Original Article

Induction of ß Cell Replication by Small Molecule-Mediated Menin Inhibition and Combined PKC Activation and TGF-ß Inhibition as Revealed by A Refined Primary Culture Screening

Saghar Pahlavanneshan, Ph.D.[1], Mehrdad Behmanesh, Ph.D.[1*], Yaser Tahamtani, Ph.D.[2], Ensiyeh Hajizadeh-Saffar, Ph.D.[3], Mohsen Basiri, Ph.D.[2], Hossein Baharvand, Ph.D.[2, 4*]

1. Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
2. Department of Stem Cells and Developmental Biology, Cell Science Research Centre, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
3. Department of Regenerative Medicine, Cell Science Research Centre, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
4. Department of Developmental Biology, University of Science and Culture, Tehran, Iran

**Corresponding Addresses: P.O.Box: 14115-154, Department of Genetics, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*
P.O.Box: 16635-148, Department of Stem Cells and Developmental Biology, Cell Science Research Centre, Royan Institute for Stem Cell Biology and Technology, ACECR, , Tehran, Iran
Emails: behmanesh@modares.ac.ir, baharvand@royaninstitute.org

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Abstract

Objective: Pancreatic β cells are recognized as central players in the pathogenesis of types 1 and 2 diabetes. Efficient and robust primary culture methods are required to interrogate β cell biology and screen potential anti-diabetic therapeutics. The aim of this study was to refine monolayer culture of beta cells and to investigate potential inducers of beta cell proliferation.

Materials and Methods: In this experimental study, we compared different culture methods to optimize conditions required for a monolayer culture of rat pancreatic islet cells in order to facilitate image analysis-based assays. We also used the refined culture method to screen a group of rationally selected candidate small molecules and their combinations to determine their potential proliferative effects on the β cells.

Results: Ham's F10 medium supplemented with 2% foetal bovine serum (FBS) in the absence of any surface coating provided a superior monolayer β cell culture, while other conditions induced fibroblast-like cell growth or multilayer cell aggregation over two weeks. Evaluation of candidate small molecules showed that a menin inhibitor MI-2 and a combination of transforming growth factor-β (TGF-β) inhibitor SB481542 and protein kinase C (PKC) activator indolactam V (IndV) significantly induced replication of pancreatic β cells.

Conclusion: Overall, our optimized culture condition provided a convenient approach to study the cultured pancreatic islet cells and enabled us to detect the proliferative effect of menin inhibition and combined TGF-β inhibition and PKC activation, which could be considered as potential strategies for inducing β cell proliferation and regeneration.

Keywords: Menin, Pancreatic β Cells, Proliferation, Protein Kinase C, Transforming Growth Factor-β

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Introduction

Diabetes is a chronic metabolic disease characterised by increased blood glucose levels and inadequate insulin secretion from β cells. Type 1 diabetes is caused by autoimmune destruction of pancreatic β cells, whereas type 2 diabetes is associated with inadequate insulin secretion and β cell dysfunction (1). This central feature of diabetes has raised the hypothesis that restoration of a functional β cell mass would be a potential treatment for diabetes (2-4). Based on this concept, regenerative biology approaches, such as triggering the proliferation of the remaining β cells, have been introduced and are being widely investigated. Induction of pancreatic β cell proliferation is considered a potentially useful strategy for treatment of both type 1 and type 2 diabetes (1, 5).

Insulin producing cells are highly quiescent. Thus, the percentage of proliferative β cells is very low - less than 0.5% of β cells in humans and 0.5-3% of β cells in rodents actively proliferate (6). It is required to study signalling pathways in insulin producing cells that control β cell propagation and screen potential chemicals that can expand or reprogram residual functional β cells (2, 7). The results of previous studies suggest that multiple endocrine neoplasia type 1 (MEN1) protein (8) and transforming growth factor β (TGF-β) signalling (9) are negative regulators of β cell proliferation, while protein kinase C (PKC) activity is required for of cell cycle progression in these endocrine cells (10). However, little is known about the effect of combined targeting of these pathways and the potential synergistic effect of small molecules that target these pathways.

