Research article

Arg-Gly-Asp-modified elastin-like polypeptide regulates cell proliferation and cell cycle proteins via the phosphorylation of Erk and Akt in pancreatic β-cell

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ABSTRACT

Objective: Enhancement of β-cell proliferation plays an important role in maintaining β-cell mass and function, and in improving pancreatic β-cell survival before transplantation. Extracellular matrix (ECM) components increase the adhesion and proliferation of β-cells, and the RGD-modified elastin-like polypeptide (RGD-ELP, REP) has been described as a bioactive matrix. In this study, we investigated whether REP could enhance β-cell adhesion and proliferation and elucidated the signaling pathways involved.
Methods: We investigated the effect of REP on cell adhesion, proliferation and insulin secretion via assays using Rin-m and rat islets. Crystal violet, CCK-8, and BrdU assay, FACS, western blot, real time q-PCR analyses and insulin ELISA were examined. To explain the associated mechanisms, phosphorylation of Akt and extracellular signal-regulated kinase (Erk) were measured.
Results: REP more increased the adhesion, proliferation and survival of Rin-m cells compared to elastin-like poly peptide (ELP) without RGD-motif. The enhancement of β-cell proliferation by REP was associated with increased cyclin D1, cyclin D2 and cdk6, and decreased p27 levels. When β-cells were cultured on REP, Erk and the phosphatidylinositol 3-kinase (PI3-kinase) downstream effector, Akt was stimulated. Treatment with the Erk pathway inhibitor and PI3-kinase inhibitor decreased REP-induced β-cell adhesion and proliferation, and regulated REP-induced cell cycle proteins. Additionally, REP increased the mRNA and protein levels of insulin and its transcription factor, PDX-1, and insulin secretion.
Conclusions: Our results demonstrate that the up-regulation of the PI3K/Akt and Erk signaling pathways and the regulation of cell cycle proteins by REP could serve as effective strategies for improving pancreatic β-cell adhesion and proliferation.

1. Introduction

Pancreatic β-cells in the islets of Langerhans produce insulin in response to blood glucose levels [1]. Type I diabetes (T1D) results from the autoimmune destruction of β-cells, making the body incapable of maintaining normoglycemia in such patients [2]. Although mounting evidence indicates that islet transplantation is an effective treatment for T1D, early islet destruction is a major problem of islet transplantation [3]. Therefore, enhancing pancreatic β-cell proliferation is an important strategy for T1D treatment [4].

Extracellular matrix (ECM) molecules play an important role in regulating molecular signaling associated with proliferation, migration, differentiation and tissue repair [5]. Fibronectin, fibrillin, collagen and laminin are found in pancreatic islets and the surrounding, and are involved in cytoskeletal remodeling and differential cell adhesion [6]. ECM and cell interactions affect gene expression, influencing the survival, growth, and differentiation of β-cells [7, 8]. Enzymatic isolation of islets for transplantation, damages the ECM, which impacts islet survival and function. However, restoration of the ECM in islets (for example by adding ECM to the interior of immunoprotective capsules) has been

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shown to enhance islet function [9]. The importance of ECM in islet transplantation has also been recognized recently, because ECM was shown to maintain β-cell [10, 11] and improve islet graft survival by activating the intracellular signaling pathway involving focal adhesion kinase (FAK), Akt/protein kinase B (PKB), and Erk [12, 13].

Our group has reported that the Arg-Gly-Asp (RGD)-modified elastin like-polypeptide (REP, TGPG[VGRGD(VGVPG)$_6$]$_{20}$WPC), a bioactive matrix, has a beneficial effect on islet survival and function, and adipose stem cell adhesion and wound repair [11, 14]. REP consists of tandem repeats of the elastin-derived VGVPG pentapeptides (ELPs) and an RGD integrin ligand and is responsive to changes in temperature, pressure and salt concentration through a sol-gel phase transition. ELPs are compatible with cells and tissues, easily biodegradable, can be formulated in a variety of ways, and thus have great potential for use in biomedical applications [10]. Particularly, the RGD integrin-binding motif found in many ECMs, is recognized by integrins [15]. Integrins α3β1, α5β1, and α6β1 are expressed in pancreatic β-cells, mediates their attachment and spreading, and promote cell survival and insulin secretion via interaction with several ECMs [16, 17]. Previous reports have shown that β1, α3, α5-integrins are capable of activating not only the Fak/MAPK/Erk pathway but also the PI3-Akt pathway [12, 18, 19].

Cyclin D is a key regulator of the G1/S phase transition, and the effect of cyclins D1 and D2 in β-cells was demonstrated by reduced β-cell mass and hyperglycemia in mice deficient in G1 components [20, 21]. Several groups have shown that overexpression of cdk4 by adenoviral transfer in human islets increases their proliferation *in vitro*, and that cdk6- and cyclin D1-overexpressing human β-cells transplanted into immunodeficient diabetic mice continue to proliferate for 6–8 weeks with improved β-cell function/glycemic control [22, 23]. The family of cell cycle inhibitors (CKIs), Cip/Kip (p21Cip1, p27Kip1, and p57Kip2), primarily inhibit Cdk2 but aid in the cdk4/cyclin *D complex* assembly. Of the CKIs, p27Kip1 progressively accumulates in the nucleus of pancreatic β-cells in genetic models of insulin resistance, and deletion of p27Kip1 ameliorates hyperglycemia in these animal models of type II diabetes [24].

In the present study, we examined whether REP could increase β-cell proliferation and regulate cell cycle proteins, and investigated the intracellular pathways involved.

