 or exon 5 were deleted using different sets of gRNAs. These alternative *Rnls* deficient beta cell lines were also protected against ER stress-induced cell death, confirming that ER stress resistance was a direct result of *Rnls* deletion.

Then, we overexpressed *Rnls* in NIT-1 beta cells using a lentiviral transgene. While *Rnls* overexpression alone only marginally increased sensitivity to TG-induced killing, it

significantly accelerated the autoimmune killing of beta cells implanted into diabetic mice. We further proceeded to re-introduce *Rnls* into *Rnls*^{mut} cells using a transgene that carried a synonymous mutation within the gRNA target site to prevent CRISPR-Cas9 targeting. *Rnls* re-expression restored the sensitivity of *Rnls*^{mut} cells to ER stress and accelerated their autoimmune killing in diabetic NOD mice. Collectively, the data show that *Rnls* expression modulates the vulnerability of beta cells to ER stress and autoimmunity.

Next, we measured the UPR that mediates the cellular adaptation to ER stress. We found that the activation of critical ER stress sensors IRE1α, PERK and ATF6 and their downstream molecules, the phosphorylation of eIF2α, protein levels of ATF4 and splicing of XBP1 were all markedly reduced in *Rnls*^{mut} cells following TG treatment. The expression of *Chop* and *Txnip*, both implicated in ER stress-induced apoptosis, was also diminished (**Figure 3B**). The protective effect of *Rnls* deletion was not limited to ER stress, because *Rnls*^{mut} cells also better withstood oxidative stress compared to control NIT-1 cells. Consistent with this finding, *Rnls* deficiency increased the expression of a key regulator of the oxidative stress response, NRF2.

Figure 3B. Unfolded protein response to ER stress



Together these results strongly indicated that *Rnls* deficiency increases the ability of beta cells to withstand the cellular stress involved in their destruction during T1D.

The FDA-approved MAO-B inhibitor, Pargyline, phenocopies the protective effects of Rnls deletion

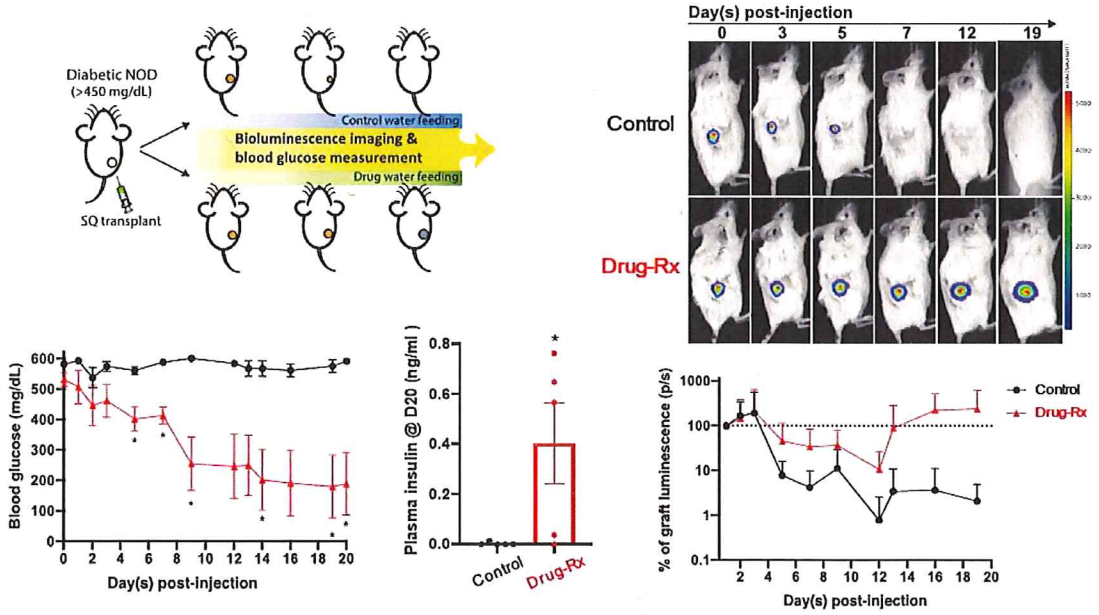
Rnls is a flavoprotein oxidase whose cellular function has not yet been elucidated. However, the crystal structure of human RNLS was solved several years ago. Based on structure-based molecular modeling, we predicted that one of the MAO-B inhibitors, Pargyline, would bind to *RNLS*. To test this prediction, we measured the thermal stability of human recombinant RNLS in the presence or absence of Pargyline and found that Pargyline decreased the thermal stability of RNLS in a dose-dependent manner, suggesting a direct interaction between Pargyline and RNLS.

Significantly, Pargyline decreased caspase-3 activation and increased the survival of NIT-1 cells following TG treatment. Moreover, the protective effect was also evident in primary islet cell cultures subjected to TG stress.

Then, we opted to evaluate the efficacy of Pargyline in a stringent beta cell transplantation model. Recently diabetic NOD mice with severe hyperglycemia were transplanted with NIT-1 beta cells with or without continuous feeding of Pargyline. Untreated mice remained hyperglycemic and rapidly lost their beta cell graft, while Pargyline treatment allowed transplanted beta cells to survive in diabetic mice, produce insulin and reverse hyperglycemia (**Figure 4**). Pancreas histology three weeks after diabetes onset and beta cell transplantation showed that Pargyline-treated mice still harbored a significant number of insulin-rich islets. In contrast, the pancreas of untreated diabetic mice was devoid of insulin staining. These observations suggest that Pargyline not only protected grafted NIT-1 beta cells but also endogenous beta cells against autoimmunity, recapitulating the protective effect of *Rnls* deletion.