Thus far, different cell lines like insulin expressing (INS)-1, MIN6, and βTC1 (from rodents) and EndoC-βH1 (from humans) have been used in screening experiments (11, 12). Despite their ease of culture and expansion, these cell lines are either derived from pancreatic endocrine tumours or immortalized by transgenic oncogenes, which potentially affect cell cycle regulation mechanisms (11). Moreover, a recent accumulation of data on molecular pathways that control *in vivo* proliferation of β cells as potential pharmacological targets suggest that it is possible to employ more appropriate approaches for drug screening (13). In one approach, primary culture platforms that retain natural cell cycle regulation mechanisms can be employed for evaluation of rationally selected candidate compounds. This underlines the need for efficient primary culture methods for subsequent screening of compatible quantitative assessments. Experiments that entail two-dimensional (2D) cultures of dispersed islet cells on plastic surfaces or coverslips that use different media and coatings with extracellular matrix (ECM)-mimicking compounds have been reported (12, 14-17). However, it is difficult to maintain pancreatic endocrine cells in primary culture, and further systematic studies on different culture methods are warranted to address the comparative effects of media and supporting surfaces.

In this study, we compared different media and surface coatings to introduce an optimized culture condition to produce monolayers of dissociated islet cells with adequate adhesion and the ability to spread over time. Then, we used the optimized culture condition to examine proliferative potential of a rationally selected group of small molecules. We hypothesized that the MEN1 inhibitor, TGF-β inhibitor, and PKC activator small molecules or their combinations could induce β cell replication. We treated primary cultured rat islet cells with different combinations of MI-2 (MEN1 inhibitor), SB431542 (TGF-β inhibitor), and indolactam V (IndV, PKC activator) to test this hypothesis.

Materials and Methods

Islet cell culture media

Complete RPMI medium was prepared by the addition of 10% foetal bovine serum (FBS, Gibco, Darmstadt, Germany), 50 μM βmercaptoethanol (Gibco, Germany), and 200 mM L-glutamic acid (Gibco, Germany) to RPMI1640 medium (Corning Inc., Corning, NY, USA). Complete Ham's F10 medium was prepared by supplementing Ham's F10 medium (Gibco, Germany) with 2% FBS, 50 μM βmercaptoethanol, 200 mM L-glutamic acid, 1.2 g/L sodium bicarbonate, 1% sodium pyruvate, and 11 mM Dglucose (all from Gibco, Germany). In all cultures, the media were changed every other day and replaced with freshly prepared complete media.

Rat pancreatic islet isolation

All of the animal studies were approved by the Royan Institute Ethics Committee (IR.ACECR.ROYAN.REC.1396.34). Rat pancreatic islets were isolated from the pancreata of 8-10 week-old Wistar rats that weighed 250-350 g by using collagenase V (Sigma-Aldrich, Vienna, Austria) for pancreas digestion. The isolated islets were hand-picked twice under a stereomicroscope in order to obtain a pure population of islets. Pure islets were incubated for at least one hour at 37°C in RPMI1640 medium (Corning) supplemented with 10% FBS, 200 mM L-glutamic acid, and 1% penicillin/streptomycin (all from Gibco, Germany) in 10 cm non-adherent cell culture dishes (one rat pancreas per dish) before dissociation.

Preparation of defined surface-coated multiwell plate with poly-L-ornitine and laminin

We diluted 0.01% poly-L-ornitine (PLO, Sigma-Aldrich, St. Louis, MO, USA) three times in phosphate buffer saline (PBS) and subsequently added 100 μl of this solution to each well of the 96-well adherent plates (Corning), which were then kept for one hour at 37°C. Thereafter, the coated wells were washed with PBS and incubated with 100 μl of 1 mg/L laminin (Gibco, Germany) for two hours at 37°C. The wells were filled with 50 μl of the desired medium and kept at 37°C until seeding of the dissociated islet cells.