2. Materials and methods

2.1. Preparation of REP

ELP and RGD-ELP (REP) was prepared as previously described [10]. REP material was designed and corresponded by Won Bae Jeon. Those were expressed in *Escherichia coli* BLR(DE3) using pET-25b (+)-1 plasmids, and was purified by inverse transition cycling. The molecular compositions of hydrophilic RGD motifs and hydrophobic VG (VGVPG) domains were computed using Compute pI/MW software (ExPASy Bioinformatics Resource Portal). Purified protein was dissolved in phosphate buffered saline (PBS, pH 7.4; Gibco, USA). To characterize thermal transition, the changes in temperature and absorbance at 350 nm were monitored via the Cary Win-UV software. The transition temperature, T_t, was defined as the temperature at which 50% of the maximal turbidity was observed.

2.2. Cell culture

The Rin-m rat insulinoma cell line (ATCC, USA) was cultured in 5% CO_2–95% air at 37 °C in an ATCC-formulated RPMI-1640 medium containing 25 mM glucose and 2 mM L-glutamine. The medium was supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Islet isolation

Pancreatic islets were isolated from the pancreata of 10–12 week aged-SD rats by the collagenase digestion technique. The Animal Care and Use Committee of DGIST approved all animal protocols. Briefly, the animals were anesthetized and sacrificed by cervical dislocation. Collagenase type V (Sigma, USA) was dissolved to a concentration of 1 mg/ml in Hank's balanced salts solution and transfused into the pancreatic ducts via the common bile duct. The dissected pancreas was then incubated for 10–15 min at 37 °C in a water bath with shaking every 3 min. The islets were isolated under a stereoscope (15×magnification) and placed in Krebs-Ringer bicarbonate buffer (KRBB) containing 0.1% BSA, penicillin (100units/ml), and streptomycin (0.1 mg/ml). The isolated islets were cultured overnight in RPMI-1640 containing 10% FBS and 11.2 mmol/l glucose to ensure optimal recovery and were used for each condition.

2.4. Cell adhesion assay

A 96-well plate coated with 50 μl of 1 μM ELP, REP (indicated concentration) or fibronectin (FN) for 16 h at 4 °C. Rin-m cells (5 × 10^4) were seeded in each well and cultured for 24h. After removing the culture medium, the wells were rinsed twice with PBS to eliminate unattached cells. Attached cells were incubated with 50 μl staining solution (0.5%w/v crystal violet, 4%w/v formaldehyde, 30% v/v ethanol, and 0.17%w/v NaCl) and incubated for 20 min at 37 °C. After gently washing out the staining solution with tap water, cells were dried for 1 h at 50 °C. The stained cells were photographed using a Leica DMI 3000B microscope. The blue dye was dissolved in 200 μl of acetic acid solution (33% v/v), and optical density (OD) was measured at 570 nm using a microplate reader Multiskan (Thermo Scientific, USA). For the adhesion inhibition assay, cells were treated with 50 μM PD98059 or 100 nM wortmannin for 30 min before seeding into wells.

2.5. Cell survival and proliferation assays

Cell survival was measured using the Cell Counting Kit-8 (CCK-8) (Dojindo Lab., Japan). Rin-m cells (5 × 10^4) were seeded into a 96-well plate coated with ELP, REP or FN, and cultured for 24h. CCK-8 (10μl) solution was added to each well, and incubated for another 3 h and then measured at OD 450 nm using the Multiskan microplate reader. Cell proliferation was measured using the 5′-bromo-2′-deoxyuridine (BrdU) Cell Proliferation Assay Kit (BioVision, USA) according to the manufacturer's instructions. Rin-m cells were incubated with BrdU for 4h before harvesting, and then fixed. A monoclonal anti-BrdU antibody was then added, and finally the OD at 450 nm was measured.

2.6. Cell cycle analysis

The Rin-m cells cultured in plated coated with ELP or REP for 24 h were harvested and fixed using 70% ethanol. The fixed cells were incubated in 1.12% Citrate buffer containing 100 μg/ml RNase for 30 min at 37 °C, and then stained with 50 μg/ml Propidium Iodide solution (Sigma-Aldrich, USA) for 20 min at 37 °C. The percentage of cells in different phases was analyzed using the BD FACSAria™ III (Becton Dickinson, USA) and the BD FACSDiav (7.0) software (Becton Dickinson).

2.7. Western blot analysis

Cell lysates were prepared using RIPA buffer (Sigma) containing Complete, Mini Protease Inhibitor Cocktail (Roche, USA) and Halt Phosphatase Inhibitor Cocktail (Thermo) according to the manufacturer's instructions. Proteins were separated by electrophoresis and then electrophoretically transferred to PVDF membranes (Millipore, USA). The membranes were blocked by incubation in blocking buffer containing 5% BSA (Sigma) and then probed with the following antibodies: anti-cyclin D1, anti-cyclin D2, anti-p21, anti-p27, and anti-BETA2 (Abcam, UK); anti-cdk6, anti-PDX-1 (Santacruz, USA), anti-phospho-Erk antibody, anti-ERK, anti-phospho-Akt, and anti-Akt antibody (Cell signaling, USA). After washing out the antibodies, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody, and the signals

were visualized using Super Signal West Dura Extended Duration Substrate (Thermo) according to the manufacturer's instructions. The membrane was then re-probed with an anti-actin antibody (Santa Cruz) to verify equal loading of proteins in each lane. Immunoblot images were captured using ChemiDoc™XRS+ (BIO-RAD) and the intensity of bands was quantified using Image Lab™ Software (Bio-Rad).

