Molecular and Cellular Endocrinology 382 (2014) 178–189

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

The *PARK2* gene is involved in the maintenance of pancreatic β-cell functions related to insulin production and secretion

Hyun-Seok Jin [a,1], Jeonghyun Kim [a,1], Soo-Jin Lee [b,1], Kyunga Kim [c], Min Jin Go [d], Jong-Young Lee [d], Hye-Ja Lee [e], Jihyun Song [e], Byeong Tak Jeon [f], Gu Seob Roh [f], Sung-Jun Kim [g], Bo-Young Kim [a], Kyung-Won Hong [h], Young-Hyun Yoo [i], Beomseok Oh [j], Yup Kang [b,*], Seon-Yong Jeong [a,*]

[a] Department of Medical Genetics, Ajou University School of Medicine, Suwon, Republic of Korea
[b] Institute for Medical Sciences, Ajou University School of Medicine, Suwon, Republic of Korea
[c] Department of Statistics, Sookmyung Women's University, Seoul, Republic of Korea
[d] Centre for Genome Science, National Institute of Health, Osong Health Technology Administration Complex, Chungcheongbuk-do, Republic of Korea
[e] Division of Metabolic Diseases, Centre for Biomedical Sciences, National Institute of Health, Chungcheongbuk-do, Republic of Korea
[f] Department of Anatomy and Neurobiology, Institute of Health Sciences, Gyeongsang National University School of Medicine, Jinju, Republic of Korea
[g] Drug Evaluation Centre, CJ CheilJedang Corporation, Icheon, Republic of Korea
[h] Division of Epidemiology and Health Index, Centre for Genome Science, Korea Centres for Disease Control & Prevention, Chungcheongbuk-do, Republic of Korea
[i] Department of Anatomy and Cell Biology and Mitochondria Hub Regulation Centre, College of Medicine, Dong-A University, Republic of Korea
[j] Department of Biomedical Engineering, School of Medicine, Kyung Hee University, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 10 July 2013
Received in revised form 18 September 2013
Accepted 24 September 2013
Available online 3 October 2013

Keywords:
Type 2 diabetes
Pancreatic β-cell
Insulin secretion
Genetic association
PARK2
Mitochondria

ABSTRACT

Several association studies have implicated the *PARK2* gene that encodes parkin – the key molecule orchestrating the mitochondrial quality control system – as a candidate susceptibility gene for diabetes. A total of 7551 unrelated Korean KARE cohort subjects were analyzed to investigate the association between the *PARK2* single nucleotide polymorphism (SNP) and quantitative glycemic traits. Two SNPs, rs10455889 and rs9365294, were significantly associated with fasting plasma glucose level ($p = \sim 1.2 \times 10^{-4}$) and insulin secretion indices ($p = \sim 7.4 \times 10^{-5}$) in male KARE subjects. Parkin was expressed predominantly in the rat pancreatic islets. Downregulation of the *Park2* gene in rat INS-1 β-cells resulted in a significant decrease in the glucose-stimulated insulin secretion, intracellular insulin gene expression, and intracellular ATP level. The *Park2*-depleted β-cells also exhibited increased mitochondrial fragmentation and ROS production and decreased mitochondrial membrane potential. Both population-based statistical evaluation and experimental evidence demonstrated a fundamental role of the *PARK2* gene in the maintenance of β-cell function.

1. Introduction

Type 2 diabetes (T2D) is characterized by hyperglycemia that results from concomitant defects in insulin secretion from the pancreatic islet β-cells (evolving β-cell failure) and insulin action in the peripheral tissues (chronic insulin resistance) (Stumvoll et al., 2005). The pathogenesis of T2D is therefore determined both by strong environmental factors and by multiple genetic factors (Bonnefond et al., 2010; Prokopenko et al., 2008). Robust genetic studies of T2D heritability have identified many susceptibility loci/genes for T2D-related quantitative traits and/or T2D status (Bonnefond et al., 2010; Huyghe et al., 2013). However, these identified genetic factors only explain the etiology of a small proportion of T2D cases. The vast majority of genetic risk factors for T2D-related traits and/or T2D still remain to be identified (Saxena et al., 2007).

In addition to genetic variations, epigenetic changes in response to environmental stimuli have been implicated in the pathogenesis of T2D. Emerging evidence raises the possibility that the epigenetic regulation of gene expression caused by alterations in DNA methylation and histone modifications may play an important role in β-cell dysfunction (Gilbert and Liu, 2012). Changes in microRNA expression have also been reported to be involved in pancreatic β-cell dysfunction in prediabetic NOD mice (Roggli et al., 2012).

Progressive β-cell failure, which includes disruption of β-cell function and reduction of β-cell mass, is the central component of the onset and progression of T2D (Prentki and Nolan, 2006).

* Corresponding authors. Address: Institute for Medical Sciences, School of Medicine, Ajou University School, San 5, Wonchon-Dong, Yeongtong-Gu, Suwon 443-721, Republic of Korea. Tel.: +82 31 219 4516 (Y. Kang), Department of Medical Genetics, School of Medicine, Ajou University, San 5, Wonchon-Dong, Yeongtong-Gu, Suwon 443-721, Republic of Korea. Tel.: +82 31 219 4520; fax: +82 31 219 4521 (S.-Y. Jeong).
E-mail addresses: kangy@ajou.ac.kr (Y. Kang), jeongsy@ajou.ac.kr (S.-Y. Jeong).
[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.mce.2013.09.031

Accumulating evidence now also indicates an involvement of mitochondrial dysfunction in the development of T2D (Patti and Corvera, 2010). Defects in mitochondrial function in β-cells are closely associated with dysregulation of insulin production and glucose-stimulated insulin secretion (GSIS) (Jitrapakdee et al., 2010). Mitochondrial function, in turn, is regulated and maintained by the mitochondrial quality control (QC) system (Fischer et al., 2012).

