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Assessing the Comparative Effectiveness of Upregulation of Beta Cell Identity Genes and Downregulation of Senescence-Associated Markers for Senescence Reversal: A Research Protocol

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Abstract

Introduction: Type 1 diabetes (T1D) is a chronic condition characterized by the immune-mediated destruction of pancreatic beta cells, resulting in insulin deficiency and elevated blood glucose levels. Despite substantial advancements in understanding the pathogenesis, epidemiology and management of T1D, a treatment for the disease is yet to be discovered. Diabetic environment appears to trigger cellular senescence in a subset of beta cells resulting in proliferation arrest and drastic phenotypic and genotypic changes in these cells. It has been suggested that senescent beta cells may exacerbate T1D progression by establishing a senescence-associated secretory profile (SASP), contributing to chronic inflammation and tissue dysfunction. Despite the potential therapeutic significance of overturning senescence, the optimal approach for such intervention is largely overlooked. This proposal, therefore, aims to unveil the relative effectiveness of two predominant strategies in senescence reversal.

Methods: Senescence is induced through UV irradiation and doxorubicin treatment in beta cells, extracted from pancreases of male and female NOD mouse models. Lipid Nanoparticle (LNP) delivery is subsequently used to overexpress two beta-cell identity genes and underexpress two senescence markers in different conditions. Changes in the expression of the predominant SASP factors, IL-6, IL-8, and TNF-α are measured and compared through Nanostring technology and one-way ANOVA, respectively. This could quantify the absolute and relative effectiveness of the aforementioned strategies in senescence reversal.

Discussion: Both reversal mechanisms are anticipated to successfully overturn senescence, which can be indicated by a significant decrease in the mean levels of SASP factors post-treatment. Nevertheless, downregulation of senescence markers may be the more effective of the two, yielding more substantial results with regards to senescence reversal. It directly addresses the issue of senescence without overwhelming cellular machinery. In addition, fewer compensatory mechanisms seem to be associated with senescence-associated genes, hence the treatments are expected to be longer lasting.

Conclusion: Results of this study can contribute to the development of therapeutic regimens for diabetes prevention and reversal. Moreover, these findings have broad applicability across various contexts where senescence reversal is of value.

Keywords: diabetes; senescence; senescence reversal; SASP; lipid nanoparticle delivery; senescence markers; beta cell identity genes

Introduction

Type 1 diabetes (T1D) is an autoimmune disease marked by the depletion of insulin-producing pancreatic β-cells, which, if left untreated, can result in chronic hyperglycemia and death [1]. The global prevalence of diabetes was estimated to be 9.3% (463 million people) and is predicted to increase by 25% in 2030, with approximately 10% of diagnosed cases presenting with T1D [2].

To this date, no definitive cure has been discovered for either of the DM subtypes. Since insulin deficiency is the underlying issue behind T1D, lifelong exogenous insulin administration, islet transplantation and insulin analogs are the prominent treatment regimens for T1D. However, severe episodes of hypoglycemia, insulin resistance, and psychiatric disorders are occasionally observed in patients undergoing insulin-centered approaches, calling for alternative strategies to achieve glycemic control [3]. New efforts focus on developing stem-cell derived and immunotherapeutic regimens to target several subcategories of diabetes mellitus, including T1D. However, such interventions do not appear to be equally responsive across all patient demographics and have yet to be optimized and translated into the clinic [4].

Cellular senescence is a phenomenon where cells cease to proliferate and undergo phenotypic changes that compromise their function [5]. Aguayo-Mazzucato et al. [6] reported that a subset of β-cells exhibit unique senescence-associated secretory profiles (SASP) which can be triggered by the diabetic environment. More specifically, senescent β-cells demonstrated increased expression of aging markers and genes that are typically repressed in β-cells, in addition to the loss of β-cell identity genes when compared to their normal counterparts. Previous studies indicate that senescent beta cells might accelerate T1D progression by inciting a stronger immune attack against insulin-producing islet cells, impairing glucose metabolism [5,7].

While current research efforts focus on using senolytics to remove senescent β-cells [8], a more optimal approach would involve the restoration of healthy β-cells to preserve overall β-cell mass. Indeed, β-cell senescence may be partially reversible; removal of metabolic stressors has been proven to restore β-cell identity and function, re-establishing insulin homeostasis and normoglycemia [6]. Here, we propose to reverse β-cell senescence by using lipid nanoparticles (LNPs) for the delivery of therapeutics that i) upregulate the expression of β-cell identity markers or ii) downregulate the expression of senescence-associated markers.