2.8. mRNA expression analysis by RT- qPCR

Total RNA was isolated from cells using Trizol Reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNAs were synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), and 2 μg of total RNA according to the manufacturer's instructions. We performed q-PCR using the SYBR Green PCR master mix kit (Applied Biosystems) and an ABI 7500 Real Time PCR System. Cycling conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 95 °C for 15 s, and at 60 °C for 1 min. Primers were designed using Primer Express Software ver. 3.0 (Applied Biosystems) as follows: Insulin-1 forward, 5′-ACCCACCCAGGCTTTTGTC-3′, Insulin-1 reverse, 5′-TCCCCACA-CACCAGGTAGAGA-3'; Insuin-2 forward, 5′-GCTGGCCCTGCTCTTCCT-3'; Insulin-2 reverse, 5′-TCCCCACACACCAGGTAGAGA-3'; PDX-1 forward, 5′- TTGAACTTGACCGAGAGACACATC-3′, PDX-1 reverse, 5′-CGTTTCTTATCTTCCTCTTTTTTCCA-3'; BETA2 forward, 5′-CCATCCGCCGAGTTTGAA-3′, BETA2 reverse, 5′- AGAA-GATTGATCCGTGGCTTTG-3'; 3'; GAPDH forward,5′-AGGTTGTCTCCTGCGACTTCA-3′ and GAPDH reverse, 5′-CAGGAAAT-GAGCTTGACAAAGTTG-3'. *Gapdh* was used as an internal standard.

2.9. Glucose-stimulated insulin secretion (GSIS)

To examine the effects of REP on GSIS in islets, 5 islets (3 wells per each condition) were cultured on 1 μM REP-coated plates for 24 h. The islets were then starved in medium containing 3 mM D-glucose for 5 h and subsequently incubated for 1 h in KRBB supplemented medium with 3 mM or 16.7 mM D-glucose. The supernatant was carefully collected and subjected to insulin measurement using rat insulin ELISA Kit (ALPCO, USA).

2.10. Statistical analysis

Data were evaluated using ANOVA followed by a post-hoc multiple comparisons least significant difference test and expressed as the means ± SEM. Values of $P < 0.05$ were considered to be statistically significant. All experiments were performed at least three times.

3. Results

3.1. REP increases Rin-m cell attachment, proliferation and survival

B-cell-ECM interactions play critical role in maintaining β-cell viability and function [16]. To determine whether REP could increase Rin-m cell adhesion, a crystal violet assay was performed with cells cultured on non-coated (control), ELP-coated (ELP), REP-coated or RGD-motif containing ECM (fibronectin, FN)- coated plates. As shown in Suppl. Figure 1A, cell adhesion to REP-coated plates increased on a concentration dependent manner. Compared to the results with control and ELP-coated plates, Rin-m cell adhesion was increased in the context of REP-coated plates (Figure 1A). Similar to the results with 1 μM FN-coated plates, Rin-m cell adhesion was found to increase on 1 μM REP-coated plates (Suppl. Figure 1B).

Next, we analyzed the effects of REP on Rin-m cell survival and proliferation via CCK-8 colorimetry and BrdU incorporation, respectively. The cell survival and proliferation of Rin-m cells were higher in the context of both REP- and FN-coated plates compared with those observed in control and ELP-coated plates (Figure 1B&C and Suppl. Figure 2).

3.2. REP regulates cyclinD1, cyclin D2, and p27 levels in Rin-m cells

The rate of cell proliferation and growth is modulated by cell cycle progression which is controlled by the expression and function of three major classes of proteins: cyclins, cyclin-dependent kinases (cdks), and cyclin-dependent kinase inhibitors (CKIs). Cyclin D1 and cyclin D2 are expressed in β-cells and are important regulators of β-cell proliferation [20]. To investigate the potential effect of REP on the cell cycle, Rin-m cells cultured on ELP- or REP-coated plates were analyzed by flow cytometry. Compared with cells cultured in the presence of ELP, Rin-m cells cultured in the presence of REP, showed a reduced frequency of cell in the G1 phase and increased the frequencies of S and G2/M phases (Figure 2A). As shown in Figure 2B, when Rin-m cells were cultured on an REP-coated plate, the protein level of cyclin D1 were increased markedly and the cyclin D2 also increased meaningfully compared to ELP. The expression of p27, a CKI, was decreased significantly in cells on REP-coated plates, whereas p21 levels were similar between cells cultured on REP and on ELP. Besides, the expression of cdk6, a cdk, was enhanced in cells cultured on REP-coated plates. These results suggest that REP regulates the cell cycle in β-cell by regulating cell cycle proteins, such as cyclin D1, cyclin D2, cdk6, and p27.

3.3. REP increases the expression of integrins and impacting the downstream intracellular signaling pathway

To determine the fundamental mechanism of REP on cell attachment and proliferation, we investigated the expression of cell surface adhesion receptors such as integrins and studied the intracellular signalings in response to ECM. The levels of Integrin α3, α5 and β1 were higher in cells cultured on REP compared to cells cultured on control and ELP (Figure 3A). Importantly, when Rin-me cells were cultured in the presence of REP, the level of p-FAK increased significantly (Figure 3B). Activation of the MAPkinase/Erk and PI 3-Kinase/Akt pathways is known to mediate the survival of β-cells, as well as β-cell proliferation via cell cycle protein regulation [24, 25]. Of note, the levels of p-Erk and p-Akt in cells cultured in REP-coated plates were elevated compared with those cells in the context of control and ELP-coated plates (Figure 3B).