Recently, mitophagy – the selective autophagy of mitochondria – has been identified as a key component of the mitochondrial QC system (Tanaka, 2010). The selective clearance of damaged and dysfunctional mitochondria during mitophagy is initially mediated by two proteins, PINK1 (a serine/threonine-protein kinase PTEN-induced putative kinase 1) and parkin (an E3 ubiquitin-protein ligase) (Jin and Youle, 2012). Two genes, *PINK1* and *PARK2* encoding the PINK1 and parkin proteins, respectively, are known to be mutated in certain cases of early-onset familial Parkinson's disease (PD), thereby indicating a critical role of PINK1/parkin-mediated mitophagy in neuronal degeneration (Deas et al., 2011). Interestingly, results from several population-based statistical associations suggest that either of these two genes may be a strong candidate as susceptibility genes associated with T2D-related traits and/or T2D. The nonsynonymous SNP rs1043424 (Asn521Thr) in the *PINK1* gene was associated with T2D in a Northern Chinese population (Qu et al., 2011). Altered *PINK1* expression was found in T2D patients and inhibition of *PINK1* induced the impairment of basal glucose uptake in human neuronal cells (Scheele et al., 2007). In the *PARK2* gene, three independent linkage or genome-wide association (GWA) studies in nondiabetic Mexican-Americans (Duggirala et al., 2001) and in diabetic Pima Indians (Lindsay et al., 2001) both suggested a possible new locus for susceptibility to T2D status. The *PARK2* gene was later revealed to be the responsible locus (Leak et al., 2008). A further study using epistatic multi-locus interaction analysis reported that two intronic SNPs (rs1893551 and rs6924502) in the *PARK2* gene were associated with T2D in a UK population (Wongseree et al., 2009).

In the present study, our aim was to investigate whether genetic variation in the *PARK2* gene is associated with quantitative glycemic traits in Korean cohorts and to further clarify the role of parkin in pancreatic β-cell function, focusing on insulin production and secretion in response to glucose stimulation.

2. Materials and methods

2.1. Study subjects

2.1.1. KARE cohort

The subjects of the Korean Association Resource (KARE) study (also known as Ansan-Ansung community based cohort) have been described in a previous report (Cho et al., 2009). Briefly, a total of 8842 participants (4183 men and 4659 women) aged from 40 to 69 years were recruited from two community-based epidemiological cohorts, the rural community of Ansung city and the urban community of Ansan city. To analyze the accurate plasma glucose and insulin levels, 1291 subjects who had been treated with any drugs in general likely to modify underlying trait values, including antidiabetic drugs and folk medicine, were excluded, and the remaining 7551 subjects (3747 men and 3804 women) were finally included in this study. The basic characteristics of the study subjects are described in Table 1. This study was approved by the institutional review board of the Korean National Institute of Health. Written informed consent was obtained from all subjects.

2.1.2. Health2 T2D cohort

The Health2 T2D cohort, a part of a Korean rural community based cohort, recruited a total of 8500 participants aged 40–69 years old. Among a total of 1305 non-diabetic T2D controls, 633 subjects were selected from the Health2 cohort according to the following inclusion criteria: (1) no past history of diabetes, (2) no anti-diabetic medicine intake, (3) fasting plasma glucose <5.6 mmol/L and plasma glucose 2 h after ingestion of 75 g oral glucose load <7.8 mmol/L, and (4) subjects older than 60 years of age. The Health2 T2D cohort (Cho et al., 2012) was used in another research article for a meta-analysis of genome-wide association studies on type 2 diabetes in East Asians.

2.1.3. KoCAS cohort

A total of 484 Korean volunteer children, aged 8–10 years old and enrolled between 2009 and 2010, were selected from the Korean Child & Adolescent Cohort Study (KoCAS). Subjects following a specific diet program or taking any medications known to affect appetite were excluded from this study. The study protocol was approved by the Institutional Review Board of the Clinical Research Institute at Inje University's Seoul-Paik Hospital. Informed consent was obtained from all subjects. All study subjects were examined the morning after an overnight fast. Blood samples were used for biochemical measurements, including fasting serum glucose, fasting serum insulin, total cholesterol, triglycerides, high-density lipoprotein cholesterol, and DNA extraction. Genotyping was performed using the Illumina Omni1 genotyping system, and data quality was assessed using duplicate DNA samples ($n = 10$).

2.1.4. MAGIC database

The Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) was established for identifying locus impacts on glycemic and metabolic traits. All participants were adults of white European ancestry from the United States or Europe. MAGIC investigators initially studied diabetes-related traits, such as fasting glucose, 2 h glucose, glycosylated hemoglobin A1c (HbA1c), fasting insulin, insulin secretion, and insulin sensitivity. All studies were approved by local research ethics committees, and all participants gave informed consent. These results can be accessed through the homepage of the MAGIC investigators (http://www.magicinvestigators.org). In this study, we analyzed the genotyped data for five traits, including fasting glucose, HbA1c, fasting insulin, fasting proinsulin, and homeostasis model assessment of β-cell function (HOMA-B) (Dupuis et al., 2010; Soranzo et al., 2010; Strawbridge et al., 2011).

2.2. Oral glucose tolerance test

All subjects in the KARE and Health2 T2D cohorts underwent an oral glucose tolerance test (OGTT), which is commonly used to test for diabetes and insulin resistance, according to the World Health Organization criteria (Alberti and Zimmet, 1998). An OGTT involves the administration of 75 g of glucose and measuring blood glucose after 1–2 h, to determine how quickly it is cleared from the blood. Blood samples were taken from the subjects once prior to the OGTT and twice at 60 min and 120 min of the OGTT. The blood plasma was separated for the analysis of glucose and insulin concentration at the specified times.

The β-cell function indices were calculated using the following equations (Stumvoll et al., 2000; Stumvoll et al., 2001); first-phase insulin secretion (IS-1ph) = $728 + 3.537 \times \text{Ins0 (pmol/l)} - 120.3 \times \text{Glu60 (mmol/l)} + 1.341 \times \text{Ins60 (pmol/l)} + 21.27 \times \text{BMI}$, second-phase insulin secretion (IS-2ph) = $208 + 0.335 \times \text{Ins60 (pmol/l)} - 26.33 \times \text{Glu60 (mmol/l)} + 0.887 \times \text{Ins0 (pmol/l)} + 3.933 \times \text{BMI}$, and HOMA-B = $\text{Ins0 (pmol/l)} \times 3.33/[\text{Glu0 (mmol/l)} - 3.5]$. Although the hyperglycemic and euglycemic hyperinsulinemic clamp methods are well-validated for the measurement of insulin secretion, we used the OGTT method because this method is desirable for large-cohort epidemiological studies, and because IS-1ph

Table 1
Basic characteristics of the subjects in the KARE study cohort.

