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Journal of Cellular Biochemistry 116:1741–1754 (2015)

Journal of Cellular Biochemistry

Photoactivation of Akt1/GSK3β Isoform-Specific Signaling Axis Promotes Pancreatic β-Cell Regeneration

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ABSTRACT

Promotion of insulin-secreting β-cell regeneration in patients with diabetes is a promising approach for diabetes therapy, which can contribute to rescue the uncontrolled hyperglycemia. Low-power laser irradiation (LPLI) has been demonstrated to regulate multiple physiological processes both in vitro and in vivo through activation of various signaling pathways. In the present study, we showed that LPLI promoted β-cell replication and cell cycle progression through activation of Akt1/GSK3β isoform-specific signaling axis. Inhibition of PI3-K/Akt or GSK3 with specific inhibitors dramatically reduced or increased LPLI-induced β-cell replication, revealing Akt/GSK3 signaling axis was involved in β-cell replication and survival upon LPLI treatment. Furthermore, the results of shRNA-mediated knock down of Akt/GSK3 isoforms revealed that Akt1/GSK3β isoform-specific signaling axis regulated β-cell replication and survival in response to LPLI, but not Akt2/GSK3α. The mechanism by which LPLI promoted β-cell replication through Akt1/GSK3β signaling axis involved activation of β-catenin and down-regulation of p21. Taken together, these observations suggest that Akt1/GSK3β isoform signaling axis play a key role in β-cell replication and survival induced by LPLI. Moreover, our findings suggest that activation of Akt1/GSK3β isoform signaling axis by LPLI may provide guidance in practical applications for β-cell regenerative therapies. J. Cell. Biochem. 116: 1741–1754, 2015.

KEY WORDS: LPLI; Akt; GSK3; β-CELL; DIABETES

Diabetes is one of the most common and serious chronic diseases affecting human health in the twenty-first century. Diabetes is characterized by uncontrolled hyperglycemia and relevant to pancreatic β-cell loss and dysfunction, leading to an insulin-insufficient state. Pancreatic β-cells are killed by auto-immune attack in type 1 diabetes [Brezar et al., 2011], whereas peripheral insulin resistance and β-cell failure are observed in type 2 diabetes [DeFronzo and Abdul-Ghani, 2011]. Defective insulin secretion in both forms of diabetes is caused in part by loss of β-cell mass [Butler et al., 2003]. For this reason, β-cell regeneration and survival play a pivotal role in preventing the pathogenesis of diabetes. Recent studies suggest that diabetes can be reversed in type 1 and type 2 diabetes by replacement of β-cell mass, as demonstrated by pancreas and islet transplantation [Shapiro et al., 2006; Dean et al., 2008]. Moreover, β-cell replication can be readily demonstrated in fetal and neonatal islets of rodents [Dor et al., 2004] and human beings [Meier et al., 2008]. This replication ability is reduced with aging, but a low rate of β-cell replication has been observed in vivo in adult islets [Reers et al., 2009]. It has been shown that β-cell replication can be evoked in adult rodents and that this is a major

Abbreviations: Akt, protein kinase B; BrdU, 5-bromo-2-deoxyuridine; CCK-8, cell counting kit-8; ER, endoplasmic reticulum; FoxO1, forkhead box protein O1; GLP-1, glucagon-like peptide-1; GSK3, glycogen synthase kinase; HA, hemagglutinin; IRS, insulin receptor substrate; InsR, insulin receptor; LPLI, low-power laser irradiation; MAPK/ERK, mitogen-activated protein kinase/extracellular regulated protein kinase; NEFA, nonesterified fatty acid; PDX-1, pancreas/duodenum homeobox protein-1; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TCF/LEF, T-cell factor/lymphoid enhancer factor; TPKR, tyrosine protein kinase receptor.

Conflict of interest: The authors declare that there is no duality of interest associated with this manuscript.

Grant sponsor: National Basic Research Program of China; Grant numbers: 2011CB910402, 2010CB732602; Grant sponsor: Program for Changjiang Scholars and Innovative Research Team in University; Grant number: IRT0829; Grant sponsor: National Natural Science Foundation of China; Grant number: 81101741.

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Manuscript Received: 18 July 2014; Manuscript Accepted: 6 February 2015

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 3 March 2015

DOI 10.1002/jcb.25133 • © 2015 Wiley Periodicals, Inc.

pathway responsible for the increased β-cell mass [Wang et al., 1995]. Therefore, one promising approach for deficit in β-cell mass in diabetic patients is to manipulate β-cell replication.

In recent years, low-power laser irradiation (LPLI) with light spectrum from the visible to near-infrared range is considered to be a nondamaging physical therapy. LPLI has been found to regulate various biological processes in cells, animal models and clinical conditions. At the cellular level, LPLI has been shown to induce activation of diverse signaling pathways, such as tyrosine protein kinase receptor (TPKR), mitogen-activated protein kinase/extracellular regulated protein kinase (MAPK/ERK), protein kinase C (PKC) and phosphoinositide 3-kinase (PI3-K)/Akt [Wu and Xing, 2014]. The mechanisms of these biological effects caused by LPLI have been explored. Light energy is absorbed by intracellular chromophores, which accelerate electron transfer reactions, leading to increase the intracellular levels of Ca^{2+} and reactive oxygen species as versatile second messengers [Lan et al., 2012]. The current studies have shown that LPLI is involved in multiple cellular physiological functions, including gene transcription and protein expression [Huang et al., 2013], cytoskeleton reorganization [Ricci et al., 2009], and cell migration [Karunarathne et al., 2013]. In addition, it has been widely employed in neck pain care [Chow et al., 2009], skin anti-aging [Ling et al., 2014], and wound healing [Ozcelik et al., 2008]. Thus, the effect of LPLI on β-cell regeneration may provide potential therapeutic strategy for diabetes. Nevertheless, the underlying molecular mechanisms still remain elusive.

Protein kinase B (also named Akt) is required for different cellular processes, from the regulation of cell cycle, survival and growth to control of glucose and protein metabolism [Mason and Rathmell, 2011]. In mammals, three highly conserved isoforms of Akt (PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3) encoded by three separate genes have been identified. All three gene products share a high degree of amino acid identity and seem to be regulated by similar mechanisms [Hay, 2011]. Among them, Akt1 is the predominant Akt isoform and widely expressed in most tissues; Akt2 is usually expressed in insulin-responsive tissues, and Akt3 expression is restricted to the brain and testes [Garofalo et al., 2003]. In most situations, activation of Akt is dependent on PI3-K and then activated Akt in turn phosphorylates downstream substrates that in general promote cell proliferation and survival [Boppart et al., 2011]. One of the major downstream targets of Akt is the glycogen synthase kinase 3 (GSK3), which is a well-known target of insulin signaling. There are two GSK3 isoforms (GSK3α and GSK3β) encoded by distinct genes. In insulin signaling, GSK3 was inhibited by PI3-K/Akt-mediated phosphorylation, promoting glycogen synthesis and glucose disposal [Morral, 2003]. Furthermore, knockdown of GSK3 by RNA interference protected β-cells against apoptosis induced by endoplasmic reticulum stress [Srinivasan et al., 2005]. Conversely, activation of GSK3 negatively affected β-cell functions through controlling the stability and subcellular localization of pancreatic and duodenal homeobox-1 (Pdx-1) [Boucher et al., 2006].

In this study, we attempted to clarify the effects of Akt/GSK3 isoform signaling axis on pancreatic β-cell proliferation induced by LPLI. Based on various techniques and approaches, we found that LPLI promoted pancreatic β-cell proliferation and cell cycle progression through activating Akt1/GSK3β isoform-specific signaling axis, but not Akt2/GSK3α. Furthermore, we found that β-catenin and p21 were involved in LPLI-mediated β-cell proliferation. Our data suggest that LPLI has potential value in β-cell regenerative therapies.

MATERIALS AND METHODS

CELL CULTURE

RIN-m and INS-1 cells were grown in RPMI 1640 (GBICO, Co. Ltd., Grand Island, NY) medium containing 10% fetal calf serum (FCS, GBICO, Co. Ltd.), 50 U/ml penicillin, and 50 μg/ml streptomycin. HEK 293T cells were cultured in DMEM (GBICO, Co. Ltd.) containing 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were maintained in a humidified, 37°C incubator with 5% CO_2 and 95% air.

PLASMIDS AND REAGENTS

Hemagglutinin (HA)-tagged wild type GSK3β (WT-GSK3β) and dominant-negative GSK3β (DN-GSK3β) expression plasmids were kindly provided by Dr Mien-Chie Hung [Ding et al., 2007]. TOPflash and Renilla reporter plasmids were kindly provided by Dr Yoonseok Kam [Kam and Quaranta, 2009] and Dr Rob Michalides [Michalides et al., 2004], respectively. Wortmannin (50 nM) was procured from BIOMOL Research Laboratories, Inc. (Plymouth, PA). LY294002 (10 μM), Akt inhibitor IV (10 nM), SB216763 (20 μM), LiCl (5 mM), and glucagon-like peptide-1 (GLP-1, 10 nM) were procured from Sigma (St, Louis, MO). Akt inhibitor V (1 μM) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA). The following antibodies were used: anti-phospho-Thr^{308}-Akt, anti-phospho-Ser^{473}-Akt, anti-Akt1, anti-Akt2, anti-total-Akt, anti-phospho-Ser^{21}-GSK3α, anti-phospho-Ser^{9}-GSK3β, anti-GSK3α, anti-GSK3β, anti-β-catenin, anti-cyclin D1, and anti-GAPDH (Cell Signaling Technology, Beverly, MA). Antibodies for p21, Histone H3, and β-actin were obtained from Santa Cruz Biotechnology. HA tag antibody was obtained from Sigma.

ISOLATION OF RAT ISLETS

Pancreatic islets were isolated from male (200 g) Wistar rats by type V collagenase (Sigma) digestion and handpicked under a stereomicroscope using methods previously described [Buteau et al., 2003]. The isolated islets were seeded into 60-mm plates and cultured in RPMI medium containing 11.1 mM glucose and 10% FCS for 24 h. The islets were then washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and cultured for a period of 24 h in minimal RPMI medium. These islets were cultured for 24 h in minimal RPMI medium containing 3 mM glucose and then subjected to different treatments.