3.4. REP enhances β-cell adhesion and survival through adhesion-induced pro-survival signaling

We have examined whether the effect of REP on cell attachment and survival in β-cells is related to the Erk and Akt pathways. As shown in Suppl. Figure 3 and Figure 3, Erk and Akt phosphorylation were increased in Rin-m cells cultured on REP-coated plates. When Rin-m cells were cultured for 24h on REP (-) or REP (+) in the presence of a MAPK inhibitor, PD98059 or a PI3K inhibitor, wortmannin, REP-induced phosphorylation of Erk and Akt was decreased significantly with 50 μM PD98059 or, 100 nM wortmannin in Rin-m cells. Treatment of Rin-m cells with PD98059 and wortmannin significantly prevented the REP-stimulated cell adhesion (Figure 4A). Likewise, addition of PD98059 and wortmannin in Rin-m cells inhibited the REP-induced survival (Figure 4B). These results suggest that REP increases β-cell attachment and survival via the Erk and Akt pathway.

3.5. REP regulates cell cycle proteins through the Erk or Akt signaling pathways

To understand the regulatory role of REP on the cell cycle by the activation of Erk and Akt, we focused on the activation status of Erk and Akt because it is a mediator of cell cycle progression in in β-cells [26]. Inhibition of Erk phosphorylation by treatment with 50 μM PD98059 inhibited the upregulation of REP-induced cyclin D1 and cyclin D2 protein expression (Figure 5A–C). On the contrary, the protein expression of CDK6 and p27 was not altered by inhibition of Erk activation (Figure 5A&D&E&F). When Rin-m cells were cultured on REP-coated
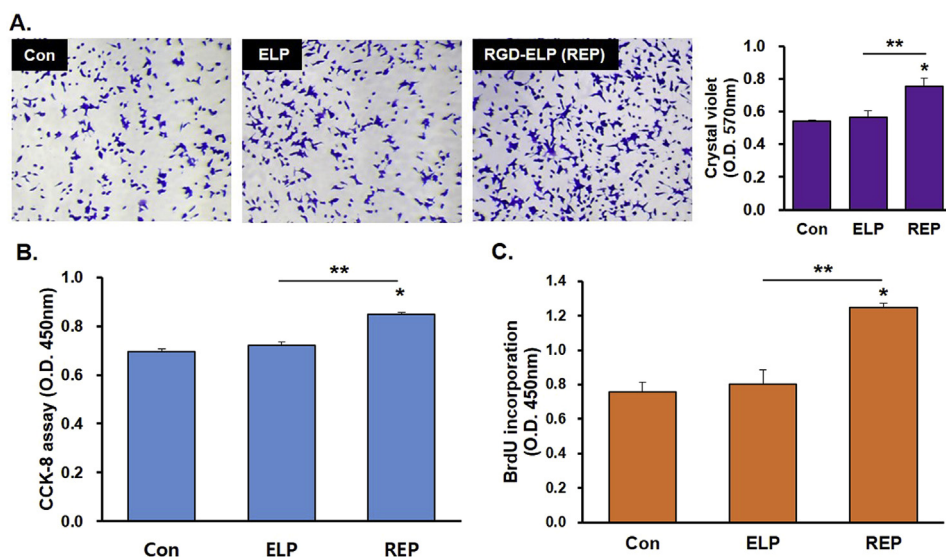
plates with 100 nM wortmannin, the protein levels of REP-induced cyclin D1, cyclin D2 and CDK6 were found to decrease significantly (Figure 6A–D). In contrast, the down-regulation of REP-induced p27 protein was not affected by Akt inhibition (Figure 6A&E&F). These results suggest that REP regulates the cell cycle in β-cells, at least by regulating the Erk and Akt phosphorylation, and the levels of cyclin D1, cyclin D2 and CDK6.

3.6. REP upregulates insulin and its transcription factors in Rin-m cells

Previously we reported that the insulin secretory function and its related transcription factors were increased in REP-coated islets [11]. Thus, we investigated the effect of REP on insulin gene expression and secretion in Rin-m cells or rat islets cultured on REP coated plates. The mRNA expression of insulin-1 and insulin-2 was increased in cells cultured in REP-coated paltes (Figure 7A). Moreover, glucose-stimulated insulin secretion (GSIS, insulin secreted at 16.7 mM D-glucose) was significantly increased in islets cultured in the presence of REP (Figure 7B, versus controls). Importantly, the mRNA and protein expression of PDX-1, a major insulin transcription factor, were significantly upregulated in cells cultured on REP (+), whereas BETA2 expression was similar in cells plated on REP (-) (Figure 7C&D). Similarly, the mRNA or protein expression of insulin-1, insulin-2, and PDX-1

was increased in cells cultured in the presence of FN (Suppl. Figure 4). Overall, these results suggest that REP improves the major function of β-cells as well as β-cell proliferation and survival.