Dissociation and culture of islet cells

The islets were washed with Ca^{2+}/Mg^{2+}-free PBS and suspended in 0.5 ml Trypsin solution (0.25%) for five minutes at 37°C in a 1.5 mL microtube. During this period, the islets were gently mixed every two minutes. After five minutes, we took a small volume of the digestion reaction each minute and examined it under a phase contrast microscope to check the islet cells. The incubation was stopped after complete digestion by the addition of 0.5 mL cold RPMI1640 medium that contained 10% FBS to the cell suspension. The single cell suspension was centrifuged at room temperature at 1200 rpm for five minutes; next, the cell pellet was resuspended in 1 mL PBS and re-centrifuged under similar conditions. The pellet of the islet cells was then suspended in 0.5 mL PBS and counted before plating, under appropriate conditions. For each condition, 3×10^4 cells were seeded in each well of a 96-well plate that contained 50 μL of medium. The cells were incubated for 20 minutes at 37°C in a humidified atmosphere that contained 5% CO_2. Next, the medium was increased to 100 μL in each well and the plates were re-incubated.

Treatment with small molecules

In this stage, cells derived from dispersed rat islets were seeded and monolayers were established during the first four days of culture in Ham's F10 medium that contained 11 mM glucose. On day 5, the cells were treated with fresh medium that contained small molecules. The media was changed every other day and the cells were treated for seven days. For each small molecule, an optimized concentration was selected from different doses based on MTS assay results (data not shown). The optimized

concentrations of the small molecules were: 1 µM for MI-2 (Selleckchem, Munich, Germany), 1 µM for SB431542 (Sigma-Aldrich), and 50 µM for IndV (Sigma-Aldrich). All the stock solutions were prepared by dissolving the specific chemical in dimethyl sulphoxide (DMSO, Sigma-Aldrich). Control groups were treated with an equal volume of DMSO. Cells were fixed after 11 days of culture and underwent immunocytochemical analysis and quantifications.

Immunofluorescence staining

The 14-day cultured cells or cells treated for 11 days with small molecules were washed three times with PBS (Gibco, Germany) and subsequently fixed in 4% cold paraformaldehyde (Sigma-Aldrich) for 20 minutes at room temperature. Cells were washed and permeabilised in 0.5% Triton X-100 (Sigma-Aldrich) for 15 minutes at room temperature. After washing with PBS-Tween, the cells were incubated with 10% donkey serum in PBS for one hour at 37°C. The cells were incubated overnight with primary antibodies at 4°C. We used the following primary antibodies: 1:200 dilution of guinea pig anti-ins (ab7842, Abcam, Cambridge, MA, USA) and a 1:500 dilution of goat anti-Ki67 (Sc7846, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterwards, the cells were incubated with the secondary antibodies - donkey anti-guinea pig Alexa Fluor 488 (706546148, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and donkey anti-goat Alexa Fluor 568 (A-11055, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for two hours. After a washing step, the cells were counterstained with DAPI and examined under an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan). Quantification was done using ImageJ software (National Institute of Health, Bethesda, MD, USA).

Statistical analysis

All experiments were performed in at least three replications. The results are expressed as mean ± SD unless otherwise specified. Data were analysed for statistically significant differences among groups by two-way or one-way ANOVA followed by Dunnett multiple comparison test. All statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software, CA, USA). $P<0.05$ were considered as statistically significant.

Results

Effects of culture media on two-dimensional primary islet cell culture

We examined different media and surface conditions to obtain the best condition to culture dissociated rat islet cells in an adherent 2D culture setting. Two different culture media, RPMI1640 and Ham's F10 media, were used. Each culture medium was tested in the presence or absence of PLO and laminin (PLO/Lam) coatings. The initial number of seeded islet cells was assessed after 24 hours by immunostaining for insulin and image analysis. At this early time point, there were no detectable differences observed in the appearance and distribution pattern of insulin expressing (INS+) cells cultured under different conditions (Fig.S1, See Supplementary Online Information at www.celljournal.org).