CELL TRANSFECTION AND LPLI TREATMENT

Transient transfection was carried out using X-tremeGENE HP DNA reagent (Roche Applied Science, Mannheim, Germany) according to manufacturer's recommendations. RIN-m cells were seeded on 22-mm culture glasses or 60-mm plates 1 day prior to transfection. Cells were maintained in Opti-MEM medium (Invitrogen, Carlsbad, CA) during transfection, and replaced with fresh culture medium 6 h later. Twenty-four hours after transfection, cells were subjected to different treatments.

RIN-m and INS-1 cells were serum-starved for 24 h in serum-free medium. The primary islets were serum-starved overnight. These cells were irradiated with a He–Ne laser (632.8 nm, HN-1000; Laser Technology Application Research Institute, Guangzhou, China) in the dark with the corresponding fluence of 0.2, 0.4, 0.8, and 1.2 J/cm^2, respectively. Throughout each experiment, these cells were kept either in a complete dark or a very dim environment, except when subjected to the light irradiation, to minimize the ambient light interference. The power intensity was maintained at 10 mW/cm^2.

RNA INTERFERENCE

Knockdown of Akt and GSK3 isoforms were performed using Akt-shRNAs and GSK3-shRNAs plasmids, respectively. Akt1-shRNA, Akt2-shRNA, and Akt1/2-shRNA were kindly provided by Dr Nicolas Tricaud [Cotter et al., 2010] and Dr Juan P. Bolaños [Delgado-Esteban et al., 2007], respectively. GSK3α-shRNA and GSK3β-shRNA were gifts from Dr David L. Turner [Yu et al., 2003]. Briefly, RIN-m cells, INS-1 cells, and primary islets were infected with lentiviral vectors expressing Akt1-shRNA or Akt2-shRNA. After 48 h, these cells were selected with 2 μg/ml puromycin (Sigma) for 1 day before different treatments. Akt1/2-shRNA, GSK3α-shRNA, and GSK3β-shRNA were delivered into RIN-m cells, INS-1 cells, and primary islets using X-tremeGENE HP DNA reagent (Roche Applied Science) according to the manufacturer's instructions. After 48 h, these cells were subjected to the next experiments. In order to inhibit PI3-K and Akt expression, small interfering RNA (siRNA) down-regulation of PI3-K and Akt were achieved with SignalSilence® PI3-K/Akt siRNA kit according to the manufacturer's instructions (Cell Signaling Technology). Briefly, cells at 80% confluence were incubated with PI3-K, Akt, and nonspecific siRNA. At 48 h posttransfection, cell proliferation was measured.

CELL VIABILITY AND PROLIFERATION ASSAY

RIN-m cells, INS-1 cells, and primary islets were seeded on 96-well microplate at a density of 1×10^4 cells or 20 islets per well. RIN-m and INS-1 cells were serum-starved for 24 h and primary islets were serum-starved overnight prior to LPLI or GLP-1 treatment. Forty-eight hours after treatments, cell viability and proliferation were assessed by cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Rockville, MD) and 5-bromo-2-deoxyuridine (BrdU) incorporation ELISA kit (Roche, Indianapolis, IN), respectively, according to the manufacturer's protocol. Cell viability and proliferation detection were determined by absorbance at 450 and 492 nm, respectively, using an Infinite 200 plate reader (TECAN, Mönnedorf, Switzerland).

CELL CYCLE ANALYSIS

For cell cycle analysis, RIN-m cells, INS-1 cells, and primary islets were rinsed with PBS and fixed in ice-cold 70% ethanol in PBS. After washing in PBS, the cells were resuspended in PBS containing 250 μg/ml RNase A (Sigma) at 4°C overnight. To stain DNA, cells were incubated for 30 min with propidium iodide (PI; Sigma) at 10 μg/ml in PBS. The DNA-PI contents were analyzed using a FACScanto II flow cytometer (Becton Dickinson, Mountain View, CA) with excitation at 488 nm. Fluorescent emission of DNA-PI complexes was measured at 564–606 nm. Cell debris was excluded from the analysis by an appropriate forward light scatter threshold setting. Data were analyzed with the ModFit (Verify Software House, Inc., Mansfield, MA) software.

IMMUNOFLUORESCENCE

RIN-m cells were cultured on glass cover slips and incubated overnight to establish adherence. The cells were serum-starved for 24 h and then subjected to different treatments. The treated cells were fixed with 4% paraformaldehyde for 15 min, followed by permeabilization in methanol for 10 min at −20°C. The cells were incubated in blocking buffer (5% bovine serum albumin in PBS) for 1 h at room temperature followed by incubation with anti-phospho-Ser^{473}-Akt and anti-β-actin antibody or β-catenin antibody (diluted 1:100 in blocking buffer) at 4°C overnight. Cells were washed three times for 5 min in PBS, and then incubated for 1.5 h with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody for β-actin and Alexa Fluor 555-conjugated goat anti-rabbit secondary antibody for phospho-Ser^{473}-Akt or β-catenin (diluted 1:300 in blocking buffer; Invitrogen) at room temperature. Nuclei were stained with PI for 30 min at room temperature before observation. The fluorescence of β-actin or phospho-Ser^{473}-Akt was excited with a 488 nm Ar-Ion laser or 543 nm He–Ne laser, and the fluorescence emission was detected through a 505–550 or 560–615 nm band pass filter, respectively. The fluorescence of β-catenin and PI were excited with a 488-nm Ar-Ion laser, and the fluorescence emission was detected through a 505–550 nm band pass or 600–650 nm band pass filter, respectively. Images were acquired using an LSM510 META confocal microscope (Zeiss, Jena, Germany) through a 40× oil objective (NA = 1.3) and the data were analyzed with Zeiss Rel 3.2 image processing software (Zeiss).

SUBCELLULAR FRACTIONATION

In order to isolate cytosolic and nuclear extracts, RIN-m cells, INS-1 cells, and primary islets were washed twice with ice-cold PBS and added with 250 μl of lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.05% Nonidet P-40, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 nM okadaic acid). Cytosolic and nuclear extracts were obtained as described previously [Huang et al., 2013].

WESTERN BLOT ANALYSIS

Proteins were extracted from RIN-m, INS-1, HEK 293T cells, and primary islets using lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1% Triton X-100, 0.1% SDS, and 100 mM phenylmethylsulfonyl fluoride). Western blot analysis was performed as described previously [Huang et al., 2011]. Briefly, the extracted proteins were separated in SDS–polyacrylamide gels and transferred to PVDF membranes. The membranes were washed three times for 10 min each time with Tris Buffered Saline with Tween 20 and incubated with the indicated primary antibodies at 4°C overnight. After incubation, the membranes were labeled with goat anti-mouse conjugated to IRDye™ 680 or goat anti-rabbit conjugated to IRDye™ 800 secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA). Detection was performed

using LI-COR Odyssey Scanning Infrared Fluorescence Imaging System (LI-COR, Inc., Lincoln, NE). Data were analyzed using LI-COR Image Studio Software (LI-COR, Biosciences, Lincoln, NE).

TRANSCRIPTIONAL REPORTER GENE ASSAY

T-cell factor/lymphoid enhancer factor (TCF/LEF)-dependent transcription activity was assessed by using TOPflash reporter plasmid. RIN-m and HEK 293T cells were seeded on 24-well dishes and co-transfected with TOPflash and Renilla reporter plasmids using Lipofectamine^{TM} 2000. After 24 h serum starvation, the cells were stimulated by LPLI (1.2 J/cm^2). Luciferase activity in transfected cells was measured with the Dual Luciferase Reagent Assay Kit (Promega, Madison, WI) according to manufacturer's instructions. Firefly and Renilla luciferase activity were detected using an Infinite 200 plate reader (TECAN). The relative luciferase activity was calculated by firefly activity (TOPflash) normalized to Renilla activity. In order to determine the effect of WT-GSK3β or DN-GSK3β on the TCF/LEF-dependent transcriptional activity induced by LPLI, they were co-transfected into cells with TOPflash reporter plasmid and the luciferase activity was measured as described above.

STATISTICAL ANALYSIS

All experiments were carried out in triplicate under identical conditions and data were represented as means ± SEM. Differences between two groups were analyzed by two-tailed Student's test. Difference with $P < 0.05$ was considered statistically significant. One-way analysis of variance (ANOVA) was used in multiple group comparisons. Bars with different characters are statistically different at $P < 0.05$ level.

RESULTS

LPLI PROMOTES PANCREATIC β-CELL PROLIFERATION AND CELL CYCLE PROGRESSION

It remains unclear whether LPLI could promote pancreatic β-cell proliferation and cell cycle progression. To this end, RIN-m and INS-1 cells were treated with different doses of LPLI or GLP-1. GLP-1 is a well-known factor which induces pancreatic β-cell proliferation and survival through activation of PI3-K/Akt signaling axis. Therefore, GLP-1 exposure was set as a positive control. Cell viability and proliferation were significantly increased by LPLI (≥0.8 J/cm^2) or GLP-1 treatment compared to control group (Fig. 1A and B). LPLI at 0.8 or 1.2 J/cm^2 caused marked 1.26- or 1.4-fold increase in cell proliferation in RIN-m cell, respectively; caused marked 1.29- or 1.55-fold increase in cell proliferation in INS-1 cells, respectively; GLP-1 exposure caused marked 2.17- or 1.94-fold increase in cell proliferation in RIN-m and INS-1 cells, respectively. Then, LPLI at 1.2 J/cm^2 was chosen for the further experiments. To evaluate the proliferative effect of LPLI on the primary islets, the isolated pancreatic islets were treated with LPLI or GLP-1. As shown in Figure 1C and D, cell viability and proliferation were significantly increased by LPLI or GLP-1 treatment in a time-dependent manner. LPLI treatment caused marked 1.24- or 1.36-fold increase in cell proliferation at 24 or 48 h, respectively; GLP-1 exposure caused marked 1.52- or 1.78-fold increase in cell proliferation at 24 or 48 h, respectively. Next, the effect of LPLI on pancreatic β-cell cycle progression was determined by flow cytometry analysis using PI staining. We found that LPLI treatment as well as GLP-1 exposure dramatically decreased cells in G0/G1 phase and increased cells in S and G2/M phase (Fig. 1E–H). Collectively, these results demonstrate that LPLI promotes pancreatic β-cell proliferation and cell cycle progression in a dose and time-dependent manner.