Characteristics	Total	Men	Women	*p*-Value*
Number of subjects	7551	3747	3804	
Age (*M* years ± *SD*)	51.44 ± 8.78	51.27 ± 8.68	51.59 ± 8.89	0.113
Body mass index (BMI) (*M* kg/m^2 ± *SD*)	24.42 ± 3.07	24.14 ± 2.90	24.68 ± 3.22	<0.0001
Blood glucose (M mg/dL ± SD)				
Fasting plasma glucose	87.21 ± 21.51	89.89 ± 23.29	84.58 ± 19.24	<0.0001
Plasma glucose after 60 min of OGTT	150.09 ± 52.99	156.72 ± 56.83	143.63 ± 48.10	<0.0001
Plasma glucose after 120 min of OGTT	124.30 ± 49.89	123.33 ± 54.78	125.24 ± 44.61	0.104
HbA1c (%)	5.77 ± 0.91	5.80 ± 0.94	5.74 ± 0.88	0.008
Blood insulin (M μIU/ml ± SD)				
Fasting plasma insulin	7.48 ± 4.74	7.03 ± 4.39	7.93 ± 5.03	<0.0001
Plasma insulin after 60 min of OGTT	31.43 ± 31.47	31.01 ± 31.60	31.85 ± 31.34	0.261
Plasma insulin after 120 min of OGTT	27.78 ± 27.09	24.33 ± 23.88	31.13 ± 29.50	<0.0001
Insulin secretion (M pmol/L ± SD)				
First phase insulin secretion	720.34 ± 483.40	654.73 ± 505.40	784.26 ± 451.97	<0.0001
Second phase insulin secretion	203.62 ± 112.87	188.97 ± 117.27	217.89 ± 106.52	<0.0001
HOMA-B	177.56 ± 170.18	151.34 ± 159.77	203.39 ± 176.09	<0.0001

* Significant differences in characteristics between the male and female subjects were determined by a two-tailed Student's *t*-test. *Abbreviations:* HbA1c, glycosylated hemoglobin A1c; HOMA-B, homeostasis model assessment of beta cell function; KARE, Korean Association REsource; *M*, mean value; OGTT, oral glucose tolerance test; *SD*, standard deviation.

and IS-2ph indices derived from an OGTT are well correlated with the clamp method (*r*-values of >0.75) (Huyghe et al., 2013; Stumvoll et al., 2000).

2.3. Genotyping and SNP selection in the KARE study

The genotype data were provided by the Center for Genome Science, the Korea National Institute of Health. The detailed genotyping and quality control processes have been described in a previous report (Cho et al., 2009). Briefly, most DNA samples were isolated from the peripheral blood of participants and genotyped using the Affymetrix Genome-Wide Human SNP array 5.0 (Affymetrix; Santa Clara, CA, USA). The accuracy of the genotyping was calculated by Bayesian Robust Linear Modeling using the Mahalanobis Distance (BRLMM) genotyping algorithm. Samples that had lower genotyping accuracy (⩽98%), high missing genotype call rates (⩾4%), high heterozygosity (>30%) or gender biases were excluded from this study. A total of 242 SNPs in the *PARK2* gene were selected based on their locations within the gene boundary region (5 kb upstream and downstream of the first and last exons, respectively) according to the NCBI human genome build 36 (Table A1). The location of the SNPs was validated by the Ensembl BioMart database (http://www.ensembl.or/biomart).

2.4. Cell culture

Rat insulinoma INS-1 β-cells were grown in RPMI 1640 media (Life Technology Inc.; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich; St. Louis, MO, USA), 100 U/ml penicillin (Duchefa; RV Haarlem, the Netherlands) and 100 μg/ml streptomycin (Duchefa). All cultured cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.5. Isolation of rat tissues and pancreatic islet cells

Rat tissues were isolated from 8-week-old male Sprague-Dawley (SD) rats. For isolation of pancreatic islet cells, after injecting 10 ml of Hank's balanced salt solution (HBSS) containing 0.75 mg/ml collagenase P (Roche Diagnostics Corporation; Indianapolis, IN, USA) into the bile ducts, swollen pancreases were excised and then incubated in water bath at 37 °C for 10 min. The collagenase digestion reaction was stopped by addition of cold HBSS (Delle et al., 2007). The cells were washed with HBSS and then the tissues were dissociated by repetitive pipetting and passing through a 400 μm mesh. The cells were then separated by gradient centrifugation at 2000*g* for 10 min on 25%, 23%, 21.5%, and 11.5% Ficoll (Amersham Biosciences; Uppsala, Sweden). Islets at the interface between the 21.5% and 11.5% Ficoll fractions were collected and washed with HBSS. Islets of appropriate size were hand-picked under a stereomicroscope (Leica zoom 2000; Buffalo, NY, USA). Animal experiments were conducted in accordance with the institutional guideline established by the Institutional Animal Care and Use Committee of the Ajou University School of Medicine.

2.6. Gene silencing by small interfering RNAs

The small interfering RNAs (siRNAs) were synthesized by Genolution Pharmaceuticals, Inc. (Seoul, South Korea). The target sequences for the siRNAs were as follows: 5′-TGCAAGGAAGCATAC CAT-3′ for rat *Park2* (GenBank: NM_020093) (Chen et al., 2010) and 5′-GTTCAGCGTGTCCGGCGAG-3′ for *egfp* (GenBank: U55762), the nonspecific negative control (scrambled siRNA). Another previously reported target sequences for the *Park2* siRNAs (Cui et al., 2010) were also used. The siRNAs were transfected into INS-1 β-cells using the Neon™ electroporator transfection system (Invitrogen; Carlsbad, CA, USA). The siRNAs in 100 μl of R buffer (Invitrogen) were transfected into 1×10^7 INS-1 β-cells under conditions of 1650 V and 10 ms width. After transfection, 2×10^5 cells were seeded into each well of a 24-well plate and incubated for 48 h.

