Brief Report

Stem Cell INVESTIGATION

The effect of short-term necrostatin-1 treatment on the differentiation of human induced pluripotent stem beta cells

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Abstract: Despite ongoing effort over the past decade, current protocols have been unable to generate mature functional beta cells from human induced pluripotent stem cells (hiPSCs). Based on our previous findings that short-term necrostatin-1 (Nec-1) treatment enhanced pancreatic endocrine cell differentiation and insulin secretion capacity of young porcine islets, we assessed whether short-term treatment of Nec-1 can improve the differentiation and function of hiPS beta cells. After 3 days of culture, hiPS beta cells, cultured in either control differentiation media (n=3) or supplemented with Nec-1 (100 µM, n=3), were evaluated for viability, cellular composition, GLUT2 expression in beta cells, and glucose-stimulated insulin expression. While the viability and levels of beta-, alpha-, and GLUT2-positive beta cells were unaffected, the level of insulin- and glucagon-positive bi-hormonal cells was significantly lower in Nec-1 treated hiPS beta cells. Short-term Nec-1 treatment also did not affect the insulin secretion ability of hiPS beta cells. The addition of Nec-1 to the differentiation media of hiPS beta cells reduced the number of bi-hormonal cells after short-term culture, suggesting that future studies should evaluate different concentrations and treatment duration of Nec-1.

Keywords: Diabetes; human induced pluripotent stem beta cells (hiPS beta cells); islets; necrostatin-1 (Nec-1); short-term culture

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Introduction

Type 1 diabetes mellitus (T1DM) is T-cell moderated disorder characterized by autoimmune destruction of insulin-producing pancreatic beta cells, resulting in severe insulin deficiency, hyperglycemia, and metabolic disorientation (1). Patients suffering from hyperglycemia as a result of T1DM experience manifestations of discomforting symptoms including polydipsia, polyuria, and diabetic ketoacidosis that could lead to death (1). To ameliorate the threatening consequences of insulin deficiency, daily self-administered exogenous insulin therapy is being used to rectify the lack of insulin produced (2). Although this method can regulate blood glucose levels, variability in the suboptimal absorption of subcutaneously absorbed insulin and imprecise dosing to achieve proper glycemic control have been reckoned as an impediment to this method of treatment (3).

To relieve patients of life-long exogenous insulin therapy, pancreatic islet transplantation has been explored as a promising cure for T1DM (4). Islet allotransplantation has proven to be a propitious method of beta cell replacement that can induce glycemic balance and healthy insulin production (5). Established in 2000 as the Edmonton Protocol, allotransplantation of pancreatic islet cells is performed through preparation of pancreases from deceased donors and transplantation into recipients to achieve sufficient glycemic control for extended periods of time (6). In 2000, seven patients who received islet allografts in conjunction with a glucocorticoid-free immunosuppressive

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regimen were able to sustain insulin dependency and euglycemia, an unprecedented success rate in the world of clinical islet transplantation (6). In 2007, islet cell allotransplantation was proven to successfully restore long-term endogenous insulin production and glycemic control in T1DM patients. Despite the achievements made, insulin independence was found to be gradually lost over time (7). Furthermore, islet transplantation requires islets from 2–4 donors per recipient in order to promote the engraftment of sufficient insulin-producing cells to achieve insulin independence. Due to the critical scarcity of donors and imperfect isolation efforts, few patients with T1DM have been able to obtain islet allografts to restore euglycemia (8).