After 14 days of culture, the cells cultured in RPMI medium, regardless of the surface coating, formed dense adherent aggregates, whereas Ham's F10-treated groups were spread (Fig.1A). Staining of the nuclei and INS cells showed that these structures consisted of dense multilayer aggregates of INS+ cells (Fig.1B). In addition, over 14 days, the number of INS− fibroblast-like cells cultured in RPMI medium dramatically increased to 8178.3 ± 253.11 cells (8-fold) in PLO/Lam-coated wells and 7247.6 ± 942.5 cells (7-fold) in non-coated wells. However, culture in Ham's F10 medium produced significantly ($P<0.0001$) lower numbers of INS− fibroblast-like cells on day 14 in both PLO/Lam coated and non-coated wells (Fig.2A). Taken together, these data showed that Ham's F10 medium provided better conditions for 2D culture of primary islet cells as evidenced by a lower rate of expansion of the fibroblast-like cells and production of a well-spread monolayer of INS+ cells which facilitated image analysis.

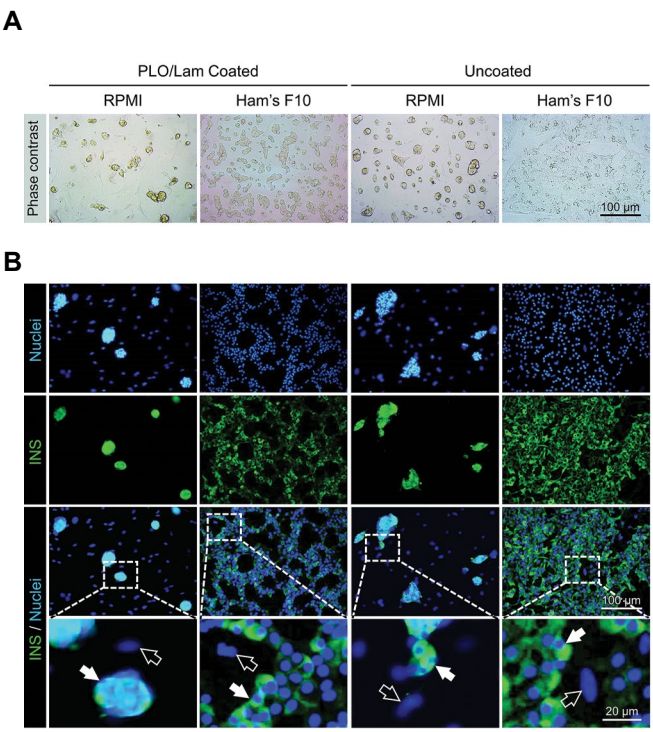

Fig.1: Rat pancreatic islet cells cultured in either RPMI or Ham's F10 media in the absence or presence of PLO/Lam coating for 14 days. **A.** Phase contrast micrograph of the cultures on day 14 showed scattered distribution of islet cells with spread fibroblast-like cells in RPMI medium, while Ham's F10 cultures had a monolayer distribution of the islet cells after 14 days of culture in the absence or presence of PLO/Lam coating. **B.** Immunostaining of insulin on day 14 confirmed that the majority of cells aggregated in RPMI cultures were multilayer INS+ cells (white arrows) while in Ham's F10 medium, they formed spread monolayer with few touching or overlapped nuclei which facilitated subsequent image analysis. INS− fibroblast-like cells (black arrows) were more frequently observed in RPMI cultures. Green signals represent insulin protein staining and blue signals represent nuclear staining with DAPI. PLO/Lam; Poly-L-ornitine and laminin coating and INS; Insulin expressing.