2.7. Quantification of glucose-stimulated insulin secretion and intracellular insulin levels

Rat INS-1 β-cells were seeded in 24-well plates at a density of 2×10^5 and were cultured for 48 h. The cells were then washed with KRB buffer (5 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM HEPES, 129 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 0.2% BSA, pH 7.4) and incubated with the same KRB buffer for 1 h. The glucose-stimulated insulin secretion (GSIS) level was elicited by incubation for 2 h with 0.2 mM (low dose) or 16.7 mM (high dose) glucose. The insulin concentration in the supernatant was determined using the Rat insulin RIA kit (Millipore Corporation; Concord Road Billerica, MA, USA). Briefly, the supernatant was mixed with ^{125}I-insulin and anti-insulin antibody, and then incubated overnight at 4 °C. After incorporating the precipitating reagents

into the mixtures and incubating at 4 °C for 20 min, the aggregates were collected by centrifugation at 2000*g* for 20 min. Radioactivity in the pellet aggregates was quantified using a gamma-counter (Perkin–Elmer; Fremont, CA, USA). To measure the intracellular insulin level, INS-1 β-cells were incubated with 200 μl of acid-ethanol (75% ethanol, 0.2 mM HCl) at 4 °C for 12 h. The supernatant was collected and used to measure the insulin concentration with the Rat Insulin RIA kit.

2.8. RNA isolation, cDNA synthesis and quantitative reverse transcribed-PCR (RT-PCR)

Total RNAs were isolated from the cultured cells using TRIzol reagent (Invitrogen; Carlsbad, CA, USA), treated with RNase-free DNase I (Invitrogen) to avoid amplification of genomic DNA, and were subsequently reverse transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Inc.; Hanover, MD, USA) with the oligo(dT)$_{15-18}$ primer. All real-time RT-PCR measurements were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems; Foster City, CA, USA). All PCR amplifications (40 cycles) were performed in a total volume of 25 μl containing 150 ng cDNA using the SYBR Green I qPCR kit (TaKaRa; Shiga, Japan) according to the manufacturer's recommendations. The specific primers used were as follows: 5′-TGGTTTCTTC TACACACCCAAG-3′ and 5′-ACAATGCCACGCTTCTGCC-3′ for the rat *Ins1* (GenBank: NM_019129), 5′-GGTTGCAGGGCTTCGAGAAGCAG-3′ and 5′-GCTTCCCGTTCATA CCACACC-3′ for the rat *Gusb* (GenBank: NM_017015.2), and 5′-GTTACCAGGGCTGCCTTCTC-3′ and 5′-GGGTTTCCCGTTGATGACC-3′ for the rat *Gapdh* (GenBank: NM_017008.3). By normalizing to *Gapdh*, relative quantification of gene expression was performed using the comparative threshold (CT) method as described by the manufacturer (Applied Biosystems). The values were expressed as fold change over control.

2.9. Cell viability assay

Cell viability was assessed using the EZ-Cytox Cell Viability Assay Kit (Daeil Labservice; Seoul, Korea). Rat INS-1 β-cells were seeded in 96-well plates at a density of 5×10^4. After 48 h incubation, 10 μl of Ez-Cytox reagent was added to each well, and the cells were incubated for another 2 h. The plate was read with an enzyme-linked immunosorbent assay microplate reader (Bio-Rad Model 680; Hercules, CA, USA) at 450 nm and 655 nm.

2.10. Measurement of intracellular ATP level

Rat INS-1 β-cells were seeded in 96-well plates at a density of 5×10^4 and cultured for 48 h. Intracellular ATP concentration was measured by an ATPLiteTM Luminescence ATP detection assay system (PerkinElmer Life Science). 50 μl of cell lysis solution was added to each well containing 100 μl of culture media. The plate was then shaken for 5 min (700 rpm). Afterwards, 50 μl of substrate solution was added to each well, and the plate was shaken for 5 min in the dark and then incubated at room temperature for 10 min in the dark. The luminescence in the medium was quantified using luminometer (Turner Design Instrument; Sunnyvale, CA, USA).

2.11. JC-1 staining and fluorescence activated cell sorter (FACS) analysis

Mitochondrial membrane potential was assessed by a mitochondrial voltage-sensitive dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1) (Invitrogen). Mitochondria with intact membrane potential concentrate JC-1 into aggregates that show red fluorescence. De-energized mitochondria cannot concentrate JC-1 and show green fluorescence. INS-1 β-cells (5×10^5 cells in 12 well plate) transfected with either 2.0 μM *Park2* siRNA or 2.0 μM nonspecific scrambled siRNA and then incubated for 48 h. Cells were collected in 1 ml of PBS and then incubated with 0.1 μg/ml of JC-1 in media for 30 min in the dark. The non-transfected INS-1 β-cells treated without and with 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) for 1 h before JC-1 staining were used as negative and positive controls, respectively, to test for mitochondrial membrane potential changes. After washing in PBS, fluorescence stained cells were subjected to the FACSAria III cell sorter (BD Biosciences; San Jose, CA, USA) using FACS Diva software (BD Biosciences) for acquisition and analysis. The ratio of the intensity of the JC-1 aggregate (PE, red fluorescence) to the intensity of the JC-1 monomer (FITC, green fluorescence) was calculated and normalized to the control.

2.12. Flow-cytometric measurement of reactive oxygen species (ROS)

INS-1 β-cells (5×10^5 cells in a 12-well plate) were transfected with either 2.0 μM *Park2* siRNA or 2.0 μM nonspecific scrambled siRNA and then incubated for 48 h. The non-transfected INS-1 β-cells treated both with and without 100 μM H_2O_2 for 1 h before the measurement of ROS levels were used as negative and positive controls, respectively, to test for ROS production. Cells were treated with ROS Detection Solution (Enzo Life Sciences; Farmingdale, NY) for 30 min and then washed according to the manufacturer's protocol. The total ROS was measured using the FACSAria III cell sorter (BD Biosciences) and FACS Diva software (BD Biosciences). The dichlorofluorescein diacetate (DCF-DA) intensity was calculated and normalized to the control.