Efforts to address the shortage of donors have introduced studies involving the utilization of human induced pluripotent stem cells (hiPSC) to produce mature insulin-producing beta cells. HiPSCs are cells derived from somatic cells through a method of reprogramming to induce conversion into pluripotent embryonic stem cells (9). HiPSCs provide the advantage of eliminating immune rejection through the utilization of patient-specific cells (10). This has, however, been an ongoing progress. Functional beta cells derived from hiPSCs have been evidenced to display an increase in the glucose threshold for insulin secretion (11). The protocol to generate mature insulin-producing beta cells from hiPSCs to successfully demonstrate such function of endogenous beta cell *in vitro* has yet to be found. Insulin expressing cells derived from hiPSCs express insulin lack the glucose responsiveness characteristic of mature endogenous beta cells (11,12). Failure to devise a successful hiPSC differentiation protocol to give rise to mature insulin-producing beta cells has resulted in the conduction of a myriad of studies to find the missing piece to current targeted differentiation protocols (11,13,14). Pathway analysis has revealed that the LXR/RXR pathway was inhibited in S7 hiPS beta cells, associating with a high proliferation index as indicated by an increased Ki67 staining compared to human islet cells (15). The Wnt/beta-catenin and PCP signaling pathways have also been identified as promising targets for improving *in vitro* beta cell maturation (15,16). Despite strong evidence suggesting that Wnt signaling could induce the final maturation of S7 hiPS beta cells, a recent study has shown that Wnt modulation at the S7 stage of hiPSCs only modestly promoted beta-cell-like maturation with no improvement in the glucose-stimulated insulin secretion (17). Thus, efforts must be made to identify molecules that could promote the final differentiation step of hiPSCs into mature functional beta cells.

Necrostatin-1 (Nec-1) is a small-molecule allosteric inhibitor of the death domain receptor-interacting protein kinase 1 (RIPK1) (18). The protective effects of short-term Nec-1 treatment on preventing necroptosis and reducing cell death in multiple cell types and animal models of ischemic injury have been studied extensively (19,20). However, the function of Nec-1 in pancreatic islets and beta cells has not been well characterized. In one study, Nec-1 treatment of beta cells lines and isolated mouse islets for 24 hours decreased the release of DAMPs and improved cell survival after nitric oxide exposure (21). Our recent study has demonstrated the novel effects of 3-day Nec-1 treatment to enhance the islet insulin content, pancreatic endocrine cell differentiation, GLUT2 expression, and insulin secretion of young porcine islets (22). Furthermore, prolonged 7-day culture with Nec-1 did not significantly improve the level of beta cells and insulin secretion stimulation index (SI) compared to 3-day culture (22). As young porcine islets have been reported to contain a major stem-cell like population similar to hiPSCs that is capable of differentiating into mature endocrine cells, we hypothesized that short-term exposure to Nec-1 may affect the *in vitro* differentiation of hiPSCs into mature functional beta cells (23). In this present study, we aimed to determine whether short-term Nec-1 treatment during *in vitro* culture could improve the differentiation and function of hiPS beta cells.

Materials and methods

Cell source, culture, and Nec-1 treatment

HiPS beta cells, derived from ChiPSC22 cells that were reprogrammed from normal healthy skin fibroblasts, was purchased from Cellartis (cat# Y10100, Takara Bio, Mountain View, CA, USA). Cryopreserved hiPS beta cells were thawed and cultured for maturation according to the manufacturer's protocol. In brief, thawed cells were cultured in Beta Cell Maintenance Medium at a cell density of 2.0×10^5 viable cells/cm^2 in a 37 ℃, 5% CO_2 humidified incubator (cat# 3110, Thermo Forma Series II 3120 Water Jacketed CO_2 Incubators, Carlsbad, CA, USA). On day 12 of culture, cells were divided into 2 groups for culture in Beta Cell Medium 2 alone (n=3) or supplemented with Nec-1 (100 μM, Abcam, Cambridge, UK, cat# ab141053, n=3) until day 15 (22). A full media change was performed on day 1 after thawing and every other day thereafter. Cells were collected after thawing, before Nec-1 treatment (day

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12 of culture) and after Nec-1 treatment (day 15 of culture) for assessment.