We also found that Pargyline treatment was protective against diabetes induced by several approaches including cyclophosphamide injection, PD-1 blockade and immune cell transplantation. Furthermore, the drug also delayed diabetes induced by multi-low-dose streptozotocin in C57BL/6 mice, a distinct model for immune-mediated diabetes. These data suggest that Pargyline may carry potential as a preventive therapeutic for T1D.

Figure 4. The FDA-approved MAO-B inhibitor phenocopied *Rnls* deletion



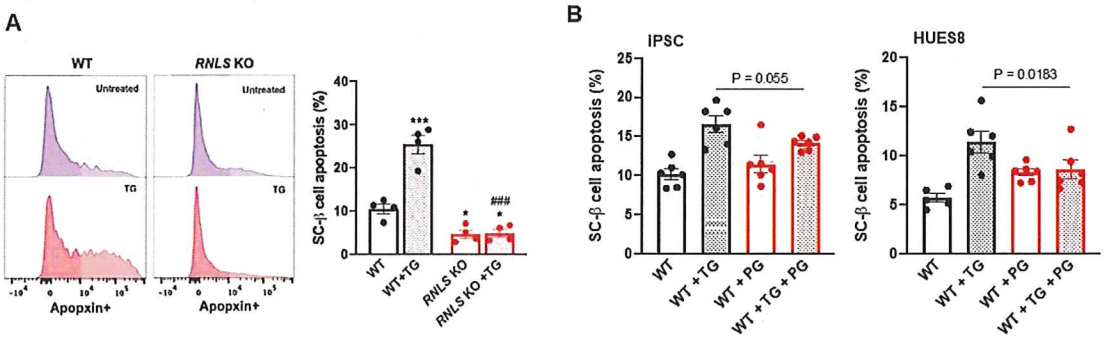
RNLS deletion confers ER stress resistance to human stem cell-derived beta cells

Then, we generated *RNLS* knockout (KO) human induced pluripotent stem cells (SC)

by CRISPR-Cas9 gene targeting. Significantly, *RNLS* KO human SC-beta cells were resistant to TG-induced apoptosis, reproducing the phenotype of *Rnls*^{mut} mouse beta cells (**Figure 5A**).

We also extended findings in the mouse beta cells by testing if the MAO-B inhibitors would replicate the protective effects of *RNLS* deletion in human SC-beta cells. We found that Pargyline (PG in figure 5) decreased ER stress-induced cell death in both induced pluripotent and embryonic SC-beta cells following TG treatment (**Figure 5B**), indicating that Pargyline phenocopies the protective effects of *RNLS* deletion in both mouse and human beta cells.

Figure 5. ER stress response in *RNLS*-KO human stem cell-derived beta cells



As I summarized above, we have found novel and significant findings for beta-cell protection against autoimmune diabetes, which would have a potential impact in this field and facilitate the development of diabetes research and its clinical applications. We put these novel findings in a manuscript and this manuscript was accepted and published in *Nature Metabolism*[6]. Our work was well appreciated by one of the prestigious journals and got a chance to be seen by a lot of readers, which will eventually lead to an advance in the relevant fields.

Also, I presented our work at several international conferences both in forms of poster and oral as I described in from 10-1.

Further suggestions for future experiments

Followings are what we planned for the next steps after publishing the paper. Although I was not able to carry out most of the experiments due to the COVID-19 outbreak and a subsequent shut-down of our research center, the Lab had started to re-open in mid-summer and the project was still ongoing when I left the Lab. Thus, those are worth a brief mention in this final report.

Since the detailed mechanism by which *RNLS* impacts on the cellular response to ER

and oxidative stress remains still obscure, we are still working on the detailed mechanisms as I proposed in the initial application.

NIT-1 cells are tumor cell lines and thus they proliferate in the recipient mice after transplantation, which eventually caused hypoglycemia after ~2 months of transplantation. Thus, a long-term observation is still missing in our data. In addition, considering the relatively low efficiency of lentiviral transduction, *Rnls*-KO primary islets will be an ideal source both to test in primary islets and to conduct long-term experiments. To this end, we are now generating *Rnls*-KO NOD mice using CRISPR-Cas9 system.

We are also planning to test the drug efficacy on primary islets against autoimmune killing by transferring primary islets into diabetic NOD mice treated with or without the drug. The effect on recognition by diabetogenic CD8+ cells are going to be tested using ELISPOT assay as we did on NIT-1 cells.

Most of the findings summarized above were obtained from the murine primary islet or its cell-line and the effect of *RNLS* deletion on human beta cells is relatively lacking. We are going to test whether RNLS deletion affect human CD8+ T-cell response as we tested on murine islets.

Together these experiments will further elucidate the detailed mechanism of protection of *RNLS* deletion in human beta cells, clarify a missing link between resistance to cellular stress including ER stress, oxidative stress, or cytokine stress, and protection against autoimmunity, and accumulate further evidence of drug development with higher specificity.

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