4. Discussion

Enhancing β-cell proliferation and survival before islet transplantation is very important technique for the treatment of T1D. ECM has been reported to increase cell attachment and proliferation, as well as, protect β-cells from apoptosis [12]. Our laboratory has generated REP, an elastin-like polypeptide with the sequence TGPG $[VGRGD(VGVPG)_6]_{20}$WPC that contains both the RGD integrin ligand sequences and the elastin-derived VGVPG pentapeptide sequences [10]. Previously, we demonstrated that REP plays a positive role in enhancing cell survival on the transplanted islets [11]. Several ECMs is reported to contain multiple protein binding domains, including RGD sequences, and play a critical role in β-cell proliferation [11, 12, 13]. We examined whether REP could significantly induce β-cell attachment and proliferation similar to other ECMs, and investigated the intracellular pathways involved. REP more elevated Rin-m-cell adhesion and proliferation compared with ELP. Like fibronectin, REP significantly increased Rin-m-cell adhesion and proliferation. Moreover, the enhancement of REP- induced β-cell attachment and proliferation was accompanied by
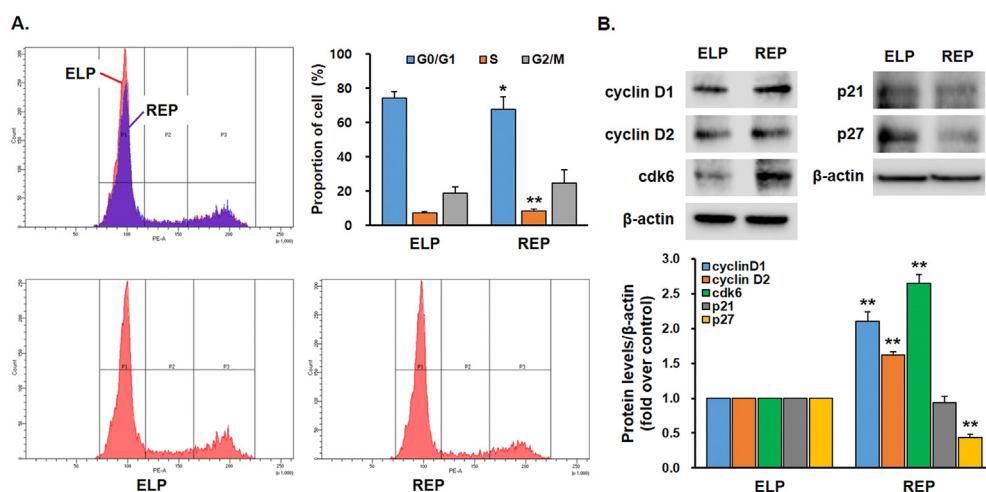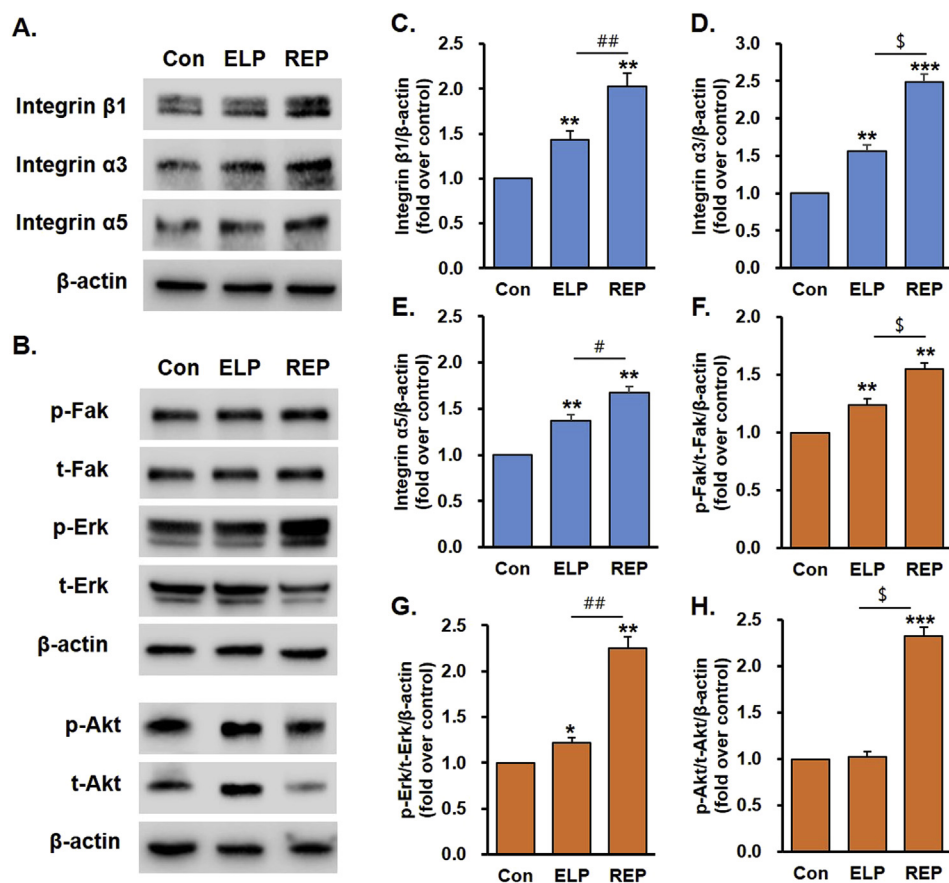
Figure 3. The effect of REP on integrin mediated intracellular signaling. Rin-m cells were cultured on non-coated (control, Con), 1 μM ELP- or REP-coated plates for 24 h. (A) Western blot analysis of the protein expression of integrin β1, α3, and α5. (B) Western blot analysis of the phosphorylation of Fak, Erk and Akt. (C–H) Quantification of western blot analysis. *P < 0.05, **P < 0.01 and ***P < 0.001 compared with control. #P < 0.05, ##P < 0.01 and $P < 0.001 compared with ELP. Data are expressed as the mean ± SEM of three independent measurements. β-actin levels were analyzed as an internal control.
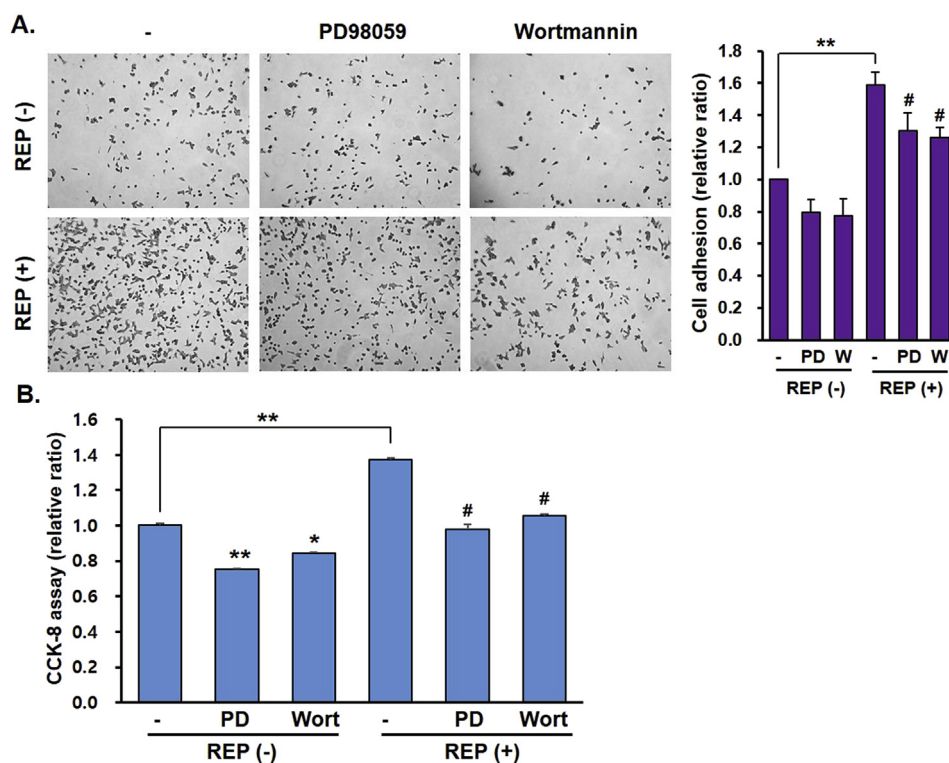