Increased fibroblast-like cell numbers following poly-L-ornitine and laminin surface coating

In order to further define the effect of PLO/Lam coating on cultured islet cells, we compared cells cultured in the presence or absence of coating in Ham's F10 medium. In wells coated with PLO/Lam, the number of INS− fibroblast-like cells increased from 1055 ± 325.47 on day 1 to 3786.66 ± 702.07 on day 14, which indicated a greater than 3.5-fold increase during this period. On the other hand, on day 14 the number of these cells in non-coated wells was significantly lower (1885.66 ± 195.91, P=0.0008) compared to the PLO/Lam-coated wells (Fig.2A). This cell count reflected an ~1.8-fold expansion of fibroblast-like cells in Ham's F10 media-treated cells cultured in non-coated wells when compared to the initial number of these cells on day 1 (1039 ± 255.78).

There was no significant difference (P=0.1164) in INS+ cell counts on day 14 between PLO/Lam-coated (14118.33 ± 75476) and non-coated (15168.33 ± 332.35) wells. However, as INS+ cell counts were similar, increased expansion of INS− fibroblast-like cells in PLO/Lam-coated wells resulted in a significantly (P=0.0168) lower percentage of INS+ cells in these wells (Fig.2B). Comparison of the total cell number of INS+ cells per well (Table S1, See Supplementary Online Information at www.celljournal.org) on day 1 (20000 ± 996.12) with day 14 (15168.33 ± 332.36) showed that INS+ cells did not grow but were largely (~75.8%) maintained in Ham's F10 medium in the absence of surface coating. Altogether, these data suggested that the use of Ham's F10 medium in the absence of surface coating provided a spread monolayer cell culture, which facilitated 2D image analysis and produced an INS+ population with fewer INS− fibroblast-like cells within a two-week period.

Screening of candidate small molecules using the optimized culture system

In order to investigate the efficacy of the optimized culture conditions, we screened the proliferative effects of three selected small molecules - MEN1 inhibitor MI-2, TGF-β inhibitor SB43, and PKC activator IndV on islet cells cultured in Ham's F10 medium in non-coated 96-well plates. Quantification of proliferative INS+ cells was based on co-staining of INS and Ki67 on day 11 followed by image analysis of randomly selected fields. The results revealed the proliferative potential of the tested small molecules. Interestingly, incubation with MI-2, a small molecule inhibitor of MEN1, led to significantly higher numbers of $Ki67^{+}INS^{+}$ cells (4.79 ± 1.68%) compared to untreated cells (1.02 ± 0.60%), which accounted for a greater than 4.5fold increase (P=0.0186) in pancreatic β cell proliferation (Fig.3A).

We also treated the islet cells with combinations of two or three small molecules in order to investigate whether a combination of these small molecules could synergize to induce β cell proliferation. Interestingly, treatment with TGF-β inhibitor SB43 along with PKC activator IndV resulted in significantly (P=0.193) increased proliferation (4.70 ± 1.75%) in INS+ cells (Fig.3A, B). Furthermore, we assessed proliferation of β cells using the latter combination concomitantly with the previously determined effective menin inhibitor MI-2. Surprisingly, the triple combination of SB43, IndV, and MI-2 small molecules did not significantly (P=0.9994) increase β cell proliferation (2.20 ± 0.70%) compared to the control group (1.45 ± 0.26%). Additionally, the combination of MI-2 with either SB43 or IndV resulted in replication rates (4.53 ± 1.36% and 4.73 ± 1.97%, respectively) roughly similar to what we observed with MI-2 alone (4.79 ± 1.68%) (Fig.3A, B). These results suggested that SB43 and IndV synergized to increase β cell replication, whereas none of them could further improve the proliferative effect of menin inhibition.