LNPs can be used for the delivery of pharmaceuticals with robust applications in modulating translational activity [9]. These spherical vesicles are composed of ionizable lipids that enable the encapsulation of nucleic acids, including messenger RNA (mRNA) and small interfering RNA (siRNA). Their small size and lipid-based nature enhance cellular uptake via endocytosis. LNPs possess a neutral charge at physiological pH but become positively charged upon exposure to the low pH of the endosomes. As a consequence, the lipid membrane of LNPs will be disrupted, resulting in their cargos to be released in the cytoplasm of host cells [10, 11]. Therefore, LNPs facilitate tissue-specific delivery of target molecules while protecting them from degradation [12]. LNPs became clinically prominent during the development of COVID-19 mRNA vaccines. Biocompatibility, biodegradability and ease of manufacture associated with LNPs make them a promising candidate for improved drug delivery in other contexts [13]. In this proposal, LNPs will serve as a vehicle for cytoplasmic transportation of mRNA or siRNA encoding proteins that will reverse β-cell senescence by restoring β-cell identity or altering β-cell SASP, respectively.

The proposed study will provide proof-of-principle of two novel approaches to reverse senescence of β-cells derived from an established mouse model of T1D. These approaches may serve not only to prevent but also reverse type 1 diabetes in mice. Because LNPs have been clinically proven as safe drug delivery systems, assessing the ability of the aforementioned techniques to reverse β-cell senescence holds significant translational value for human applications. Cellular senescence has also been identified as a contributing factor in type 2 diabetes and other age-related diseases [14]. The findings of this study may therefore eventually allow for the development of therapeutic regimens for several diseases where senescence reversal is particularly advantageous.

Methods

Culturing Pancreatic Beta Cells

Pancreatic islets will be isolated from neonatal male and female NOD mice and subsequently purified [15]. The purified islets will then be evenly distributed among three 60-mm collagen-coated culture dishes and cultured in a suitable culture medium to support their survival, growth, and function. Culture dishes should be maintained in a controlled environment at 37°C, 5% CO2 and 5% O2 [16]. To sustain optimal culture conditions, culture media will be changed frequently, with the frequency determined through monitoring the health and morphology of beta cells under the microscope. Upon detection of any signs of nutrient depletion, waste accumulation or contamination, the frequency of media changes will be increased.

Confirmation of Beta Cell Identity

Nanostring analysis will be performed on beta cells to establish the baseline expression of genes associated with beta cell identity and senescence prior to experimental manipulation. Gene expression of the predominant SASP factors [17] interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor - alpha (TNF-α), in addition to beta cell identity genes *INS1* and *NKX6.1*, will be quantified and compared to the literature.

Senescence Induction

To induce senescence *ex vivo*, media is removed from the plates and the beta cells are irradiated with UVB of 20 mJ/cm^2. The beta cells will then be subsequently incubated in 2 mL of culture media at 37°C, 5% CO2 and 5% O2 for another seven days, with the media being replaced approximately every three days. Doxorubicin-induced senescence will be performed on day 8 upon aspirating the culture media. Following that, 10 mL of 250 nM solution of Doxorubicin diluted in culture medium should be added to the plates. After a 24-hour incubation period, the plates will be re-aspirated and carefully washed with 10 mL of medium. The cells will eventually be transferred into 10 mL of media for an additional six days, with the media being refreshed every three days [18].

Confirmation of Beta Cell Senescence

To confirm beta cell senescence, Nanostring analysis will be performed to measure the expression levels of IL-6, IL-8 and TNF-α. One-way ANOVA will be used to determine if there was a statistically significant (P-value <0.05) change in the expression levels of the SASP factors under investigation. Significantly elevated levels of SASP factors will confirm beta-cell senescence and indicate their

suitability for the experimental intervention; lipid nanoparticle (LNP) delivery.

Lipid Nanoparticle (LNP) Delivery

Senescent beta cells will be treated with the following LNP formulations which would be ordered and purchased from a provider: (1) LNPs containing mRNA encoding *INS1* and *NKX6.1* to upregulate beta cell identity genes, 2) LNPs containing siRNA targeting *P16* ^{Ink4a} and *P19* ^{Arf} to downregulate senescence-associated markers, or 3) LNPs loaded with nonsense RNA oligonucleotide, each followed by a 24-hour incubation period.