Figure 4. Involvement of the Erk and Akt/PkB pathways in REP-induced β-cell attachment. Rin-m cells were cultured with or without indicated concentration of PD98059 (a specific Mek inhibitor) or wortmannin (PI3-kinase inhibitor) on a 1 μM REP-coated plate for 24 h. (A) Evaluation of the effects of 50 μM PD98059 or 100 nM wortmannin on REP-induced cell adhesion via crystal violet staining. (Bright field microscopy Image, ×200). Quantitative comparison of REP-induced cell adhesion treated with or without 50 μM PD98059 and 100 nM wortmannin after crystal violet staining. Data are expressed as the mean ± SEM of three independent measurements. *P < 0.05 and **P < 0.01 compared with REP (-). #P < 0.05 compared with REP (+). (B) CCK-8 assay showing the effects of 50 μM PD98059 or 100 nM wortmannin on REP-induced cell survival. *P < 0.01 and **P < 0.001 compared with REP (-). #P < 0.01 compared with REP (+). All data are expressed as the mean ± SEM of three independent measurements.
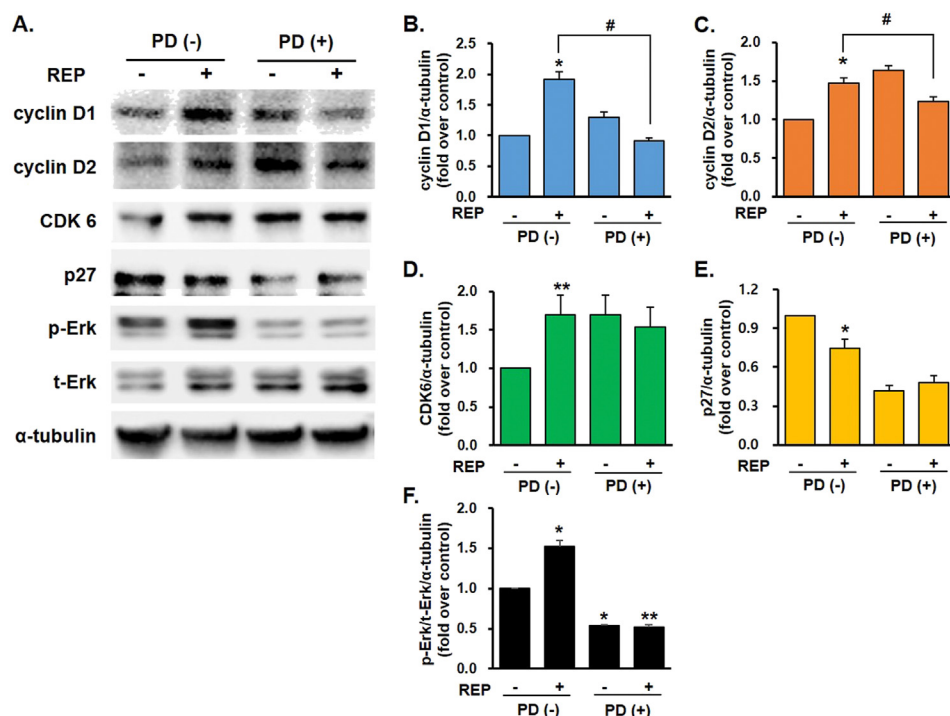
Figure 5. Involvement of the Erk pathway in REP-induced cell cycle protein regulation in β-cells. Rin-m cells were cultured with or without 50 μM PD98059 (a specific Mek inhibitor) on a 1 μM REP-coated plate for 24 h. (A) Western blot analysis of cyclin D1, cyclin D2, CDK6, p27, p-Erk and t-Erk in Rin-m cells on REP with or without Erk inhibitor. (B–F) Quantification of western blot analysis. Data are expressed as the mean ± SEM of three independent measurements. β-actin levels were analyzed as an internal control. (B–E) *P < 0.01 and **P < 0.05 compared with REP(-)/PD(-), #P < 0.01 compared with REP(+)/PD(-). (F) *P < 0.01 compared with REP(-)/PD(-), **P < 0.01 compared with REP(+)/PD(-).