2.13. Confocal microscopy

Rat INS-1 β-cells were fixed and permeabilized and then stained with MitoTracker® Red CMXRos (Invitrogen) for mitochondrial staining or with 4′,6-diamidino-2-phenylindole dihydrochloride (Invitrogen) for nuclear staining. Mitochondrial morphology was analyzed by confocal microscopy using a Zeiss LSM 710 (Carl Zeiss Microscopy; Jena, Germany). At least 100 cells were analyzed per slide. Error bars represent standard deviations calculated from three slides.

2.14. Western blotting

The homogenized rat tissues and rat pancreatic islet cells or INS-1 β-cells were lyzed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris buffer, pH 8.0). The lysate were centrifuged at 13000 rpm for 20 min at 4 °C to remove cellular debris. Protein concentration of the extracts was determined using the D_C Protein assay (BIO-RAD Laboratories; Hercules, CA, USA). Proteins were heated at 95 °C for 5 min and analyzed by SDS-PAGE on 8–12% polyacrylamide gels. The proteins were electroblotted onto PVDF membrane (Millipore). The membrane blots were blocked with 5% (w/v) nonfat dried milk or BSA, incubated with primary and secondary antibodies, and then visualized by the WEST-ZOL plus ECL Western blotting detection system (iNtRON BioTechnology; Seoul, Korea). The following antibodies were used: anti-parkin antibody (Cell Signaling; Danvers, MA, USA); anti-DLP1/Drp1 and Opa1 antibodies (BD Transduction Laboratories; Lexington, KY, USA); anti-Mfn1, anti-Mfn2, and anti-Fis1 antibodies (Epitomics; Burlingame, CA, USA); anti-α-synuclein antibody (Millipore); anti-UCP2 (N-19), anti-β-tubulin, anti-β-actin, HRP-conjugated goat anti-rabbit IgG, and HRP-conjugated goat anti-mouse IgG antibodies (Santa Cruz biotechnology; Santa Cruz, CA, USA).

Table 2
The *PARK2* SNPs significantly associated with quantitative glycemic traits in the KARE male subjects.

SNP	EA	EAF	Phenotype	Men (n = 3747)					
				Genotype			Effect size ($\beta \pm$ s.e)	p-Value	Sample size for 80% power
				NEA/NEA	NEA/EA	EA/EA			
rs10455889	G	0.534	No.	777	1834	1048			
			Glu0	88.41 ± 18.58	89.17 ± 21.28	92.25 ± 28.94	2.02 ± 0.54	2.0×10^{-4}	2093
			IS1-ph	703.0 ± 532.9	656.8 ± 484.7	615.2 ± 517.2	−44.08 ± 11.61	1.5×10^{-4}	2069
			IS2-ph	200.4 ± 125.4	189.4 ± 112.6	179.7 ± 118.4	−10.37 ± 2.70	1.3×10^{-4}	2013
rs9365294	G	0.533	No.	776	1839	1045			
			Glu0	88.22 ± 18.09	89.25 ± 21.44	92.24 ± 28.97	2.09 ± 0.54	1.2×10^{-4}	1954
			IS1-ph	703.7 ± 527.8	657.2 ± 488.9	613.8 ± 513.9	−45.53 ± 11.61	9.0×10^{-5}	1939
			IS2-ph	200.5 ± 124.3	189.5 ± 113.6	179.3 ± 117.5	−10.72 ± 2.70	7.4×10^{-5}	1883

Age, body mass index and residential area were included as covariates in the additive genetic models. The p-values lower than the Bonferroni-corrected significance level ($p < 0.00021$) are indicated. Sample size for 80% power at $\alpha = 0.05$ is based on the Korean Association REsource (KARE) cohort parameters, including minor allele frequency, effect size and mean value of the glucose and insulin secretion using the Quanto program (version 1.2) (http://hydra.usc.edu/gxe). *Abbreviations:* EA, effect allele; EAF, effect allele frequency; Glu0, fasting plasma glucose level of oral glucose tolerance test (OGTT); IS-1ph, first phase insulin secretion index; IS-2ph, second phase insulin secretion index; NEA, non-effect allele.

2.15. Statistical analysis

Most statistical analyses of the association study were performed using PLINK version 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink) and PASW Statistics version 17.0 (SPSS Inc.; Chicago, IL, USA). Linear regression was used to analyze plasma glucose, glycosylated hemoglobin and plasma insulin as the quantitative traits. The selected significant SNPs were also analyzed in the type 2 diabetes case-control study using logistic regression analysis. All association tests were based on the additive genetic model, and p-values were adjusted for multiple tests by the Bonferroni-corrected significance level. In the KARE association study, the significant p-value threshold was adjusted as $p = 0.00021$ (0.05/242 SNPs). Age, gender, and body mass index (BMI) were included as covariates in all analyses. The estimated sample sizes for 80% study power at $\alpha = 0.05$ in Table 1 were based on the KARE parameters, including minor allele frequency, effect size, and mean value of plasma glucose and insulin levels using the Quanto program (version 1.2) (http://hydra.usc.edu/gxe). In the MAGIC association study, the significant p-value threshold was adjusted as $p = 0.000025$ (0.05/1975 SNPs). For regional association plots, we used LocusZoom Version 1.1 (http://csg.sph.umich.edu/locuszoom), which is a web-based plotting tool (Pruim et al., 2010). We tested the heterogeneity between the sexes in terms of the effects of two SNPs (rs10455889, and rs9365294) on Glu0, HbA1c, IS-1ph, IS-2ph, and T2D statuses (Magi et al., 2010).