Akt/GSK3 SIGNALING AXIS IS INVOLVED IN PANCREATIC β-CELL PROLIFERATION UPON LPLI TREATMENT

We next investigated whether Akt/GSK3 signaling axis could be involved in β-cell proliferation induced by LPLI. In order to examine the activation of Akt, we used immunofluorescence staining for measuring activated Akt with phospho-Ser^{473}-Akt specific antibody. As shown in Figure 2A, phospho-Ser^{473}-Akt was barely detectable in control group (top row). Conversely, phospho-Ser^{473}-Akt substantially increased upon LPLI or GLP-1 treatment (middle and bottom row). Furthermore, the phosphorylation levels of Akt and GSK3 were significantly enhanced by LPLI or GLP-1 treatment with western blot analysis (Fig. 2B–E), whereas total levels of Akt and GSK3 were unchanged. These results suggest that activation of Akt/GSK3 signaling axis upon LPLI treatment is involved in pancreatic β-cell proliferation.

Subsequently, we investigate the role of Akt/GSK3 signaling axis in pancreatic β-cell proliferation induced by LPLI. RIN-m cells, INS-1cells, and primary islets were treated with LPLI in the presence or absence of PI3-K-, Akt-, or GSK3-specific inhibitors, respectively. We found that cell viability and proliferation were significantly increased by LPLI (Fig. 2F–I), which was dramatically reduced in the presence of PI3-K/Akt inhibitors (wortmannin, LY294002, Akt inhibitor IV, or Akt inhibitor V), revealing that PI3-K/Akt signaling was indeed involved in LPLI-induced β-cell proliferation. In addition, we found that cell viability and proliferation were lower than control group with or without LPLI treatment when Akt activity was prevented. To further verify the role of PI3-K/Akt signaling in pancreatic β-cell proliferation induced by LPLI, PI3-K/Akt siRNA were used. As shown in Figure 4J, β-cell proliferation mediated by LPLI was marked reduced, when PI3-K/Akt were down-regulated by siRNA, indicating that the PI3-K/Akt were required for β-cell proliferation and survival. Consistent with the above results, LiCl and SB216763 (two GSK3 inhibitors) significantly increased LPLI-induced cell viability and proliferation compared to LPLI treatment (Fig. 2K and L). Taken together, these results indicate that Akt/GSK3 signaling axis is involved in pancreatic β-cell proliferation upon LPLI treatment.

LPLI PROMOTES PANCREATIC β-CELL PROLIFERATION THROUGH Akt1/GSK3β ISOFORM-SPECIFIC SIGNALING AXIS, BUT NOT Akt2/GSK3α

Since Akt and GSK3 have several isoforms, we next asked if β-cell viability and proliferation were regulated by Akt/GSK3 isoform-specific signaling axis upon LPLI treatment. RNA interference technology was used to knock down the expression levels of Akt1, Akt2, Akt1/2, GSK3α, or GSK3β in RIN-m cells, INS-1 cells, and primary islets. As shown in Figure 3A and B, down-regulation of Akt1 specifically reduced LPLI-stimulated cell viability and proliferation, whereas Akt2 knockdown

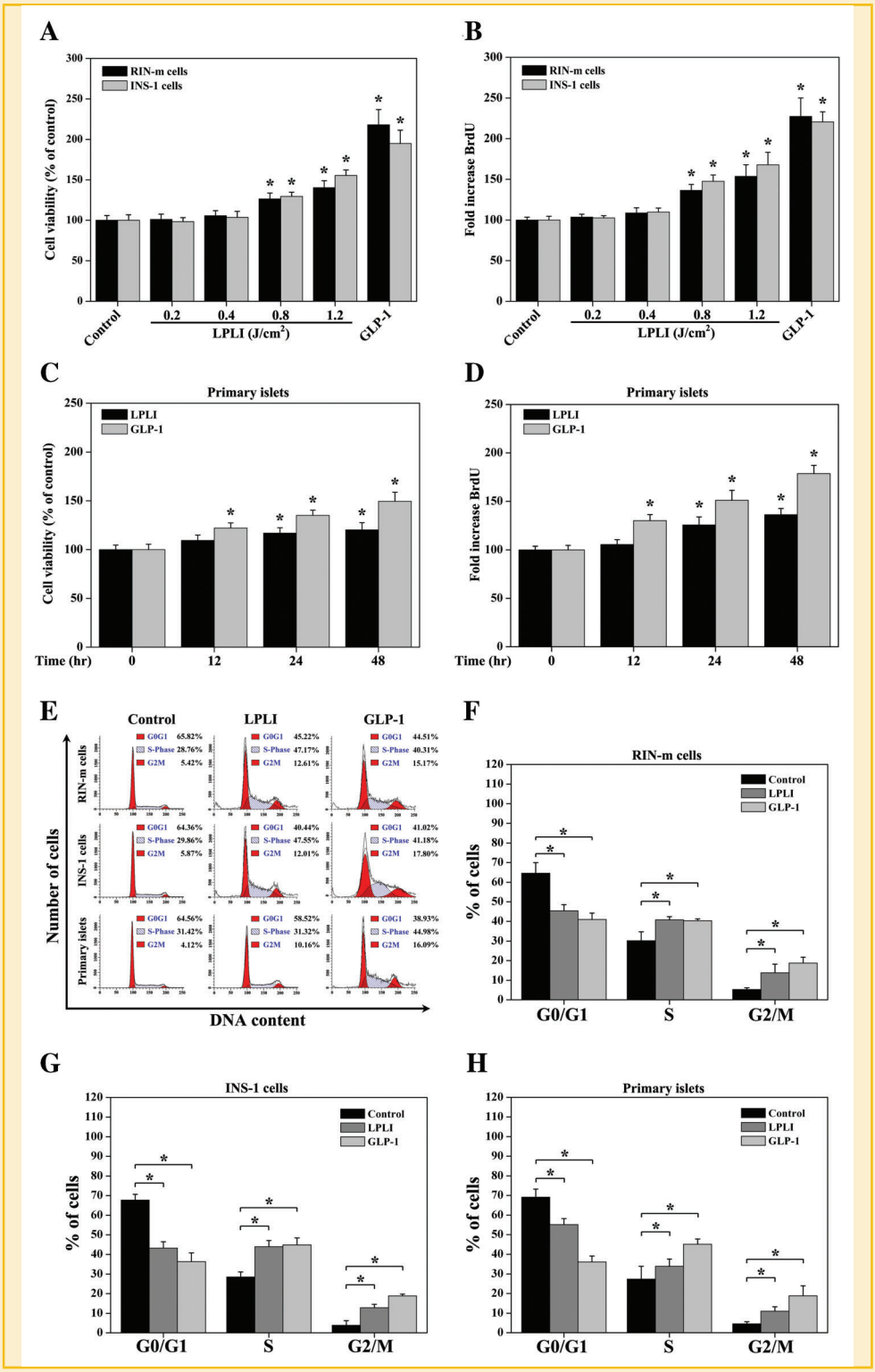


Fig. 1. LPLI induces β-cell proliferation and cell cycle progression. RIN-m and INS-1 cells were serum-starved for 24 h and then treated with different doses of LPLI at 0.2, 0.4, 0.8, 1.2 J/cm^2 or GLP-1. Forty-eight hours later, cell viability (A) and proliferation (B) were measured by CCK-8 and BrdU incorporation assays, respectively. Cells without any treatment under the same experimental procedure were set as control group. Data represent mean ± SEM (n = 3, $^*P < 0.05$). Primary islets were serum-starved overnight and then treated with LPLI at 1.2 J/cm^2 or GLP-1. Cell viability (C) and proliferation (D) were measured by CCK-8 and BrdU incorporation assays, respectively, at the indicated time. Data represent mean ± SEM (n = 3, $^*P < 0.05$). (E) RIN-m cells, INS-1 cells and primary islets were serum-starved and then treated with LPLI at 1.2 J/cm^2 or GLP-1. Twenty-four hours later, the cells were sorted and fixed, and then stained with PI. These cells were analyzed for DNA content by flow cytometry. The fractions of cells with G0/G1, S and G2/M DNA content were shown. (F–H) Quantitative analysis of the changes of cell cycle progression in treated cells. Data represent mean ± SEM (n = 3. $^*P < 0.05$ vs indicated group).