Alterations in Gene Expression of SASP and Beta Cell Markers: Quantitative Assays

Nanostring analysis will be conducted on the treated beta cells to quantify expression levels of IL-6, IL-8 and TNF-α, in addition to the targeted beta cell identity genes and senescence-associated markers, 24 hours post LNP delivery. One-way ANOVA will be initially performed on beta cell specific genes and senescence markers to assess whether the treatments have had the intended effect. A significant increase in the expression of beta cell identity genes in group 1 and a reduction in the transcriptional output of senescence markers in group 2 would indicate that any changes in SASP concentrations could be attributed to the treatments. The extent of senescence reversal will then be investigated by performing a one-way ANOVA to assess whether there are significant changes in the expression levels of SASP cytokines before and after LNP deliveries. To further evaluate the degree of reversal, another one-way ANOVA will be conducted to compare the transcriptional output of these SASP factors prior to senescence induction and following LNP treatment. This would provide insight into the effectiveness of the treatment conditions in restoring these SASP markers to their baseline expression levels. The latter ANOVA can be further used to evaluate the relative efficacy of the two genetic interventions being studied in reversing senescence: upregulation of beta cell identity genes or downregulation of senescence-inducing genes.

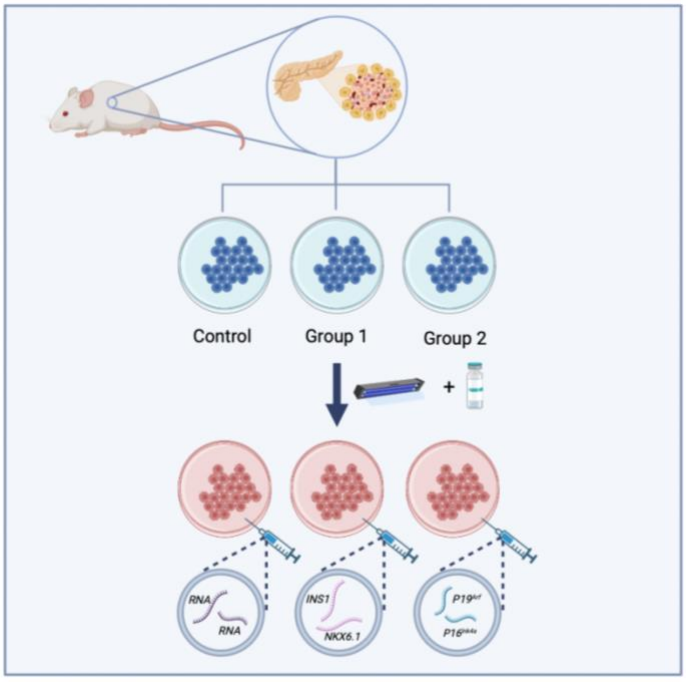

Figure 1. Overview of the experimental protocol. Pancreatic beta cells from neonatal male and female NOD mice will be isolated, purified and subsequently cultured in three dishes. After which, senescence will be induced via UV irradiation and doxorubicin treatment. Senescent beta cells will be subsequently treated with (1) LNPs loaded with nonsense mRNA oligonucleotide, 2) LNPs containing mRNA encoding *INS1* and *NKX6.1* to upregulate beta cell identity genes, or 3) LNPs containing siRNA targeting *P16* ^{Ink4a} and *P19* ^{Arf} to downregulate senescence-associated markers, with the premise of investigating their relative effectiveness in senescence reversal. The illustration was created using BioRender software.

Results

Inhibiting the expression of senescence markers and overexpression of beta cell identity genes are both expected to partially reverse senescence, however the efficacy of these treatments is unlikely to be equal. Upon LNP delivery, a statistically significant decrease in the mean levels of IL-6, IL-8 and TNF-α is expected in both conditions compared to their levels after senescence induction which will be measured via Nanostring analysis as outlined in the methodology. A greater reduction of

SASP levels is anticipated where senescence-associated markers are knocked down whereas the control group is unlikely to exhibit significant differences post-treatment. Furthermore, there is a low likelihood of post-treatment SASP concentrations returning to their baseline levels prior to senescence induction. Hence, we anticipate a statistically significant difference in the mean levels of SASP factors after senescence reversal is attempted compared to their pre-senescent concentrations.

Discussion

In this proposal, we are utilizing cutting-edge LNP technology to investigate the relative impact of upregulation of beta cell-specific genes and downregulation of senescence markers on senescence reversal. This would be accomplished through measuring changes in expression levels of predominant SASP factors: IL-6, IL-8 and TNF-α.

Any statistically significant decrease in the mean levels of the SASP factors pre- and post-experimental manipulation can be attributed to the effectiveness of the aforementioned approaches, with more drastic decreases indicating higher potency in overturning senescence. Therefore, it can shed light on the relative efficacy of the two approaches, which is the primary focus of this research proposal.