In the experimental study, results were expressed as means ± standard deviation. All experiments were repeated independently at least three times. Statistical analyses were performed with PASW Statistics version 17.0 (SPSS Inc.). Statistical significance between the groups was calculated by a Student's t-test. Probability values less than 0.05 ($p < 0.05$) were considered statistically significant.

3. Results

3.1. Association of PARK2 SNPs with quantitative glycemic traits

The basic characteristics of the KARE cohort (7551 subjects) are shown in Table 1. The sex ratio was approximately equal and the mean age was 51 ± 9 years; the mean ages of men and women were similar. The quantitative glycemic traits that were analyzed included plasma glucose traits (plasma glucose levels obtained during an OGTT and HbA1c level), plasma insulin traits (plasma insulin levels obtained during an OGTT), and insulin secretion traits (IS-1ph and IS-2ph indices obtained during an OGTT and HOMA-B).

We performed an association analysis of the 242 SNPs in the *PARK2* gene with the quantitative glycemic traits in the KARE study cohort. Information about the SNPs is shown in Table A1. No significant associations satisfying the Bonferroni-corrected significance level ($p < 0.00021$ calculated by 0.05/242 SNPs) were observed between any of the SNPs and any of the traits (Tables A2–A4). We carried out a further association study of the same SNPs with the quantitative glycemic traits, separately in men (3747 subjects) and women (3804 subjects). The association analysis in subjects divided by sex revealed two SNPs located in the intron regions of the *PARK2* gene – rs10455889 and rs9365294 – that showed a significant association with fasting blood glucose level (Glu0), IS-1ph, and IS-2ph, but in men only (Table 2). The most significant SNP was rs9365294 with Glu0 ($\beta = 2.09$; $p = 1.2 \times 10^{-4}$), IS-1ph ($\beta = -45.53$; $p = 9.0 \times 10^{-5}$), and IS-2ph ($\beta = -10.72$; $p = 7.4 \times 10^{-5}$). This SNP also showed moderate association with Glu0 ($p = 9.1 \times 10^{-4}$) in the total subject population (Table A2). Fig. 1 shows the plots for the association results of SNPs in the *PARK2* gene with fasting glucose and insulin secretion quantitative traits in the KARE male subjects. The plots for total and sex-divided subjects are shown in Fig. A1.

For the replication study, we further analyzed the association between the two discovered *PARK2* SNPs and quantitative glycemic traits in three other cohorts, including the Health2 T2D (633 Korean control subjects for T2D) and KoCAS (468 Korean children) cohorts and the MAGIC database (46186 non-diabetic European-descendent participants). The analyzed results showed that the two SNPs did not replicate in these other three cohorts (data not shown). Therefore, we further analyzed all of the available SNPs in the *PARK2* gene in the three cohorts (Table 3). The rs10806742 SNP was significantly associated with HbA1c ($\beta = 0.022$; $p = 4.8 \times 10^{-6}$) in the MAGIC database, and the association p-value was below the Bonferroni-corrected significance level ($p < 0.000025$ calculated by 0.05/1975 SNPs). Additionally, the rs4709532 SNP associated with fasting proinsulin ($p = 1.8 \times 10^{-3}$) in the MAGIC cohort showed a meaningful LD correlation ($r^2 = 0.532$ and $D' = 1$) to the two discovered *PARK2* SNPs. Collectively, an association was observed between *PARK2* and fasting glucose and insulin secretion indices in KARE cohort, but no replication between the analyzed cohorts was detected in these two SNPs.

3.2. Predominant expression of parkin in pancreatic islets in rats

The cohort results indicated that genetic variation in the *PARK2* gene is most significantly associated with insulin secretion indices in the KARE. Since these traits are closely related to pancreatic β-cell function, we investigated whether the *PARK2* gene was