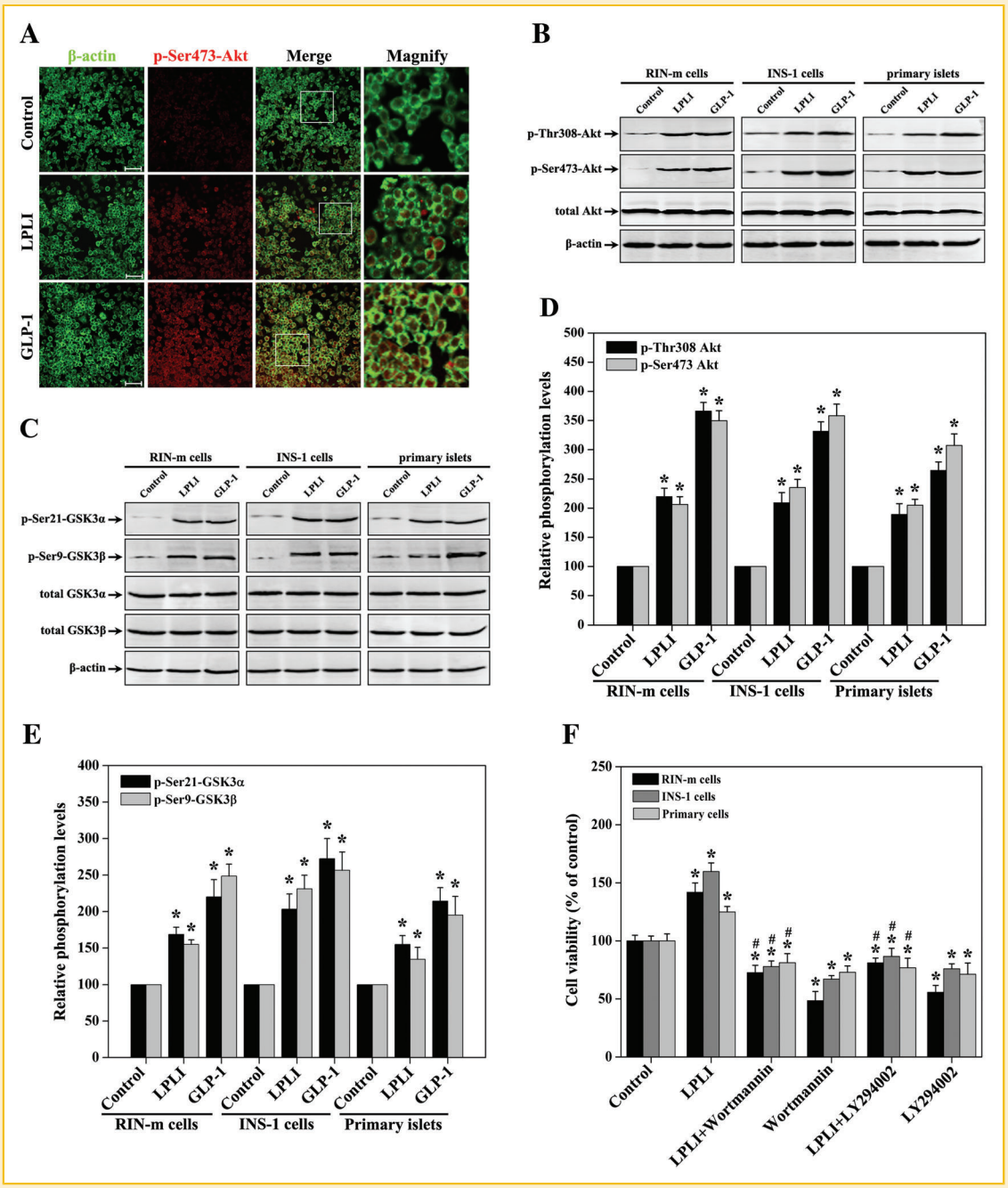
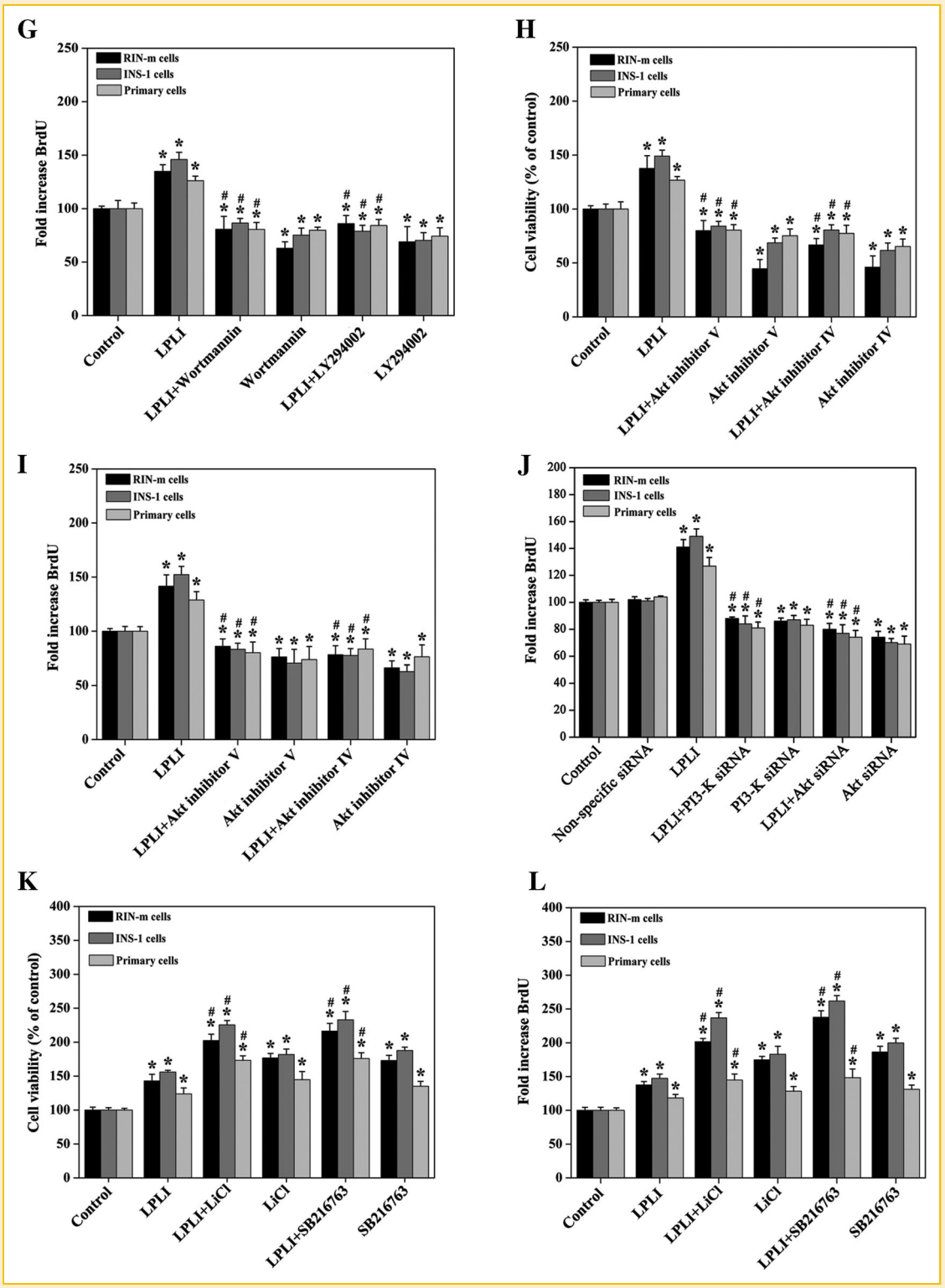


Fig. 2. LPLI induces β-cell proliferation through activation of Akt/GSK3 signaling axis. (A) Akt activation and localization were analyzed by immunofluorescence staining with phospho-Ser^{473}Akt (red emission) and β-actin (green emission) antibodies 0.5 h post LPLI treatment (1.2 J/cm^2) or GLP-1 exposure in RIN-m cells. Cells without any treatment were served as control group. Bar = 50 μm (n = 3). (B, C) Western blot analysis of RIN-m cells, INS-1 cells and primary islets upon LPLI treatment (1.2 J/cm^2) or GLP-1 exposure was performed to detect phospho-Thr^{308}-Akt, phospho-Ser^{473}-Akt, total Akt, phospho-Ser^{21}-GSK3α, phospho-Ser^{9}-GSK3β, total GSK3α and total GSK3β. β-actin served as the loading control. (D, E) Quantitative analysis of the changes of phospho-Thr^{308}-Akt, phospho-Ser^{473}-Akt, phospho-Ser^{21}-GSK3α and phospho-Ser^{9}-GSK3β in treated cells. Data represent mean ± SEM (n = 3. $^{*}P < 0.05$ vs control group). (F, G) RIN-m cells, INS-1 cells and primary islets were treated with LPLI (1.2 J/cm^2) in the presence or absence of PI3-K inhibitors (Wortmannin and LY294002). After 48 h, cell viability and proliferation were measured by CCK-8 and BrdU incorporation assays, respectively. Data represent mean ± SEM (n = 3, $^{*}P < 0.05$ vs control group, $^{\#}P < 0.05$ vs LPLI treatment). (H, I) Similar to (F, G), Akt specific inhibitors (Akt inhibitor IV or Akt inhibitor V) were used. Data represent mean ± SEM (n = 3, $^{*}P < 0.05$ vs control group, $^{\#}P < 0.05$ vs LPLI treatment). (J) PI3-K/Akt siRNA or control siRNA (100nM) were transfected into RIN-m cells, INS-1 cells and primary islets with or without LPLI treatment. After 48 h, cell proliferation was measured by BrdU incorporation assay. Data represent mean ± SEM (n = 3, $^{*}P < 0.05$ vs control group, $^{\#}P < 0.05$ vs LPLI treatment). (K, L) To determine the effect of GSK3 on cell viability and proliferation, the cells were treated with LPLI (1.2 J/cm^2) in the presence or absence of GSK3 inhibitors (LiCl and SB216763). After 48 h, cell viability and proliferation were measured by CCK-8 and BrdU incorporation assays, respectively. Data represent mean ± SEM (n = 3, $^{*}P < 0.05$ vs control group, $^{\#}P < 0.05$ vs LPLI treatment).