Flow cytometry

Flow cytometry was used to characterize hiPS beta cells after thawing, before Nec-1 treatment, and after Nec-1 treatment as previously described (22). HiPS beta cells were stained with 7-AAD viability dye (7-aminoactinomycin D; cat# A1310, Invitrogen, Carlsbad, CA, USA) for 30 minutes on ice after thawing, before Nec-1 treatment, and after Nec-1 treatment. Stained cells were fixed in 4% paraformaldehyde for 10 minutes and permeabilized using Intracellular Staining Permeabilization Wash Buffer (cat# 421002, BioLegend, San Diego, CA, USA) for 15 minutes on ice. After permeabilization, cells were incubated in Protein Block (cat# ab64226, Abcam) for 30 minutes on ice to reduce non-specific binding. Blocked cells were stained with fluorescently conjugated antibodies for 30 minutes on ice in Intracellular Staining Permeabilization Wash Buffer (cat# 421002, BioLegend) supplemented with 0.5% bovine serum albumin (cat# BAL62-0500, Equitech-Bio, Inc., Kerrville, TX, USA). PE conjugated anti-insulin (anti-insulin-PE; cat# 8508, CST, Danvers, MA, USA) antibody was used as a marker for beta-cells. APC conjugated anti-glucagon (anti-glucagon-APC; cat# NBP2-21803AF647, Novus Biological, Littleton, CO, USA) antibody was used as a marker for alpha-cells. FITC-conjugated anti-GLUT2 (cat# FAB1414G-100UG, Novus Biological) and PE conjugated anti-insulin (cat# 8508, CST) antibodies were used to identify GLUT2-positive beta cells. After staining, cells were analyzed on a NovoCyte 3000VYB Flow Cytometer (ACEA Biosciences, Inc., San Diego, CA, USA) and quantified using FlowJo software (FlowJo, Ashland, OR, USA). Gating controls included unstained, single-stained, fluorescence minus one, and matching isotype samples.

Glucose-stimulated insulin secretion

The insulin secretion in response to glucose stimulation of hiPS beta cells after thawing, before Nec-1 treatment, and after Nec-1 treatment was determined using glucose-stimulated insulin release assay (22). HiPS beta cells were incubated at 37 ℃ and 5% CO_2 for 1 hour in the following order: low glucose #1 (2.8 mM; L1), high glucose (28 mM; H), low glucose #2 (2.8 mM; L2), and high glucose plus 3-isobutyl-1-methylxanthine (28 mM + 0.1 mm IBMX; H+) media. The insulin secreted by hiPS beta cells in each glucose media was analyzed using a standard human insulin enzyme-linked immunosorbent assay (Human Insulin ELISA; cat# 10-1113-01, Mercodia, Winston Salem, NC, USA) and quantified on a microplate reader (Infinite F200, Tecan and Magellan V7, Männedorf, Switzerland). The amount of insulin was normalized to the DNA content of each sample and expressed as pg of insulin/ng of DNA/h. The SI was calculated as the ratio of the amount of insulin secreted in the high glucose media (H) over the amount of insulin secreted in the first low glucose media (L1).

DNA content

HiPS beta cells were lysed, and the DNA content was analyzed as previously described (24). In brief, hiPS beta cells were incubated in cell lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, pH 8) and sonicated (Sonics VibraCell Ultrasonic Processor Model VC70T; Sonics & Materials, Inc, Newtown, CT, USA) for 30 seconds on ice. After centrifugation, the DNA content in the supernatant was quantified using a fluorescent DNA stain (Quant-iT PicoGreen dsDNA kit; cat# Q32850, Molecular Probes, Eugene, OR, USA) and read on a microplate reader (Infinite F200, Tecan and Magellan V7, Männedorf, Switzerland).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). A one-way ANOVA followed by a post hoc Tukey's HSD test was used to determine statistical significance. A P value <0.05 was considered to be statistically significant. Statistical analysis was performed on GraphPad Prism (GraphPad Software 8.0.1, San Diego, CA, USA).

Results

Characterization of hiPS beta cells after thawing

Flow cytometry was used to characterize the viability and cellular content of hiPS beta cells after thawing. The viability of hiPS beta cells after thawing was 87.70%±0.52% (*Table 1*). The beta-cell and alpha-cell composition was 39.60%±1.16% and 3.62%±0.17%, respectively (*Table 1*). The level of insulin- and glucagon-positive bi-hormonal cells after thawing was 2.10%±0.32% (*Table 1*). The percentage of GLUT2-positive beta cells was 13.26%±5.51% (*Table 1*).