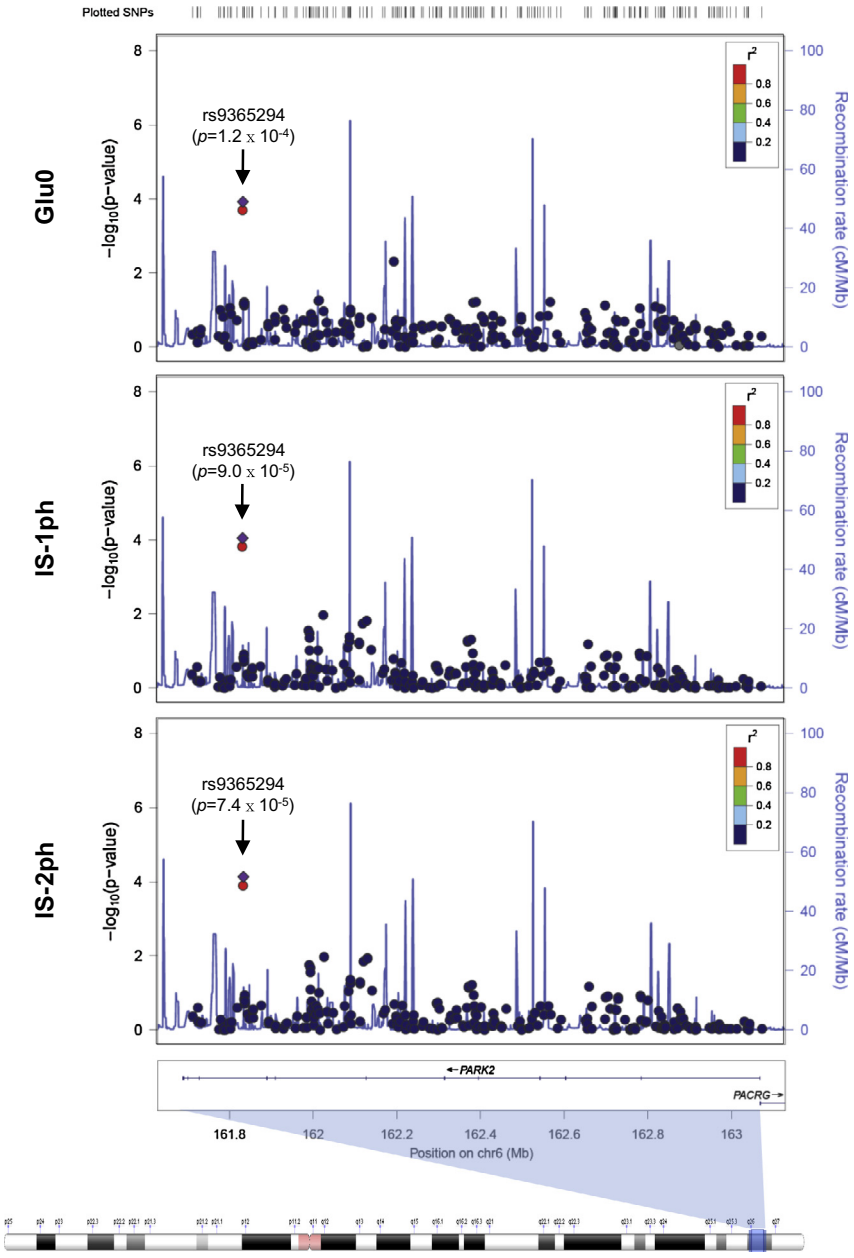

Fig. 1. Plots for the association result of the *PARK2* SNPs with quantitative glycemic traits in the KARE male subjects. The position of the SNPs is shown at the top, and association results between SNPs in the *PARK2* gene and fasting glucose (Glu0), first-phase insulin secretion (IS-1ph), and second-phase insulin secretion (IS-2ph) in male subjects from the KARE study cohort are shown in the middle. The statistical significance ($-\log_{10}$ *p*-value) of the analyzed SNPs is plotted. The recombination rate estimated from the HapMap CHB (Han Chinese from Beijing) and JPT (Japanese from Tokyo) population data is shown by a blue bar. The purple diamond with a SNP number and *p*-value represents the SNPs most strongly associated with the indicated traits, and its correlated SNPs are shown in the indicated colors according to the levels of linkage disequilibrium (r^2). At the bottom, the position (Mb) of the *PARK2* gene on chromosome 6 (NCBI build 36) is shown along with an ideogram of human chromosome 6. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expressed in pancreatic islet tissue. Western blot analysis of the expression profile of the *Park2* gene (parkin) showed ubiquitous expression in all rat tissues examined (Fig. 2). Interestingly, pancreatic islet tissue showed a predominant expression of parkin, as did brain, skeletal muscle, and liver tissues. We further performed immunohistochemical staining of parkin in rat pancreas tissues and immunocytochemical staining of parkin in rat INS1 β-cells (Fig. A3). The double staining of antibodies against parkin and insulin revealed that parkin is predominantly expressed in the insulin-producing β-cells of pancreatic islets. Taken together, these results suggest an important role of parkin in pancreatic β-cell function.

3.3. Decrease in insulin production and secretion by knockdown of *Park2* in rat INS-1 β-cells

The two significant SNPs found in the KARE are located in the intron regions of the *PARK2* gene. Therefore, we examined whether modulation of *Park2* gene expression would affect β-cell function. The *Park2* gene was initially knocked down by RNA interference in rat INS-1 β-cells by transfecting the cells with siRNAs against the *Park2* gene. Two previously reported target sequences for the *Park2* siRNAs (Chen et al., 2010; Cui et al., 2010) were used. Treatment of INS-1 cells with *Park2* siRNAs for 48 h reduced the level of parkin protein in an siRNA dose-dependent manner and no

Table 3
The best *PARK2* SNPs associated with quantitative glycemic traits in three cohorts.

Cohort	SNP	Location (bp)	EA	EAF	Effect size	Phenotype	*p*-Value	Subjects	Gender
Health2 T2D	rs2846488	162884672	C	0.967	0.021	Fasting glucose	1.5×10^{-4}	633 Korean subjects with (1) no past history of diabetes, (2) no anti-diabetic medicine, (3) fasting plasma glucose < 5.6 mmol/L and plasma glucose 2 h after ingestion of 75 g oral glucose load < 7.8 mmol/L and (4) subjects older than 60 years of age	Men and women
	rs4708909	161754979	C	0.068	−0.141	HOMA-B	1.1×10^{-2}		
	rs6901071	162440123	G	0.132	−0.024	IS-1ph	2.1×10^{-2}		
	rs6901071	162440123	G	0.132	−0.026	IS-2ph	2.0×10^{-2}		
	rs2849545	162993406	T	0.955	0.018	Fasting glucose	4.7×10^{-3}	309 Korean men of the above subjects	Men
	rs9365399	162655871	C	0.519	−0.098	HOMA-B	1.7×10^{-3}		
	rs9458225	161686381	A	0.058	−0.056	IS-1ph	4.6×10^{-3}		
	rs9458225	161686381	A	0.058	−0.063	IS-2ph	3.6×10^{-3}		
	rs2803068	162889900	T	0.986	0.029	Fasting glucose	3.5×10^{-3}	324 Korean women of the above subjects	Women
	rs993253	162000539	G	0.114	−0.142	HOMA-B	6.0×10^{-3}		
	rs6921599	162057502	A	0.038	−0.068	IS-1ph	6.9×10^{-3}		
	rs6921599	162057502	A	0.038	−0.073	IS-2ph	7.0×10^{-3}		
KoCAS	rs2022993	162165676	A	0.458	0.007	Fasting glucose	9.5×10^{-4}	468 Korean children (Insulin & HOMA-B, 290 children)	Boys and girls
	rs6455756	162017158	A	0.970	−0.159	Fasting insuln	2.6×10^{-3}		
	rs6455756	162017158	A	0.970	−0.237	HOMA-B	1.9×10^{-4}		
MAGIC	rs7738279	162174536	T	0.177	0.034	Fasting glucose	1.2×10^{-4}	46,186 non-diabetic participants	Men and women
	rs10806742	161672544	T	0.392	0.022	HbA1C	$\mathbf{4.8 \times 10^{-6}}$	46,368 non-diabetic adults of European descent	
	rs16892554	161810977	A	0.040	−0.030	Fasting insulin	3.2×10^{-3}	46,186 non-diabetic participants	
	rs4709532[a]	161831806	C	0.780	−0.220	Fasting proinsulin	1.8×10^{-3}	10,701 non-diabetic adults of European ancestry	

The locations of the SNPs were based on NCBI human genome build 36. The *p*-value lower than the Bonferroni-corrected significance level ($p < 0.000025$) in the MAGIC is bolded.