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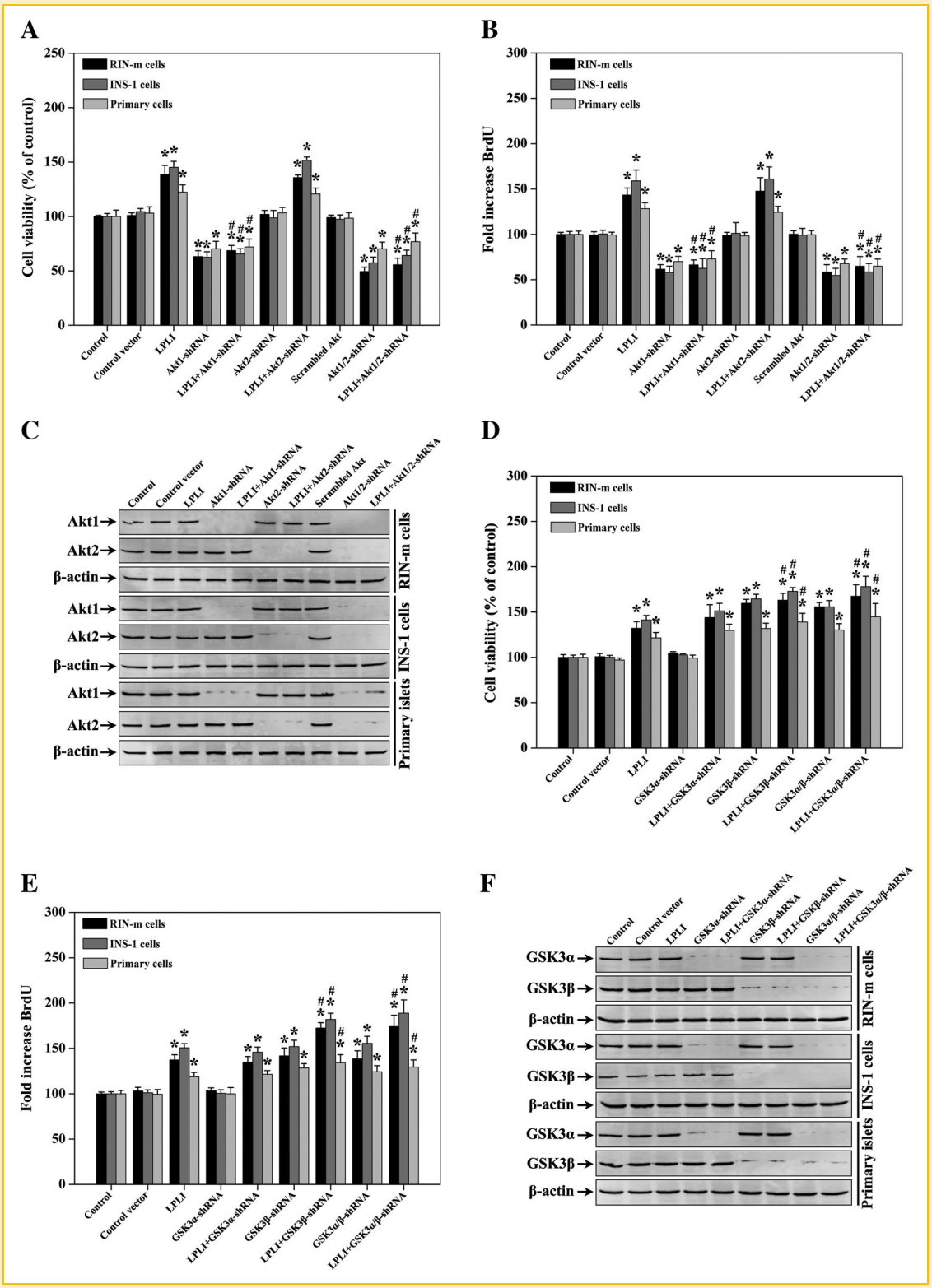

Fig. 3. Akt1/GSK3β isoform-specific signaling axis mediates β-cell proliferation upon LPLI treatment. Akt1-shRNA, Akt2-shRNA, Akt1/2-shRNA or control vector was delivered into RIN-m cells, INS-1 cells and primary islets. These cells were serum-starved and treated with LPLI (1.2 J/cm^2). After 48 h, cell viability (A) and proliferation (B) were measured by CCK-8 and BrdU incorporation assays, respectively. Data represent mean ± SEM (n = 3, $^*P < 0.05$ vs control group, $^\#P < 0.05$ vs LPLI treatment). (C) Expression levels of Akt1 and Akt2 in RIN-m cells, INS-1 cells and primary islets were detected by western blot analysis. GSK3α-shRNA, GSK3β-shRNA, GSK3α/β-shRNA or control vector was delivered into RIN-m cells, INS-1 cells and primary islets. These cells were serum-starved and treated with LPLI (1.2 J/cm^2). After 48 h, cell viability (D) and proliferation (E) were measured by CCK-8 and BrdU incorporation assays, respectively. Data represent mean ± SEM (n = 3, $^*P < 0.05$ vs control group, $^\#P < 0.05$ vs LPLI treatment). (F) Expression levels of GSK3α/β in RIN-m cells, INS-1 cells and primary islets were detected by western blot analysis.

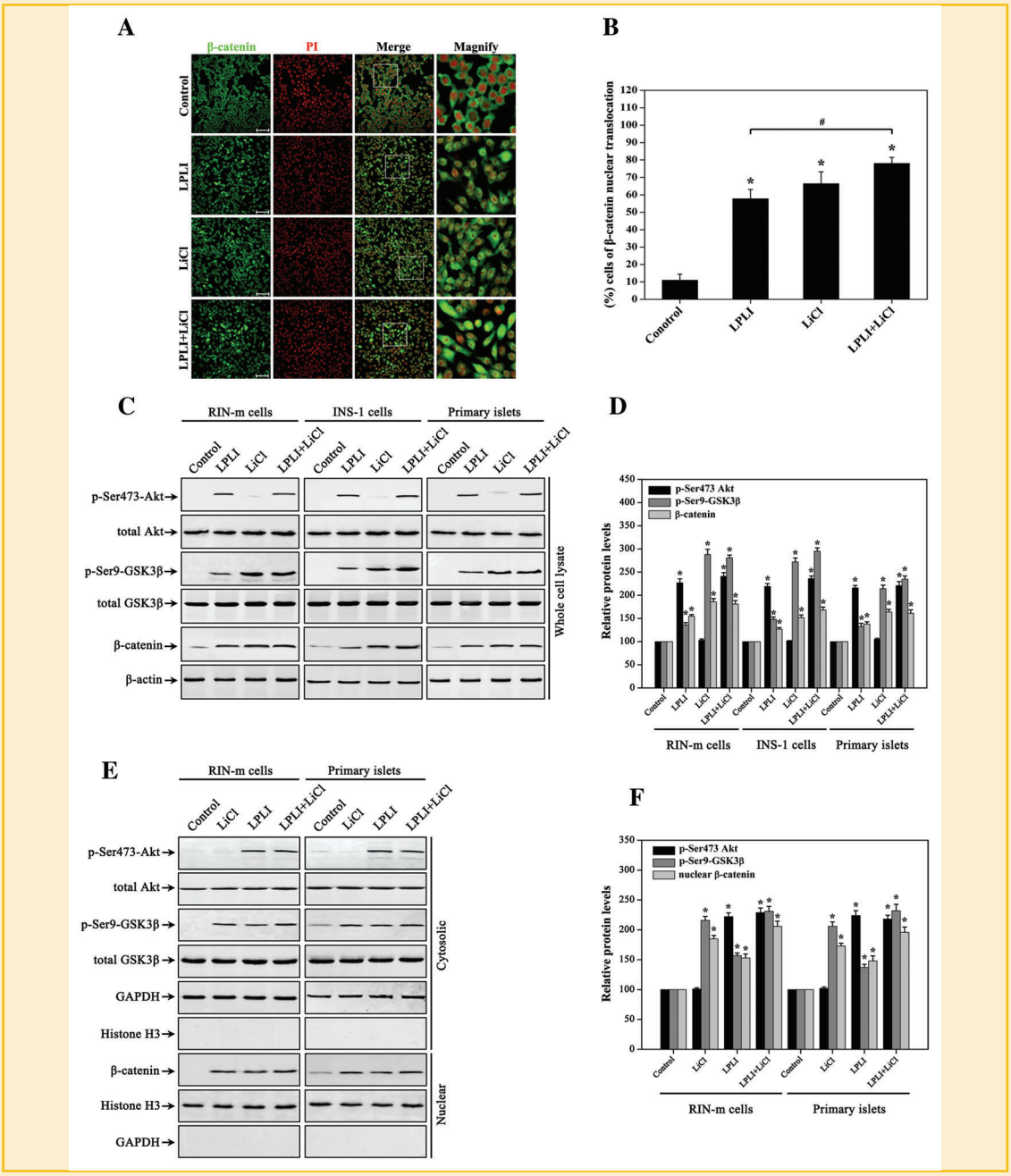

Fig. 4. LPLI induces activation of β-catenin and down-regulation of p21 in β-cells. (A) Nuclear translocation of β-catenin in RIN-m cells was analyzed by immunofluorescence staining with β-catenin (green emission) specific antibody 3 h post LPLI (1.2 J/cm^2), LiCl or LPLI plus LiCl treatment. Nuclei were stained with PI (red emission). Bar = 50 μm (n = 3). (B) The percentages of RIN-m cells with β-catenin nuclear translocation received different treatments. Data represent mean ± SEM (n = 3, $^*P < 0.05$ and $^{**}P < 0.01$ vs control group, $^\#P < 0.05$ vs. indicated group). For each group per experiment, 1,000 cells were counted. (C) Western blot analysis was performed to detect endogenous levels of phospho-Ser^{473}-Akt, total Akt, phospho-Ser^9-GSK3β, total Akt and β-catenin 0.5 h post LPLI (1.2 J/cm^2), LiCl or LPLI plus LiCl treatment. β-actin served as the loading control. (D) Quantitative analysis of the changes of phospho-Ser^{473}-Akt, phospho-Ser^9-GSK3β and β-catenin in treated cells. Data represent mean ± SEM (n = 3, $^*P < 0.05$ vs control group). (E) Akt and GSK3β activity and β-catenin stabilization were detected by western blot analysis. Cytosolic extracts were immunoblotted for phospho-Ser^{473}-Akt, total Akt, p-Ser^9-GSK3β and total GSK3β. Nuclear extracts were immunoblotted for β-catenin. Cytosolic and nuclear extracts were immunoblotted for GAPDH and Histone H3 protein to verify complete separation of the cytosolic and nuclear fractions. (F) Quantitative analysis of the changes of phospho-Ser^{473}-Akt, phospho-Ser^9-GSK3β and β-catenin in treated cells. Data represent mean ± SEM (n = 3, $^*P < 0.05$ vs. control group). (G, H) TOPflash, WT-GSK3β or DN-GSK3β expression plasmid and Renilla reporter plasmid were co-transfected into RIN-m and HEK 293T cells. After 24 h serum starvation, the cells were stimulated by LPLI (1.2 J/cm^2) and the luciferase activity was measured. Data represent mean ± SEM (n = 3, $^*P < 0.05$ vs control group, $^\#P < 0.05$ vs indicated group). (I) Exogenous expression of WT-GSK3β and DN-GSK3β in RIN-m and HEK 293T cells were measured using HA antibody. (J) RIN-m cells were serum-starved for 24 h and then treated with LPLI, LiCl or LPLI plus LiCl. Western blot analysis was performed to detect the expression of cyclin D1. (K) Quantitative analysis of the changes of cyclin D1 expression. Data represent mean ± SEM (n = 3, $^*P < 0.05$ vs control group). (L) RIN-m cells were transfected with WT-GSK3β or DN-GSK3β expression plasmid and then serum-starved for 24 h. After serum starvation, the cells were treated with LPLI (1.2 J/cm^2). Western blot analysis was performed to detect the expression of p21. Exogenous expression of WT-GSK3β and DN-GSK3β were measured using HA antibody. (M) Quantitative analysis of the changes of p21 expression. Data represent mean ± SEM (n = 3, $^*P < 0.05$ vs control group, $^\#P < 0.05$ vs indicated group).