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Table 1 Flow cytometric analysis of viability, cellular composition and GLUT2 expression in beta cells of hiPS beta cells after thawing

	Viability (%)	Beta cells (%)	Alpha cells (%)	Insulin + glucagon + cells (%)	GLUT2 + beta cells (%)
After-thawing	87.70±0.52	39.60±1.16	3.62±0.17	2.10±0.32	13.26±5.51

hiPS beta cells were thawed according to the manufacturer's protocol, stained with 7-AAD viability dye, anti-insulin, anti-glucagon, anti-GLUT2 antibodies, and analyzed by flow cytometry. hiPS, human induced pluripotent stem; 7-AAD, 7-aminoactinomycin D.

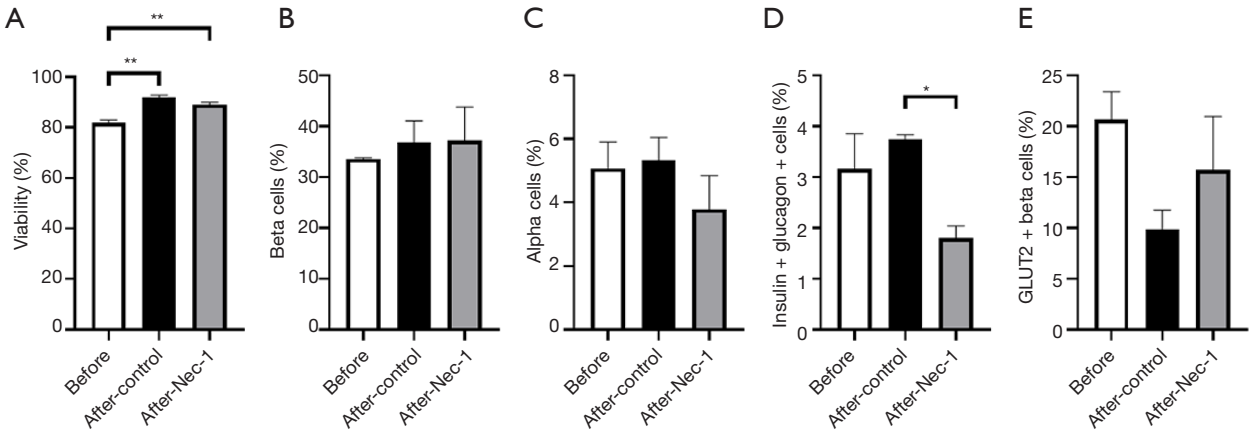

Figure 1 Flow cytometric analysis of viability, cellular composition and GLUT2 expression in beta cells of hiPS beta before and after Nec-1 treatment. hiPS beta cells were stained with 7-AAD viability dye, anti-insulin, anti-glucagon, anti-GLUT2 antibodies and analyzed by flow cytometry. (A) The viability of hiPS beta cells (n=3 for each group); (B) the percentage of beta cells (n=3 for each group); (C) the percentage of alpha cells (n=3 for each group); (D) the percentage of insulin- and glucagon-positive cells (n=3 for each group); (E) the percentage of GLUT-2-positive beta cells (n=3 for each group). *, P< 0.05; **, P<0.01. Data expressed as mean ± SEM. hiPS, human induced pluripotent stem; Nec-1, necrostatin-1.