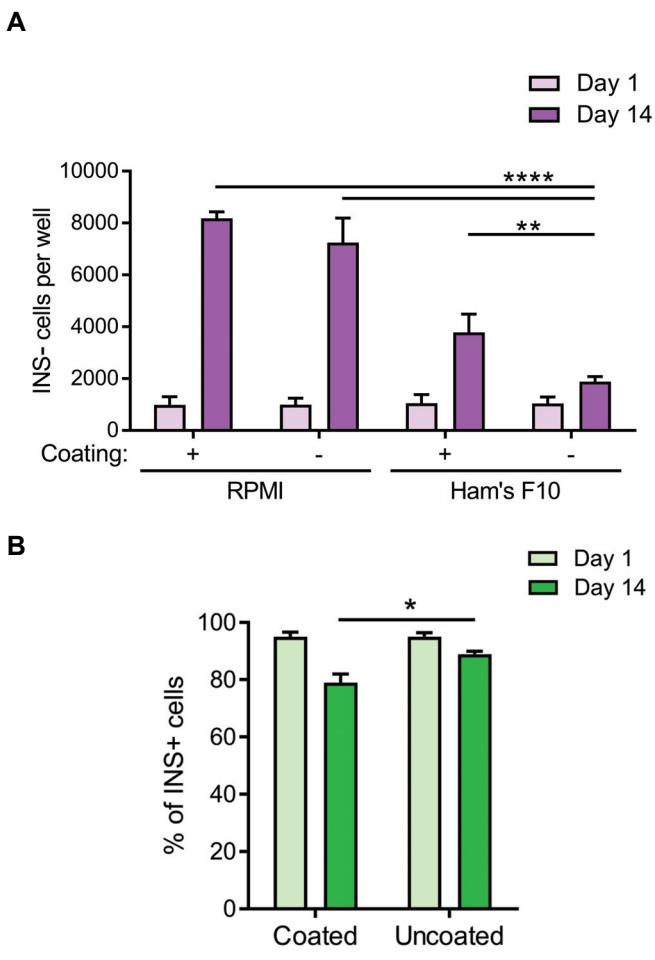

Fig.2: Quantification of INS+ and INS− cells in different culture systems. **A.** Fibroblast-like INS− cells were counted by insulin immunostaining followed by image analysis after 1 or 14 days of culture under four different conditions. Cultures in Ham's F10 medium on non-coated plates showed significantly lower number of fibroblast-like INS− cells on day 14. **B.** The graph shows the percentage of INS+ cells in Ham's F10 medium in the absence or presence of PLO/Lam. While the two cultures had similar percentages of INS+ cells on day 1, non-coated plates had a higher percentage of INS+ cells on day 14. Data are presented as mean ± SD. n=3, *; P<0.05, **; P<0.01, ****; P<0.0001, PLO/Lam; Poly-L-ornitine and laminin coating, SD; Standard deviation, and INS; Insulin expressing.