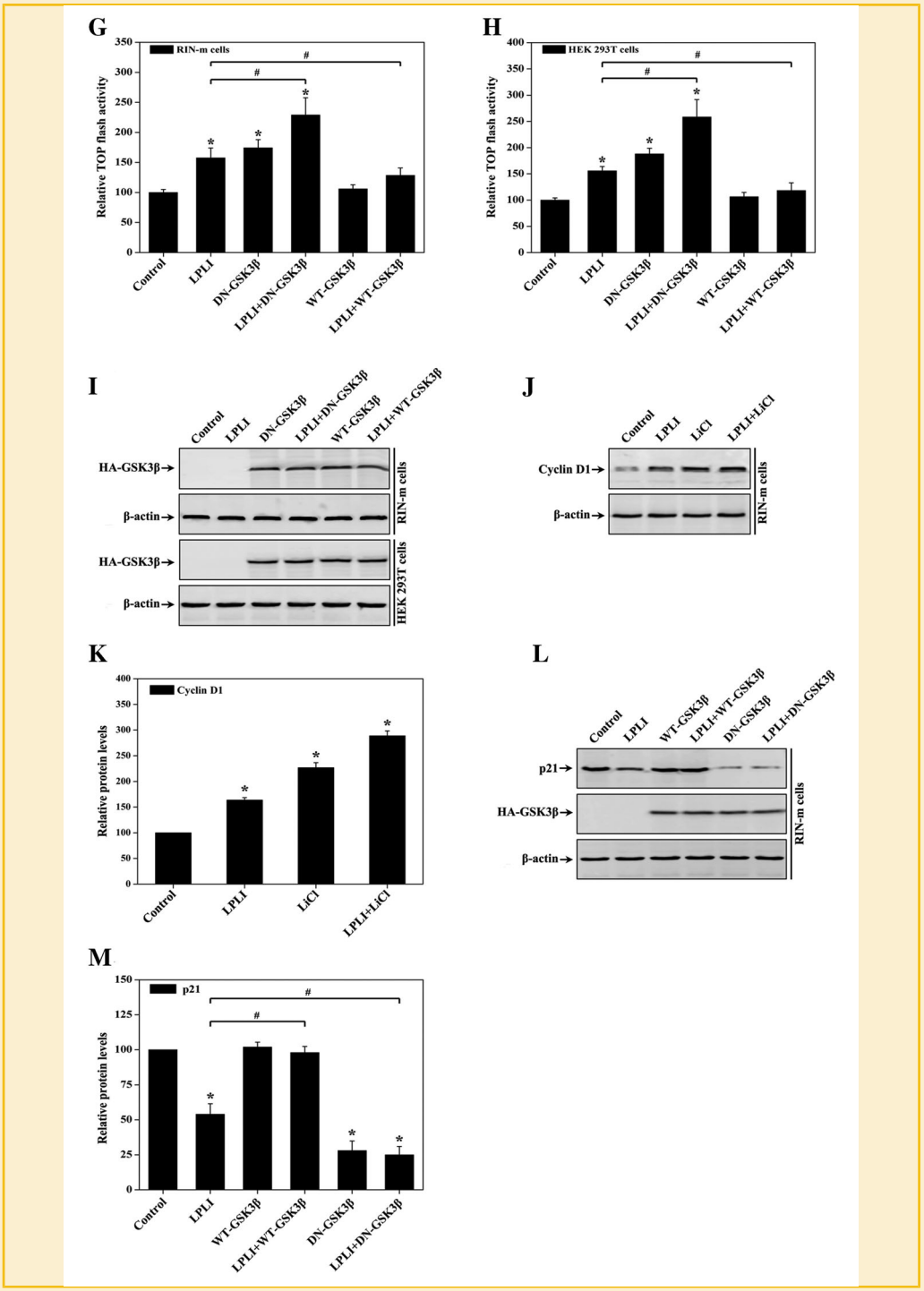

Fig. 4. Continued.

had no effect. When Akt1 and Akt2 were simultaneously knocked down, cell viability and proliferation induced by LPLI were dramatically reduced, but simultaneous knockdown of both Akt1 and Akt2 had not a greater inhibitory effect than Akt1 knockdown alone. In addition, we found that cell viability and proliferation were lower than control group with or without LPLI treatment when Akt1 was down-regulated, indicating that the expression levels of Akt1 were required for β-cell proliferation and survival. The expression levels of Akt1 and Akt2 in RIN-m cells, INS-1 cells, and primary islets were detected by western blot analysis (Fig. 3C). These results demonstrate that Akt1 regulates pancreatic β-cell proliferation and survival in response to LPLI stimulation, but not Akt2.

Subsequently, the expression levels of GSK3α and GSK3β in RIN-m cells, INS-1 cells, and primary islets were down-regulated with GSK3α-shRNA and GSK3β-shRNA, respectively. As shown in Figure 3D and E, knockdown of GSK3β significantly increased LPLI-induced cell viability and proliferation compared to LPLI treatment alone, but knockdown of GSK3α had no effect. When GSK3α and GSK3β were simultaneously knocked down, cell viability and proliferation induced by LPLI were dramatically increased, but simultaneous knockdown of both GSK3α and GSK3β had not a greater promotion than GSK3β knockdown alone. The expression levels of GSK3α and GSK3β in RIN-m cells, INS-1 cells, and primary islets were detected by western blot analysis (Fig. 3F). These results demonstrate that GSK3β regulates pancreatic β-cell proliferation in response to LPLI stimulation, but not GSK3α. Altogether, these results provide the first hard evidence that LPLI induces pancreatic β-cell proliferation through Akt1/GSK3β isoform-specific signaling axis, but not Akt2/GSK3α.

LPLI INDUCES ACTIVATION OF β-CATENIN AND DOWN-REGULATES EXPRESSION OF P21 IN PANCREATIC β-CELL

Next, we explore the effects of Akt1/GSK3β isoform signaling axis on pancreatic β-cell proliferation upon LPLI treatment. To this end, the distribution and transcriptional activity of β-catenin, a key downstream target of GSK3β, were assessed. As shown in Figure 4A, we found that β-catenin was localized in the cytoplasm in control group (top row). When cells were treated with LPLI, LiCl, or LPLI plus LiCl, nuclear accumulation of β-catenin was significantly increased (middle and bottom row). In addition, nuclear translocation of β-catenin was further enhanced in response to LPLI plus LiCl treatments (Fig. 4B). To further confirm that β-catenin nuclear translocation induced by LPLI through activation of Akt and inhibition of GSK3β activity, we lysed the RIN-m cells, INS-1cells, and primary islets and purified cytosolic/nuclear fractions. Concomitant with the increased levels of phospho-Ser^{473}-Akt and phospho-Ser^{9}-GSK3β, the stability of β-catenin in cells were dramatically enhanced under LPLI and LiCl treatment (Fig. 4C and D). Furthermore, concomitant with the increased levels of phospho-Ser^{473}-Akt and phospho-Ser^{9}-GSK3β in cytosolic fractions, the nuclear fractions of β-catenin were marked increased upon LPLI and LiCl treatment (Fig. 4E and F).

Subsequently, we evaluated the effect of nuclear translocation of β-catenin on target gene transcription induced by LPLI. The TCF/LEF-dependent transcriptional activity was measured in RIN-m cells using the TOPflash reporter plasmid. We found that TCF/LEF-dependent transcriptional activity was significantly elevated under LPLI treatment or transfected DN-GSK3β compared to control group (Fig. 4G). Furthermore, overexpression of DN-GSK3β in cells further increased TCF/LEF-dependent transcriptional activity induced by LPLI. Conversely, overexpression of WT-GSK3β in cells dramatically decreased TCF/LEF-dependent transcriptional activity induced by LPLI. Similar results were obtained from HEK 293T cells (Fig. 4H). Exogenous expressions of WT-GSK3β and DN-GSK3β were detected by western blotting (Fig. 4I). Since cyclin D1, the determinant of cell cycle progression, is a key downstream target of β-catenin/TCF/LEF axis, we measured the expression of cyclin D1 upon LPLI treatment. As shown in Figure 4J and K, we found that cyclin D1 expression was dramatically up-regulated in RIN-m cells upon LPLI, LiCl, and LPLI plus LiCl treatment. Altogether, these results indicate that LPLI induces pancreatic β-cell proliferation through stabilization and activation of β-catenin.

We also examined the expression of p21 upon LPLI treatment, a critical regulator of cell cycle arrest. As shown in Figure 4L and M, we found that the expression of p21 was significantly reduced in response to LPLI stimulation, and that overexpression of DN-GSK3β further decreased the expression of p21 induced by LPLI. In contrast, overexpression of WT-GSK3β dramatically prevented down-regulation of p21 induced by LPLI. These results suggest that LPLI promotes pancreatic β-cell cycle progression through down-regulation of p21.