Characterization of hiPS beta cells before and after short-term Nec-1 treatment

Both untreated (91.73%±1.05%) and Nec-1-treated hiPS beta cells (89.13%±0.85%) on day 15 of culture had a significantly higher viability than hiPS beta cells before Nec-1 treatment (81.83%±1.16%) on day 12 of culture (P<0.01, *Figure 1A*). There was no significant difference in the beta-cell composition of hiPS beta cells before Nec-1 treatment (33.60%±0.21%) and after short-term Nec-1 treatment (control: 36.83%±4.23%, Nec-1 treated: 37.23%±6.56%) (P=NS, *Figure 1B*). Similarly, no significant difference in the level of alpha cells was observed in hiPS beta cells before Nec-1 treatment (5.08%±0.82%) and after 3-day culture in either control (5.32%±0.74%) or Nec-1-treated media (3.79%±1.06%) (P=NS, *Figure 1C*). On day 15 of culture, hiPS beta cells cultured in media supplemented with Nec-1 (1.80%±0.25%) had a significantly lower composition of insulin- and glucagon-positive bi-hormonal cells compared to untreated hiPS beta cells (3.75%±0.083%) (P<0.05, *Figure 1D*). The percentage of insulin- and glucagon-positive bi-hormonal cells in hiPS beta cells before Nec-1 treatment (3.16%±0.70%) was similar to hiPS beta cells cultured in either control or Nec-1 treated media on day 15 of culture (P=NS, *Figure 1D*). There was no significant difference in the composition of GLUT2-positive beta cells between hiPS beta cells before (20.67%±2.75%) and after short-term Nec-1 treatment (control: 9.90%±1.86%, Nec-1 treated: 15.73%±5.23%) (P=NS, *Figure 1E*).

Glucose-stimulated insulin secretion of hiPS beta cells before and after short-term Nec-1 treatment

Nec-1 treated hiPS beta cells (L1: 0.067±0.0044 pg insulin/ng DNA/h, H: 0.065±0.0042 pg insulin/ng DNA/h, L2: 0.060±0.0037 pg insulin/ng DNA/h, H+: 0.070±0.0091 pg insulin/ng DNA/h) secreted a similar amount of insulin during glucose stimulation in comparison to control hiPS beta cells (L1: 0.073±0.0017 pg insulin/ng DNA/h,

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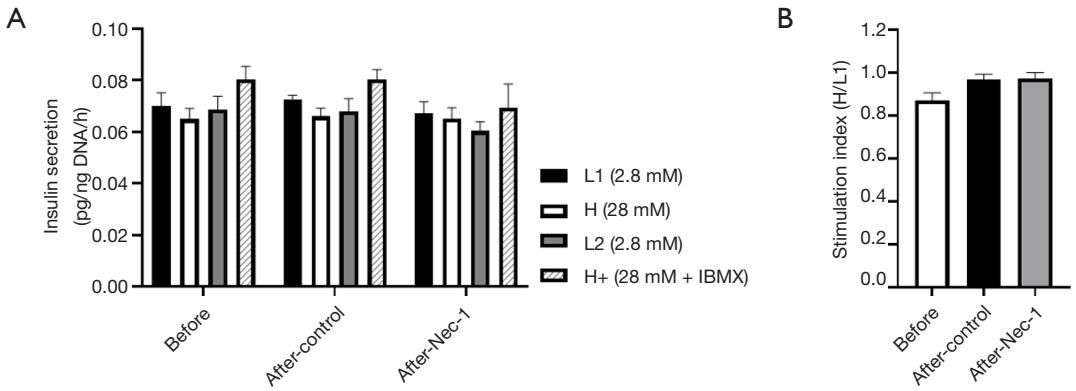

Figure 2 Insulin secretion of hiPS beta cells in response to glucose stimulation before and after Nec-1 treatment. Glucose stimulated insulin release assay was used to evaluate the insulin secretion of hiPS beta cells in response to glucose stimulation. hiPS beta cells were incubated for one hour in the following order: 2.8 mM (L1), 28 mM (H), 2.8 mM (L2), and 28 mM + 0.1 mM IBMX (H+) glucose media. The amount of insulin secreted was quantified using standard human insulin ELISA and normalize to the DNA content in each sample. (A) Insulin secretion of hiPS beta cells during glucose stimulation (n=3 for each group); (B) stimulation index of hiPS beta cells—calculated as the amount of insulin secreted in H glucose media over L1 glucose media (n=3 for each group). Data expressed as mean ± SEM. hiPS, human induced pluripotent stem; Nec-1, necrostatin-1.