the REP-dependent activation of both MAP kinase Erk and PI3-kinase/Akt pathways.

Cell surface adhesion receptors such as the integrins α3β1 and αvβ3, bind to the RGD sequence present on ECM molecules including type I and IV collagen, and fibronectin, and consequently transduce ECM signals that promote cell adhesion and proliferation in a variety of cell types, including β-cells [7, 8, 27]. Here, we observed that integrins α3, α5, and β1 were elevated in cells cultured in the presence of REP. Moreover, the interaction of those integrins with REP triggered downstream pathways via focal adhesion assembly and the subsequent Erk and Akt activation [12, 13, 15, 28, 29]. Fak (particularly the phosphorylated form) plays a major role in the regulation of intracellular signals and responses to ECM/integrin interaction [30]. Of note, MAP kinases such as Erk, are activated by p-Fak [31] and regulates cell proliferation and cell cycle progression [32, 33]. Our results showed that REP increased p-Fak and p-Erk levels in Rin-m cells, and support the hypothesis REP mediates the activation of Fak/Erk signals.
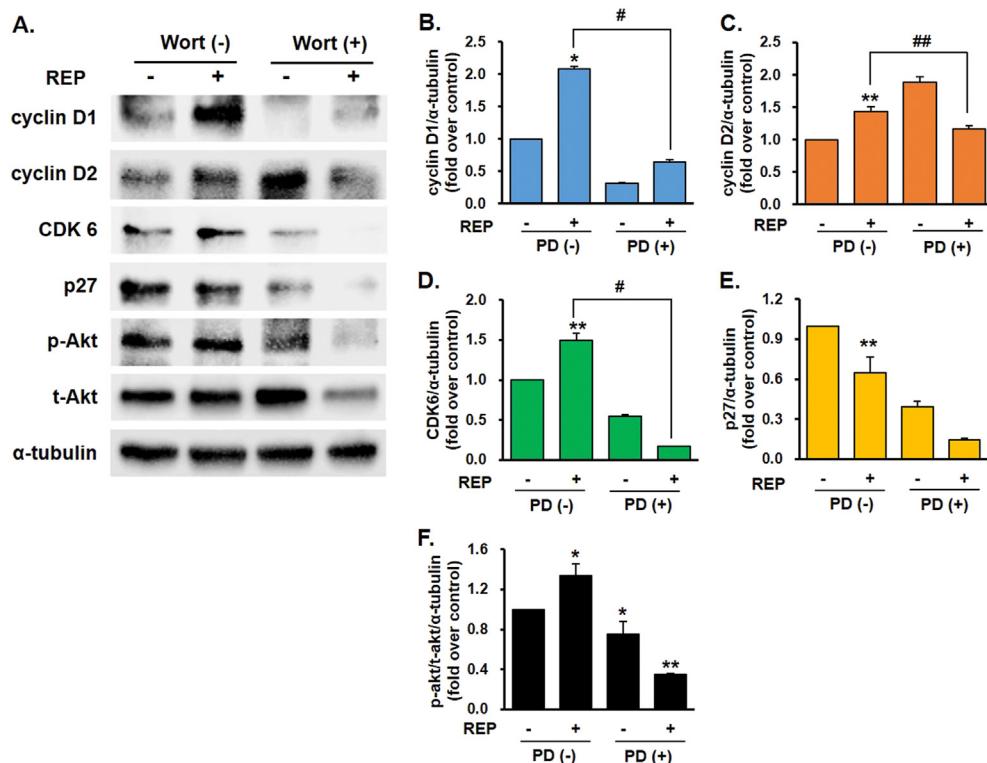