Overexpression of beta cell identity genes is anticipated to facilitate senescence reversal by enhancing beta cell functionality. However, a potential drawback is that it can overwhelm the cellular machinery, imposing a burden on these cells. In addition, beta cells are associated with various compensatory mechanisms, hence any attempts to modulate the transcriptional activity of these genes may be countered by the cells to some extent. Contrastingly, inhibiting the expression of senescence markers directly addresses the issue of senescence in beta cells without putting a strain on them. Since senescence markers are not typically active in beta cells, there are likely fewer compensatory mechanisms associated with these genes. Therefore, SASP levels are predicted to decrease more profoundly upon implementing this strategy compared to upregulating beta cell-specific genes.

Comparing SASP levels prior to senescence induction and post-LNP treatments would further determine the degree of senescence reversal. This would establish the absolute effectiveness of the experimental strategies, which would be an intriguing discovery regardless of the relative efficacy of the two methods. No significant difference in the mean expression levels of these cytokines implies a complete reversal, which is highly unlikely, given that we are only manipulating transcriptional activity of a few genes in each condition. The more likely result would be observing a significant difference in the baseline SASP levels, and their concentrations post treatment, hinting at a partial reversal.

The results of this study could lead to designing treatment regimens targeted at diabetes reversal, which is a complex and relatively unexplored area of diabetes research. This would be paramount considering that most therapeutic approaches are currently directed towards diabetes prevention. In addition, LNP-induced transcriptional changes render this treatment clinically promising due to the ease of administration and successful prior experience with LNPs. Furthermore, the findings of this study can be applied to various anti-aging contexts where senescence and its reversal are of interest. Acknowledging that these findings may relate specifically to beta cells, it is more likely that they are universal, given that the underlying pathways for senescence appear to be shared across tissue types. Thereby, it can provide insights into the most effective way to restore cellular functionality in light of senescence.

Conclusions

Senescence in beta cells has long been associated with drastic changes in the phenotypic profiles of these cells, hindering their function and exacerbating diabetes progression. Consequently, overturning senescence is paramount in re-establishing insulin homeostasis, arresting disease advancement, and potentially reversing it. The premise behind this proposal is to delineate the optimal strategy for senescence reversal by using novel LNP technology. We are aiming to determine the extent to which alterations in the transcriptional output of beta cell identity genes and senescence-associated markers would reverse beta cell senescence. The findings of this study have the potential to be translated into the clinic, leading to development of therapeutic regimens targeted towards diabetes prevention and reversal. Additionally, results of this project can be extended to numerous contexts where senescence seems to be detrimental, and its reversal is desirable.

A potential limitation of this proposal may be the number of selectively regulated genes in each experimental condition. Even though modifying expression levels of two genes per group is anticipated to leave a considerable impact on the senescent beta cells, increasing the number of targeted genes in each group may yield more substantial results. Additionally, we have proposed measuring changes in the expression levels of predominant SASP factors, IL-6, IL-8, and TNF-α to quantify senescence. In future studies, measurements could be extended to include additional SASP factors such as Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), and Transforming Growth Factor-beta (TGF-β) to obtain a more comprehensive understanding of senescence. It would be particularly intriguing to merge the two suggested strategies and examine their combined effects on senescence reversal. Furthermore, upon obtaining significant results *in-vitro*, it is crucial to replicate this experiment in an *in vivo* setting. This would enable us to assess the effectiveness of these LNP-centered treatments when confronted with challenges and complexities pertinent to the whole organism.

It is noteworthy that in our proposal, the process of senescence reversal is initiated immediately following

induction. However, such immediacy is not feasible within clinical settings. T1D patients are typically situated at various points along the senescence spectrum. Therefore, future experiments should aim to determine the optimal timeframe following senescence induction during which reversibility is still achievable. Moreover, it is imperative to explore whether senescence reversal is sufficient to reverse diabetes prior to clinical translation as there are various genetic and environmental factors involved in T1D progression.

List of Abbreviations Used
T1D: type 1 diabetes
SASP: senescence-associated secretory profile
UV: ultraviolet
NOD: non-obese diabetic
LNP: lipid nanoparticle
IL-6: nterleukin-6
IL-8: interleukin-8
TNF-α: tumor necrosis factor – alpha
mRNA: messenger ribonucleic acid
siRNA: small interfering ribonucleic acid
UVB: ultraviolet B
IL-1α: interleukin – 1 alpha
IL-1β: interleukin – 1 beta
TGF-β: tumor growth factor – beta

Conflicts of Interest
Author(s) declare that they have no conflict of interests.

Ethics Approval and/or Participant Consent
This research protocol did not require ethics approval and/or participant consent.

Authors' Contributions
AHK: Contributed exclusively to the design of the study, drafted and revised the manuscript, and gave final approval of the version to be published.

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