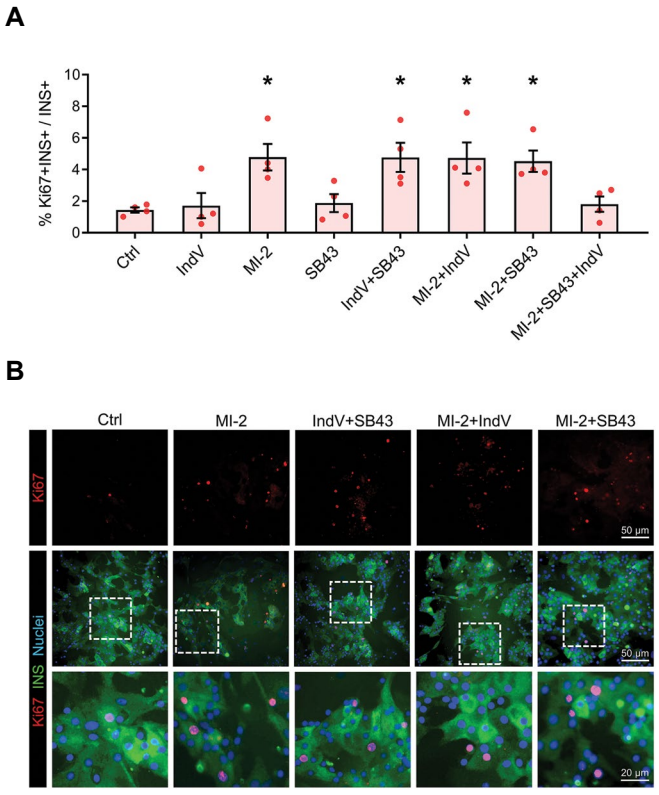

Fig.3: Proliferative effects of candidate small molecules on rat pancreatic islet cells. **A.** Dissociated islet cells cultured in Ham's F10 media were treated with one or combination of candidate small molecules or DMSO (untreated control) for seven days. The percentage of proliferative INS+ cells was assessed by Ki67 and insulin immunostaining. Experimental groups treated with MI-2, IndV+SB43, MI-2+IndoV or MI-2+SB43 showed significantly increased proliferation of INS+ cells compared to the untreated control. **B.** Representative micrographs of INS and Ki67 costained cells from MI-2, IndV+SB43, MI-2+IndoV, MI-2+SB43, and untreated control cultures show increased Ki67-stained nuclei in the treated cells. Data presented as mean ± SEM. *; P<0.05. Green signals represent insulin protein staining; red signals represent proliferative Ki67 nuclei; and blue signals represent nuclear staining with DAPI. Ctrl; Control untreated group, IndV; Indolactam V, SB43; SB431542, and DMSO; Dimethyl sulphoxide.

Discussion

Here, we reported a simple straightforward method optimized for culturing dissociated rat islet cells and its application for screening drug-induced proliferation of pancreatic β cells. Although different methods, including the application of PLO and laminin (16, 17) as supporting matrix in Ham's F10 (18, 19) or RPMI (14, 20) media have been previously reported for primary culture of these cells, no study has systematically compared the efficacy of these different culture conditions. In the present study, our side-by-side comparison of culture conditions showed that selection of culture medium and surface coating could dramatically impact the culture characteristics in terms of islet cell distribution in culture and propagation of INS− fibroblast-like cells. We showed that in Ham's F10 medium supplemented with 11 mM D-glucose and FBS with non-coated surfaces, pancreatic islet cells could be maintained in an adherent monolayer culture for at least two weeks. This time period was enough for performing the *in vitro* assays; however, this culture system was not intended to expand β cells for subsequent transplantation.

Monolayer culture systems facilitate microscopic investigation and image analysis of a relatively small number of pancreatic islet cells. In contrast, methods that use intact islets or reaggregated pseudo-islets rely on special facilities such as confocal microscopy (21), time-consuming histologic techniques (22), or flow cytometry (23) which is challenging when working with small numbers of cells. Our results have shown that although RPMI1640 medium has been routinely used for an adherent culture of pancreatic islet cells (23-26), it promotes a scattered multilayered cell culture. In accordance with our observation, a scattered distribution has been reported for islet cells cultured in RPMI1640 medium with and without laminin coating (24). Furthermore, RPMI1640 showed cytotoxic effects on pancreatic islet cells, which was probably due to the presence of high amounts of neurotoxic amino acids (27). This problem can be overcome by culturing primary dissociated islets with neuron-compatible culture medium as well as laminin-coated surfaces for four days (24); however, longer culture periods were not investigated. Improved monolayer distribution of insulin-expressing islet cells in our proposed Ham's F10 culture system facilitated visualization and quantification of pancreatic β cell-like proliferation during a two-week culture. Additionally, in our selected culture condition, from 20000 ± 996.12 INS+ cells on day 1, around 75.8% of these cells (15168.33 ± 332.36) survived over a two-week period without the loss of INS expression. More importantly, the 24.2% drop in INS+ cell number was not accompanied by a significant increase in the number of INS- cells, which highlighted the fact that there was no detectable functional dedifferentiation in this culture condition.

Propagation of fast-growing fibroblast-like cells in a primary culture of pancreatic endocrine cells can hamper subsequent treatments and biological analysis. Although dedifferentiation of human pancreatic β cells into fibroblast-like cells in prolonged primary culture was previously reported, this phenomenon was not seen for murine β cells (28). Therefore, fibroblast-like cells are most probably produced by islet stromal cells that contaminate the primary culture of pancreatic endocrine cells. Researchers have attempted to eliminate these cells from pancreatic islet cell cultures using chemicals such as thimerosal (14) and ARA-C (24) or mitotoxin-conjugated fibroblast growth factor (29). We observed that culture medium and surface coating could dramatically affect propagation of these fibroblast-like cells. Moreover, we could prevent the propagation of these cells without application of any toxic treatment and only by using Ham's F10 medium supplemented with a low FBS concentration (2%) on non-coated 96-well plates. The minimized expansion of fibroblast cells in our proposed culture condition led to higher purity of β cells after 14 days of culture.