DISCUSSION

In recent years, LPLI is becoming a valuable means to therapy of several diseases, such as nerve repairs [Chen et al., 2011], wound healing [Ozcelik et al., 2008], and reduction of pain and inflammation in a variety of pathologies [Khuman et al., 2012]. LPLI has also shown good effects on treatment of stroke, traumatic brain injury, and neurodegenerative disease [Naeser and Hamblin, 2011]. Previous studies have shown that LPLI modulated different biological processes through activation of diverse signaling pathways, such as RTK/PKCs, and MAPK and ultimately affected cell physiological processes [Wu and Xing, 2014]. Nevertheless, it is still unclear whether LPLI could promote pancreatic β-cell proliferation and survival and the underlying mechanisms are as yet obscure. In this study, we found that pancreatic β-cell proliferation and survival caused by LPLI was controlled by Akt1/GSK3β isoform-specific signaling axis, but not Akt2/GSK3α (Figs. 1–3). Another important observation was that activation of β-catenin and down-regulation of p21 were involved in LPLI-mediated β-cell proliferation (Fig. 4). Understanding the molecular mechanisms and functional significance of LPLI-induced pancreatic β-cell proliferation may lead to development of a new approach for β-cell regeneration therapies.

LPLI with light spectrum from the visible to near-infrared range is considered to be a nondamaging physical therapy, which has been used clinically to treat diverse diseases [Wu and Xing, 2014]. As we know, pancreatic β-cells are the only cells which can secrete insulin and control blood glucose in mammals. Therefore, promotion of moderate β-cell mass in diabetic patients may help to ameliorate diabetes and its complications. In this study, we found that LPLI promoted pancreatic β-cell replication and survival by activating Akt/GSK3 isoform-specific signaling pathway. Although LPLI was often used for treatment of different diseases, the effects of laser irradiation on β-cell growth and proliferation for clinical applications remain unknown. We believe that LPLI can be delivered into pancreatic islets by optical fiber in minimally invasive surgery and maintain β-cell mass in the patients with diabetes, although the relevant studies have not yet been reported.

Akt is considered to be an important downstream target of insulin receptor substrate (IRS)/PI3-K signaling in regulation of pancreatic β-cell mass and function [Rains and Jain, 2011]. However, the available data are contradictory. Our study is designed to evaluate the relevance of Akt isoforms in control of pancreatic β-cell proliferation upon LPLI

treatment. In the present study, we found that LPLI not only induced activation of Akt, but also increased pancreatic β-cell proliferation (Fig. 2A–H). Knockdown of Akt isoforms by shRNAs revealed that Akt1 mediated LPLI-induced β-cell proliferation, but not Akt2 (Fig. 3A–C), which suggested the functions of Akt isoforms in β-cells were different, though their structures were very similar in mammalian cells. Recent studies suggested that Akt2 was required for glucose metabolism [Buzzi et al., 2010] and deficiency of Akt2 led to insulin resistance [Dummler et al., 2006]. Despite Akt3 expression in β-cells, but metabolic parameters and pancreas morphology had no difference in Akt3$^{-/-}$ mice [Buzzi et al., 2010], suggesting that Akt3 was dispensable for glucose metabolism and β-cells proliferation. To our knowledge, the present study is the first to identify the Akt isoforms specificity in regulation of pancreatic β-cell proliferation induced by LPLI.

GSK3 is a well-defined downstream target of the insulin receptor (InsR)/PI3-K/Akt signaling pathway. A previous study suggested that up-regulation of GSK3 activity reduced islet β-cell mass and the resultant diabetic phenotype observed in mouse models with deficiency in receptors for insulin or IRS2 [Liu et al., 2008]. In this study, we found that inhibition of GSK3β significantly increased LPLI-induced β-cell proliferation with GSK3β inhibitors or GSK3β-shRNA (Figs. 2J–K and 3D–F). Of course, GSK3β may not be the only regulator of β-cell proliferation because other key downstream targets of the PI3-K/Akt signaling could be also involved in the process. For instance, forkhead box protein O1 (FoxO1), an important transcription factor for β-cell proliferation, reduced Pdx-1 expression through competing with FoxA2 binding at the *Pdx1* promoter [Kitamura et al., 2002]. Moreover, FoxO1 nuclear translocation activated transcription of the cell cycle inhibitors such as $p21^{cip1}$, $p27^{kip1}$, $p15^{INK4b}$, and $p19^{INK4d}$, which played important roles in modulating the cell cycle by IRS2/Akt/FoxO1 signaling pathway [Ho et al., 2008]. These studies emphasized the importance of PI3-K/Akt signaling pathway in maintenance of pancreatic β-cell growth and function, with two of the direct Akt targets, both GSK3β and FoxO1 leading to these complex regulations.

The functions of GSK3 in pancreatic β-cells could be closely related to cell proliferation and apoptosis than to its effects on glycometabolism in previous studies. It has been shown that down-regulation of GSK3β protected MIN6 cells from endoplasmic reticulum (ER)-stress-induced apoptosis [Srinivasan et al., 2005]. Recent study has also shown that inhibition of GSK3 protected pancreatic β-cell from nonesterified fatty acid (NEFA)-induced apoptosis in primary islets [Mussmann et al., 2007]. These investigations, in part along with the results of the present study, thus indicate a predominant role of GSK3β in pancreatic β-cell proliferation and survival as opposed to an effect on glucose metabolism. Conversely, GSK3α had no effect on LPLI-induced pancreatic β-cell proliferation, although it was inhibited in response to LPLI treatment (Figs. 2C and 3E). This observation suggests that GSK3α is a less important isoform than GSK3β in the regulation of pancreatic β-cell proliferation upon LPLI treatment.

The results of the present study suggest that a likely mechanism by which Akt1/GSK3β isoform-specific signaling axis regulates β-cell proliferation is through stabilization of β-catenin, induction of its nuclear translocation and transcriptional activity under LPLI treatment. GSK3β has been shown to phosphorylate β-catenin, leading to its proteasomal degradation in human islets [Liu et al., 2009] and mediate β-cell proliferation through regulation of Pitx2 and Cyclin D [Rulifson et al., 2007]. Our study confirmed that inhibition of GSK3β enhanced the stability and transcriptional activity of β-catenin in pancreatic β-cells upon LPLI treatment (Fig. 4A–H). Interestingly, FoxO1 has also been shown to inhibit TCF/LEF activity through interaction with β-catenin in nucleus [Jin, 2008]. Thus, in pancreatic β-cells with increased Akt1 activity, decreased GSK3β activity as well as decreased FoxO1 activity may converge to enhance the expression of β-catenin by different mechanisms. In addition to the regulation of β-catenin stability, GSK3β potentially modulated pancreatic β-cell proliferation through control of cyclin D1 stability because cyclin D1 has been shown to be phosphorylated and degraded by GSK3β [Diehl et al., 1998]. A previous study showed that inhibition of GSK3 by LiCl decreased p21 induction in human neuroblastoma [Watcharasit et al., 2002]. In the present study, we found that the expression of p21 were significantly decreased upon LPLI treatment (Fig. 4L and M), which may also explain that inhibition of GSK3β promoted pancreatic β-cell cycle progression through down-regulation of p21.

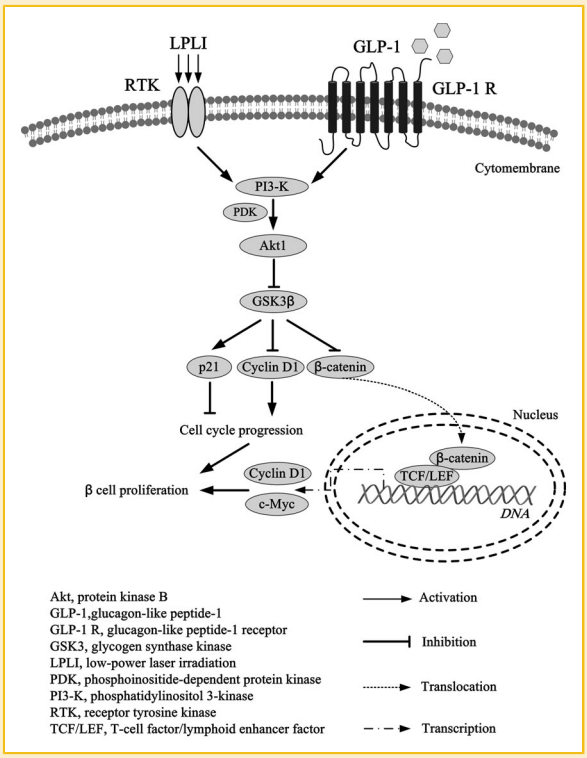

Fig. 5. The proposed model involving Akt1/GSK3β isoform-specific signaling axis to promote pancreatic β-cell proliferation upon LPLI treatment. Activated RTK/PI3-K on cell membrane by LPLI induces Akt1 activation. Subsequently, Akt1 prevents GSK3β activity through phosphorylation of serine 9, which stabilizes β-catenin and promotes β-catenin nuclear translocation. Nuclear β-catenin regulates TCF/LEF-dependent transcription, increasing cyclin D1 and c-Myc expression and accelerating cell cycle progression and proliferation. In addition, inhibition of GSK3β by Akt1 upon LPLI treatment down-regulates p21 expression, which promotes cell cycle progression.

In summary, our results revealed that Akt1/GSK3β isoform-specific signaling axis was crucial for pancreatic β-cell proliferation and survival upon LPLI treatment, but not Akt2/GSK3α. This in turn activated downstream signaling pathways that were closely related to regulation of pancreatic β-cell proliferation and cell cycle progression (Fig. 5). These findings may provide a potentially therapeutic strategy for diabetes through promotion of endogenous β-cell mass.