Figure 6. Involvement of the Akt pathways in REP-induced cell cycle proteins in β-cell. Rin-m cells were cultured with or without 100 nM wortmannin (PI3-kinase inhibitor) on a 1 μM REP-coated plate for 24 h. (A) Western blot analysis of cyclin D1, cyclin D2, CDK6, p27, p-Akt and t-Akt in Rin-m cells on REP with or without Akt inhibitor. (B–F) Quantification of western blot analysis. Data are expressed as the mean ± SEM of three independent measurements. β-actin levels were analyzed as an internal control. (B–E) *P < 0.001 and **P < 0.01 compared with REP(-)/PD(-), #P < 0.01 and ##P < 0.05 compared with REP(+)/PD(-). (F) *P < 0.05 compared with REP(-)/PD(-), **P < 0.01 compared with REP(+)/PD(-).
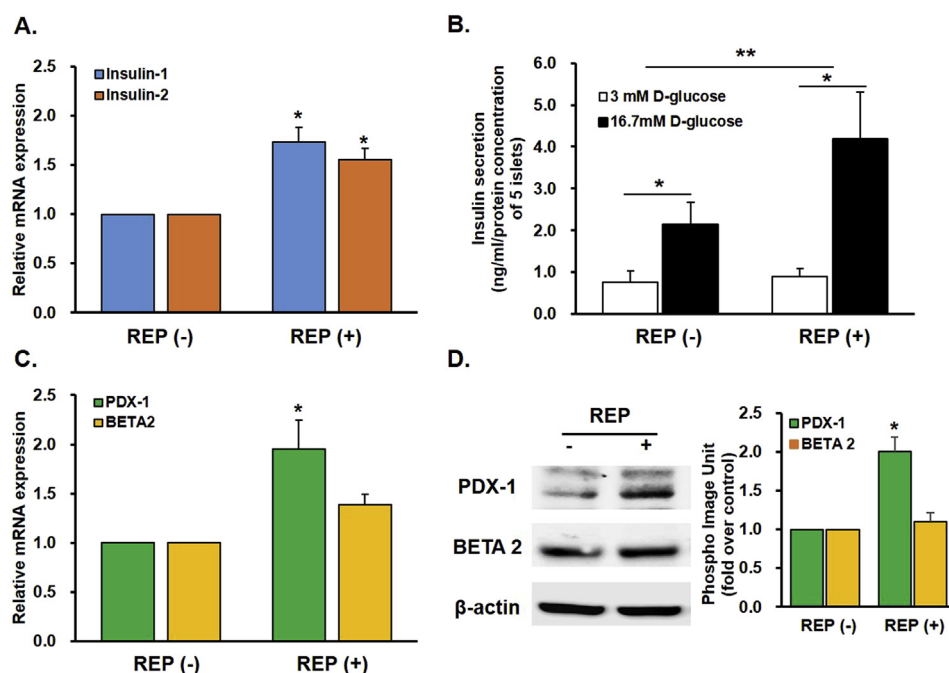
Figure 7. The effect of REP on the expression of insulin and β-cell-enriched transcription factors in β-cells. Rin-m cells were cultured on 1 μM REP for 24 h (A) RT-qPCR of the mRNA expression of Insulin-1and Insulin-2 in Rin-m cells. GAPDH mRNA was used as an internal control. *P < 0.01 compared with REP (-). (B) Quantification of GSIS of rat islets cultured on REP-coated plate. *P < 0.05 compared with 3mM D-glucose. **P < 0.05 compared with REP (-). (C) RT-qPCR of the mRNA expression of PDX-1, and BETA 2 in Rin-m cells. GAPDH mRNA was used as an internal control. *P < 0.05 compared with REP (-). (D) Western blot analysis of the protein expression of PDX-1 and BETA 2 in Rin-m cells. β-actin levels were analyzed as an internal control. *P < 0.01 compared with REP (-). All data are expressed as the mean ± SEM of three independent measurements.

In mammalian cell cycle control, cyclin D partners with cdk4 or cdk6, activating the kinase function of these cdks, and stimulating cell cycle progression [33, 34]. In contrast, p27 is an important inhibitor of the cell cycle, which restricts G1/S phase transition, downregulates CDK4/6/cyclinD holoenzyme activation, and consequently inhibits β-cell proliferation [22, 35, 36]. Here, we showed that β-cell proliferation was increased on REP-coated plates, indicating that REP induced cell cycle progression in β-cells. Additionally, the protein levels of signaling molecules that induce cell cycle progression, including Cyclin D1, Cyclin D2, and cdk6 were upregulated, whereas the protein level of p27 was down-regulated on REP coated plates. From a mechanistic point of view, the levels of Cyclin D1 and Cyclin D2 were increased in cells cultured on REP via REP-dependent Erk activation. Accumulating evidence suggests that β-cells in ECMs had increased cyclin D1 levels, as well as reduced p27 levels, which may be caused by activation of the FAK/ERK pathway [37]. Akt, a major β-cell proliferation regulator, has a number of downstream substrates that may contribute to cell cycle progression in a CDK4-dependent manner by regulation of Cyclin D1, Cyclin D2 and p21 [25]. Our results show that REP increased Cyclin D1, Cyclin D2 and CKD6 by regulating Akt phosphorylation. However, inhibition of REP-induced p27 was not related to Erk and Akt activation, and further research is needed to identify the p27 regulation responsible for REP.

PDX-1 and BETA 2 are the key transcription factors controlling insulin biosynthesis and secretion [38, 39]. PDX-1, in particular, is a key gene mediating the survival and function of β-cells [40]. Under REP-treated conditions, both the mRNA and the protein levels of PDX-1 increased significantly similar to treatment with FN. Consequently, gene expression of insulin-1 and insulin-2 was also upregulated by REP. These results suggest that REP regulates β-cell proliferation as well as functions.

In summary, our work describes possible mechanism for the regulation of β-cell proliferation by REP. REP induces enhancement of β-cell attachment and proliferation by regulating cell-cycle proteins, accompanied by integrin, Fak/MAP kinase Erk and PI3-kinase/Akt pathway stimulation. Moreover, REP increases β-cell function by regulating the gene or protein expression of insulin and its transcription factor, PDX-1. Our results provide new evidence for the mechanism of cell cycle regulation by REP and its therapeutic potential for improving pancreatic beta cell survival before transplantation.

Declarations

Author contribution statement

K. Lee: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Y. Hwang: Performed the experiments; Wrote the paper.

G. Jung: Analyzed and interpreted the data.

W. Jeon: Contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

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