We used our culture system to screen candidate proliferation inducer small molecules on INS+ pancreatic

cells. Several studies revealed important pathways that control β cell proliferation *in vivo*; thus, pharmacological targeting of such pathways by *ex vivo* drug screening strategies could be a promising approach for induction of β cell proliferation. We selected small molecules known to target some β cell proliferation regulators, namely, TGF-β receptor (2, 13, 30), PKC (10), and menin protein (31). Interestingly, only the menin inhibitor MI-2 showed a significant proliferative effect when used alone. Menin is a protein coded by the *MEN1* gene. *MEN1* loss of function mutations are known to enhance pancreatic β cell proliferation (32). The MI-2 small molecule is known to inhibit interaction of menin with the mixed lineage leukaemia (MLL) protein (5, 33). These small molecules are primarily developed for pharmacological disruption of menin-MLL interaction in leukaemia cells as potential anti-leukaemia drugs (34). In pancreatic β cells, menin containing MLL methyltransferase complexes are involved in regulation of cell function (35). It has been shown that menin recruits MLL to the promoter of cyclin-dependent kinase (CDK) inhibitors and regulates their expression through promoting histone methylation (36, 37). Our data have shown that the small molecule MI-2, which reportedly inhibits menin-MLL interaction, increased *ex vivo* proliferation of pancreatic β cells.

Our results also suggest that a combination of SB431542 (TGF-β receptor inhibitor) and IndV (activator of all α, β, γ, δ, ε, and η isozymes of PKC) can induce β cell replication synergistically and not merely additively. To our knowledge, this is the first direct evidence of the synergic proliferative effect of PKC activation and TGF-β inhibition in β cells. Mechanistically, PKC activity modulates rodent β cell proliferation through phosphorylation and activation of mTOR and inhibition of GSK3β (10). GSK3 inhibition and mTOR activation are required for downstream accumulation of cyclins, CDKs in rodent β cells both *in vivo* and *in vitro* (38). On the other hand, it has been shown that TGFβ signalling contributes to transcriptional activation of CDK inhibitor genes, including *CDKN1A*, *CDKN1C,* and *CDKN2C* (39) presumably through SMAD3-mediated recruitment of the Trithorax chromatin remodelling complex (40). Although mechanisms that underlie the synergic proliferative effect of PKC activation and TGF-β inhibition are not clear, based on the aforementioned previous data, we speculate that upregulation of cyclins and CDKs by activated PKC and downregulation of CDK-cyclin inhibitors caused by the absence of active TGF-β signalling collectively might result in higher CDK activity and β cell replication. However, further investigations are necessary to test this speculation. It is noteworthy that future *in vivo* studies on diabetic animal models may address systemic effects on blood glucose and the therapeutic potential of these small molecules. Moreover, possible adverse effects on other cell types can be checked in future *in vivo* studies.

Conclusion

Our results showed that primary culture of rat dissociated islet cells in complete Ham's F10 medium on non-coated 96-well plates could maintain pancreatic β cells for two weeks. By using this culture system, we demonstrated the proliferative effect of menin inhibitor MI2 and the combination of PKC activator IndV and TGF-β inhibitor SB431542 as potential inducers of β cell proliferation.

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Authors' Contributions

S.P., M.Ba.; Designed the research and carried out data analyses. S.P.; Conducted the experiments and wrote the manuscript. Y.T., E.H.-S., M.Ba., M.Be. H.B.; Helped with designing the experiments, data analysis, and preparing the manuscript. M.Be., H.B.; Were responsible for final approval of the manuscript. All authors read and approved the final manuscript.

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