ACKNOWLEDGMENTS

We gratefully acknowledge Dr Mien-Chie Hung (Department of Molecular and Cellular Oncology, The University of Texas M.D. Anderson Cancer Center, Houston, TX), Dr Rob Michalides (Department of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan), Dr Nicolas Tricaud (Department of Biology, Eidgenössische Technische Hochschule Zürich, Switzerland), Dr Juan P. Bolaños (Departamento de Bioquímica y Biología Molecular, Universidad de Salamanca, Spain), Dr David L. Turner (Department of Biological Chemistry, University of Michigan, Ann Arbor, MI), and Dr Yoonseok Kam (Cancer Biology Department, Vanderbilt University School of Medicine, Nashville, TN) for gifting the plasmids.

REFERENCES

Boppart MD, Burkin DJ, Kaufman SJ. 2011. Activation of Akt signaling promotes cell growth and survival in alpha7beta1 integrin-mediated alleviation of muscular dystrophy. Biochim Biophys Acta 1812:439–446.

Boucher MJ, Selander L, Carlsson L, Edlund H. 2006. Phosphorylation marks IPF1/PDX1 protein for degradation by glycogen synthase kinase 3-dependent mechanisms. J Biol Chem 281:6395–6403.

Brezar V, Carel JC, Boitard C, Mallone R. 2011. Beyond the hormone: insulin as an autoimmune target in type 1 diabetes. Endocr Rev 32:623–669.

Buteau J, Foisy S, Joly E, Prentki M. 2003. Glucagon-like peptide 1 induces pancreatic beta-cell proliferation via transactivation of the epidermal growth factor receptor. Diabetes 52:124–132.

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC. 2003. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110.

Buzzi F, Xu L, Zuellig RA, Boller SB, Spinas GA, Hynx D, Chang Z, Yang Z, Hemmings BA, Tschopp O, Niessen M. 2010. Differential effects of protein kinase B/Akt isoforms on glucose homeostasis and islet mass. Mol Cell Biol 30:601–612.

Chen AC, Huang YY, Sharma SK, Hamblin MR. 2011. Effects of 810-nm laser on murine bone-marrow-derived dendritic cells. Photomed Laser Surg 29:383–389.

Chow RT, Johnson MI, Lopes-Martins RA, Bjordal JM. 2009. Efficacy of low-level laser therapy in the management of neck pain: a systematic review and meta-analysis of randomised placebo or active-treatment controlled trials. Lancet 374:1897–1908.

Cotter L, Ozcelik M, Jacob C, Pereira JA, Locher V, Baumann R, Relvas JB, Suter U, Tricaud N. 2010. Dlg1-PTEN interaction regulates myelin thickness to prevent damaging peripheral nerve overmyelination. Science 328:1415–1418.

Dean PG, Kudva YC, Stegall MD. 2008. Long-term benefits of pancreas transplantation. Curr Opin Organ Transplant 13:85–90.

DeFronzo RA, Abdul-Ghani MA. 2011. Preservation of beta-cell function: the key to diabetes prevention. J Clin Endocrinol Metab 96:2354–2366.

Delgado-Esteban M, Martin-Zanca D, Andres-Martin L, Almeida A, Bolanos JP. 2007. Inhibition of PTEN by peroxynitrite activates the phosphoinositide-3-kinase/Akt neuroprotective signaling pathway. J Neurochem 102:194–205.

Diehl JA, Cheng M, Roussel MF, Sherr CJ. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 12:3499–3511.

Ding Q, He X, Hsu JM, Xia W, Chen CT, Li LY, Lee DF, Liu JC, Zhong Q, Wang X, Hung MC. 2007. Degradation of Mcl-1 by beta-TrCP mediates glycogen synthase kinase 3-induced tumor suppression and chemosensitization. Mol Cell Biol 27:4006–4017.

Dor Y, Brown J, Martinez OI, Melton DA. 2004. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429:41–46.

Dummler B, Tschopp O, Hynx D, Yang ZZ, Dirnhofer S, Hemmings BA. 2006. Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. Mol Cell Biol 26:8042–8051.

Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, Brees DJ, Wicks JR, McNeish JD, Coleman KG. 2003. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. J Clin Invest 112:197–208.

Hay N. 2011. Akt isoforms and glucose homeostasis—the leptin connection. Trends Endocrinol Metab 22:66–73.

Ho KK, Myatt SS, Lam EW. 2008. Many forks in the path: cycling with FoxO. Oncogene 27:2300–2311.

Huang L, Wu S, Xing D. 2011. High fluence low-power laser irradiation induces apoptosis via inactivation of Akt/GSK3beta signaling pathway. J Cell Physiol 226:588–601.

Huang L, Tang Y, Xing D. 2013. Activation of nuclear estrogen receptors induced by low-power laser irradiation via PI3-K/Akt signaling cascade. J Cell Physiol 228:1045–1059.

Jin T. 2008. The WNT signalling pathway and diabetes mellitus. Diabetologia 51:1771–1780.

Kam Y, Quaranta V. 2009. Cadherin-bound beta-catenin feeds into the Wnt pathway upon adherens junctions dissociation: evidence for an intersection between beta-catenin pools. PLoS ONE 4:e4580.

Karunarathne WK, Giri L, Patel AK, Venkatesh KV, Gautam N. 2013. Optical control demonstrates switch-like PIP3 dynamics underlying the initiation of immune cell migration. Proc Natl Acad Sci USA 110:E1575–E1583.

Khuman J, Zhang J, Park J, Carroll JD, Donahue C, Whalen MJ. 2012. Low-level laser light therapy improves cognitive deficits and inhibits microglial activation after controlled cortical impact in mice. J Neurotrauma 29:408–417.

Kitamura T, Nakae J, Kitamura Y, Kido Y, Biggs WH III, Wright CV, White MF, Arden KC, Accili D. 2002. The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. J Clin Invest 110:1839–1847.

Lan CC, Wu SB, Wu CS, Shen YC, Chiang TY, Wei YH, Yu HS. 2012. Induction of primitive pigment cell differentiation by visible light (helium-neon laser): a photoacceptor-specific response not replicable by UVB irradiation. J Mol Med (Berl) 90:321–330.

Ling Q, Meng C, Chen Q, Xing D. 2014. Activated ERK/FOXM1 pathway by low-power laser irradiation inhibits UVB-induced senescence through down-regulating p21 expression. J Cell Physiol 229:108–116.

Liu Z, Tanabe K, Bernal-Mizrachi E, Permutt MA. 2008. Mice with beta cell overexpression of glycogen synthase kinase-3beta have reduced beta cell mass and proliferation. Diabetologia 51:623–631.

Liu H, Remedi MS, Pappan KL, Kwon G, Rohatgi N, Marshall CA, McDaniel ML. 2009. Glycogen synthase kinase-3 and mammalian target of rapamycin pathways contribute to DNA synthesis, cell cycle progression, and proliferation in human islets. Diabetes 58:663–672.

Mason EF, Rathmell JC. 2011. Cell metabolism: an essential link between cell growth and apoptosis. Biochim Biophys Acta 1813:645–654.

Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC. 2008. Beta-cell replication is the primary mechanism subserving

the postnatal expansion of beta-cell mass in humans. Diabetes 57:1584–1594.

Michalides R, Griekspoor A, Balkenende A, Verwoerd D, Janssen L, Jalink K, Floore A, Velds A, van't Veer L, Neefjes J. 2004. Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer. Cancer Cell 5:597–605.

Morral N. 2003. Novel targets and therapeutic strategies for type 2 diabetes. Trends Endocrinol Metab 14:169–175.

Mussmann R, Geese M, Harder F, Kegel S, Andag U, Lomow A, Burk U, Onichtchouk D, Dohrmann C, Austen M. 2007. Inhibition of GSK3 promotes replication and survival of pancreatic beta cells. J Biol Chem 282:12030–12037.

Naeser MA, Hamblin MR. 2011. Potential for transcranial laser or LED therapy to treat stroke, traumatic brain injury, and neurodegenerative disease. Photomed Laser Surg 29:443–446.

Ozcelik O, Cenk Haytac M, Kunin A, Seydaoglu G. 2008. Improved wound healing by low-level laser irradiation after gingivectomy operations: a controlled clinical pilot study. J Clin Periodontol 35:250–254.

Rains JL, Jain SK. 2011. Oxidative stress, insulin signaling, and diabetes. Free Radic Biol Med 50:567–575.

Reers C, Erbel S, Esposito I, Schmied B, Buchler MW, Nawroth PP, Ritzel RA. 2009. Impaired islet turnover in human donor pancreata with aging. Eur J Endocrinol 160:185–191.

Ricci R, Pazos MC, Borges RE, Pacheco-Soares C. 2009. Biomodulation with low-level laser radiation induces changes in endothelial cell actin filaments and cytoskeletal organization. J Photochem Photobiol B 95:6–8.

Rulifson IC, Karnik SK, Heiser PW, ten Berge D, Chen H, Gu X, Taketo MM, Nusse R, Hebrok M, Kim SK. 2007. Wnt signaling regulates pancreatic beta cell proliferation. Proc Natl Acad Sci USA 104:6247–6252.

Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems JA, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DE, Eisenbarth G, Segal M, Preiksaitis J, Korbutt GS, Barton FB, Viviano L, Seyfert-Margolis V, Bluestone J, Lakey JR. 2006. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 355:1318–1330.

Srinivasan S, Ohsugi M, Liu Z, Fatrai S, Bernal-Mizrachi E, Permutt MA. 2005. Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3beta in mouse insulinoma cells. Diabetes 54:968–975.

Wang RN, Kloppel G, Bouwens L. 1995. Duct- to islet-cell differentiation and islet growth in the pancreas of duct-ligated adult rats. Diabetologia 38:1405–1411.

Watcharasit P, Bijur GN, Zmijewski JW, Song L, Zmijewska A, Chen X, Johnson GV, Jope RS. 2002. Direct, activating interaction between glycogen synthase kinase-3beta and p53 after DNA damage. Proc Natl Acad Sci USA 99:7951–7955.

Wu SN, Xing D. 2014. Intracellular signaling cascades following light irradation. Laser Photonics Rev 8:115–130.

Yu JY, Taylor J, DeRuiter SL, Vojtek AB, Turner DL. 2003. Simultaneous inhibition of GSK3alpha and GSK3beta using hairpin siRNA expression vectors. Mol Ther 7:228–236.