H: 0.066±0.0031 pg insulin/ng DNA/h, L2: 0.068± 0.0049 pg insulin/ng DNA/h, H+: 0.080±0.0037 pg insulin/ng DNA/h) and hiPS beta cells before Nec-1 treatment (L1: 0.070±0.0051 pg insulin/ng DNA/h, H: 0.065±0.0042 pg insulin/ng DNA/h, L2: 0.069±0.0053 pg insulin/ng DNA/h, H+: 0.080±0.0050 pg insulin/ng DNA/h) (P=NS, *Figure 2A*). The SI was also similar between hiPS beta cells before (0.87±0.036) and after short-term Nec-1 treatment (control: 0.97±0.026, Nec-1 treated: 0.97±0.028) (P=NS, *Figure 2B*).

Discussion

Inducing the final differentiation of hiPSCs into functionally mature beta cells still remains a challenge even with the current state-of-the-art differentiation protocol. Thus, the identification of a signaling pathway or a molecule to promote the final step of beta-cell differentiation is vital to generate mature insulin-producing beta cells from hiPSCs. We have previously reported that Nec-1 treatment during short-term culture can facilitate pancreatic endocrine cell differentiation and enhance GLUT2 expression, leading to an improvement in insulin secretion of young porcine islets (22). In the present study, we are the first to examine the effect of Nec-1 on the differentiation and function of hiPS beta cells after short-term *in vitro* culture. We found that short-term Nec-1 treatment reduced the percentage of insulin- and glucagon-positive bi-hormonal cells; however, the viability and composition of beta cells, alpha cells and GLUT2-positive beta cells remained unchanged. Furthermore, Nec-1 supplementation to hiPS beta-cell differentiation media did not affect glucose-stimulated insulin secretion.

Our findings that short-term Nec-1 treatment did not affect the viability of hiPS beta cells supports our previous results that Nec-1 did not alter the viability of young pre-weaned porcine islets after 3-day culture (22,24). This could be due to the high viability in both control and Nec-1 treated groups as the viability was higher than 85% on average. In accordance with our findings, the supplementation of Nec-1 to the culture media of encapsulated human islets in either low- or normal-nutrient culture condition at 20% oxygen level did not significantly improve nuclear DNA content, a measurement of cell survival (25). Another study has similarly demonstrated that the addition of Nec-1 did not affect the percentage of dead cells in normal culture condition after 24 hours as assessed by Sytox staining in βTC-6 and INS-1/832 pancreatic beta cells as well as mouse islet cells (21). However, Nec-1 has been shown to promote islet cell survival from stress induced by low oxygen culture condition or nitric oxide donors (25). Beta cells are exposed to considerable stress that is partially contributed by DAMPs after transplantation, leading to substantial loss of beta cell mass and poor

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engraftment outcomes (26,27). As Nec-1 has been shown to significantly reduce the release of DAMPs, examining the use of Nec-1 on hiPS beta cells under stressors that mimic the post-transplant environment may help to elucidate the effects of Nec-1 on the viability of hiPS beta cells after transplantation (21,25).

Various studies have attempted to improve the differentiation of hiPSCs into beta cells during *in vitro* culture (28). A recent study has demonstrated that, even with the current state-of-the-art protocol, differentiating hiPSC-derived S6 cells into S7 cells through incubation in S7 media could not improve the beta-cell content (17). In parallel, our present findings showed that the percentage of beta cells remained unchanged after culturing hiPS beta cells in beta-cell differentiation medium. Similar to previously published work, the percentage of alpha cells in our study was not improved after differentiation culture (17).

In contrary to our previous findings that Nec-1 induced the differentiation of endocrine cells in young pre-weaned porcine islets after 3-day culture, the supplementation of Nec-1 to the beta-cell differentiation medium did not impact the percentage of beta cells and alpha cells after 3 days of culture in the present study (22). A possible reason for these results is that the use of a small-molecule differentiation inducer during the final step of differentiation of hiPSCs may require more than 3 days to be effective. Thatava *et al.* have utilized DAPT, HGF, IGF-1, and GLP-1 to differentiate hiPSCs into islet-like cells over 6 days of culture (29). In an attempt to generate insulin producing cells, Walczak *et al.* have cultured hiPSCs in medium supplemented with SB431542, dorsomorphin, and retinoic acid for 7 days (30). During the final differentiation step, Rezania *et al.* incubated hiPSCs in medium containing ITS-X, T3, ALK5 inhibitor II, trolox, and AXL inhibitor R428 for 7 to 15 days (31). Another plausible explanation for the unchanged level of beta cells is that islets are exposed to considerable damages caused by prolonged ischemic time, pancreas preservation, and islet isolation (32,33). These stresses lead to the release of pro-inflammatory mediators, contributing to not only islet inflammation and islet demise but also the dedifferentiation of beta cells (34,35). Exposure of the pro-inflammatory cytokine IL-1β to both rat and human islets caused beta-cell dedifferentiation as indicated by decreased mRNA expression of insulin and impaired insulin secretion capacity (36). The combinations of pro-inflammatory cytokines, IL-1β + IFN-γ and TNF-α + IFN-γ, could reduce the expression of genes responsible for glucose-stimulated insulin secretion and maturation in purified beta cells from rat islets (37). As short-term Nec-1 exposure has been shown to inhibit the release of DAMPs that promote inflammatory responses, it is possible that Nec-1 treatment assisted in the differentiation of young pre-weaned porcine islets over 3-day culture by blocking the upregulation of inflammatory pathways induced by pancreas procurement and islet isolation (21,22,25). Since hiPSCs are exposed to substantially less insults and inflammation throughout the differentiation process, the ability of Nec-1 to facilitate endocrine cell differentiation might not have been as effective compared to our previous study.

The short-term treatment of Nec-1 in the present study resulted in a significant decrease in the percentage of insulin- and glucagon-positive bi-hormonal cells, but no significant changes in the level of insulin-positive or glucagon-positive cells. A previous study has demonstrated similar results that while differentiating S6 hiPSCs to S7 hiPSCs reduced the bi-hormonal cells, the amount of beta- and alpha cells remained unchanged (17). Furthermore, the treatment of S7 hiPSCs with a tankyrase inhibitor for 48 hours could only lower the level of bi-hormonal cells but not the percentage of insulin-positive or glucagon-positive cells normalized to total DAPI-positive cells (17). In accordance with the unaltered level of beta cells after short-term Nec-1 treatment, the insulin secretion capacity of Nec-1 treated hiPS beta cells was similar to untreated cells. These observations are in line with previous findings that Wnt-modulation and tankyrase inhibition could not improve glucose-stimulated insulin secretion of S7 hiPSCs even though the levels of bi-hormonal cells were altered (17). Our results and previous work done by others highlight the importance of assessing both the differentiation of beta cells and the insulin secretion ability in the search for a small-molecule differentiation inducer to promote the final maturation step of hiPSCs. We believe that elucidating the mechanism by which Nec-1 reduced the level of bi-hormonal cells may be helpful to determine whether prolonging Nec-1 treatment can promote the last differentiation step of hiPSCs into functional beta cells. The effect of Nec-1 has also been demonstrated to be concentration-dependent, and concentrations ranging from 10 to 300 uM have been utilized on different cell types (19,38-40). These studies suggest that different concentrations of Nec-1 should be evaluated on hiPS beta cells.

In conclusion, the current study has, to our knowledge, provided the first analysis of short-term Nec-1 treatment on the differentiation and function of hiPS beta cells during

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the final *in vitro* maturation stage. While the content of beta cells and insulin secretion capacity of hiPS beta cells remained unchanged, Nec-1 supplementation during short-term culture significantly reduced the level of insulin- and glucagon-positive bi-hormonal cells. Future studies optimizing the concentration and treatment duration of Nec-1 will be helpful to further determine whether Nec-1 can promote the final differentiation step of hiPSCs into functional mature beta cells.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/sci.2020.04.01). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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