[a] The rs4709532 SNP showed a linkage disequilibrium correlation ($r^2 = 0.532$, $D' = 1$) with two significant SNPs, rs10455889 and rs9365294, in the Korean Association REsource (KARE) cohort. *Abbreviations:* EA, effect allele; EAF, effect allele frequency; HbA1c, glycosylated hemoglobin A1c; Health2 T2D, Korean Health2 Type 2 Diabetes study cohort; HOMA-B, homeostasis model assessment of beta cell function; IS-1ph, first phase insulin secretion index; IS-2ph, second phase insulin secretion index; KoCAS, Korean Child & Adolescent cohort Study; MAGIC, the Meta-Analyses of Glucose and Insulin-related traits Consortium.

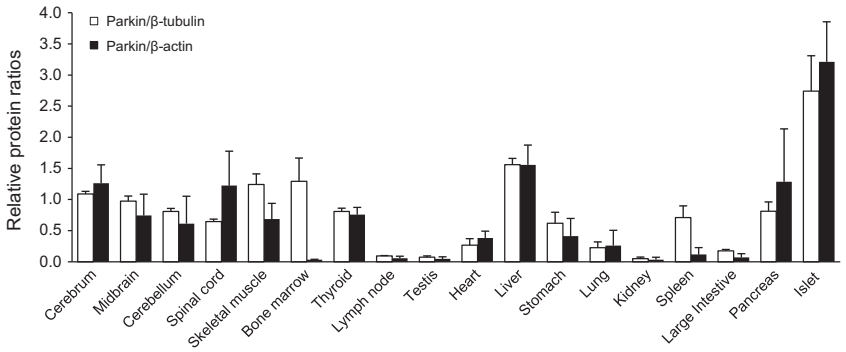

Fig. 2. Expression profile of the *Park2* gene in rats. Expression level of parkin protein in various rat tissues was evaluated in three male rats. Protein levels of parkin, β-tubulin, and β-actin were examined by Western blot analysis and the quantities of these proteins were determined using Image J software (provided by the National Institute of Health). Western blots from three independent experiments were quantified and normalized into either β-tubulin or β-actin. Relative ratios of the parkin/β-tubulin and parkin/β-actin proteins are shown.

differences were observed between the two sets of target sequences (Fig. 3A). The GSIS was dose-dependently decreased in the INS-1 cells treated with *Park2* siRNAs following both low-dose and high-dose glucose stimulations (Fig. 3B). The *Ins1* (insulin gene) mRNA and intracellular insulin protein levels were also dose-dependently reduced by *Park2* siRNA treatment (Fig. 3C and D).

3.4. Alteration in mitochondrial ATP generation by knockdown of *Park2* in rat INS-1 β-cells

Insulin production and secretion critically depend on mitochondrial respiratory capacity and appropriate ATP concentration in the β-cells (Patti and Corvera, 2010). Therefore, we also measured intracellular ATP levels in the *Park2*-depleted INS-1 cells. The ATP level was significantly reduced by knock-down of the *Park2* gene in an siRNA dose-dependent manner (Fig. 4A). We examined whether the reduction in the ATP level in the *Park2*-depleted INS-1 cells might be caused by cell damage. Cell viability assay results showed no significant alterations due to treatment of the cells with *Park2* siRNA (Fig. 4B).

ROS activate uncoupling protein 2 (UCP2), which leads to reduced ATP synthesis and content in β-cells (Ma et al., 2012). In addition, ATP production is driven by the proton-motive force that is determined by the mitochondrial membrane potential (Fujimoto

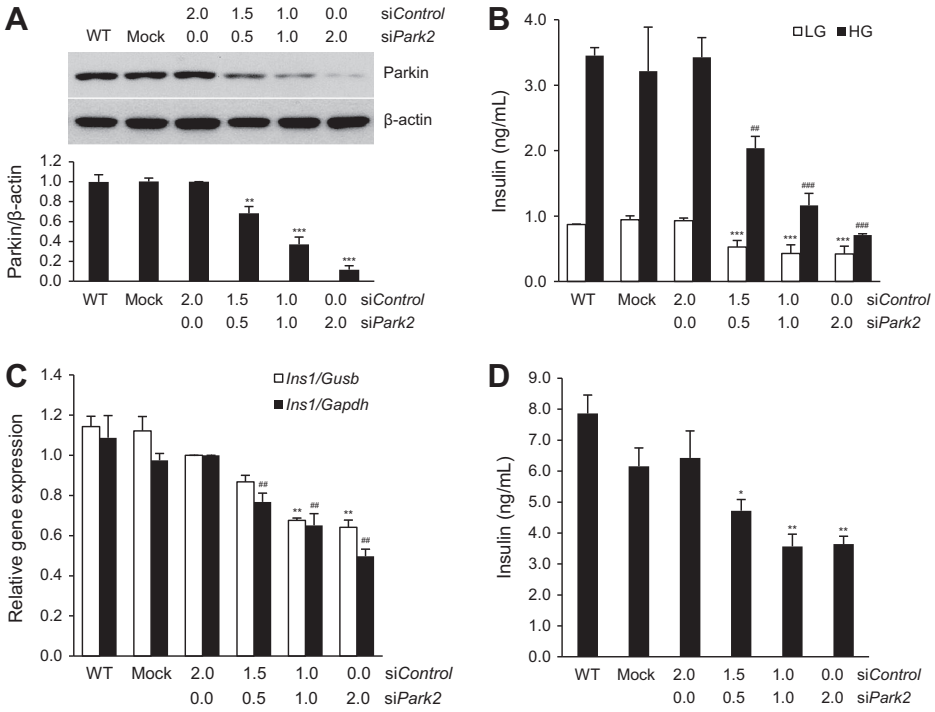

Fig. 3. Decrease in insulin production and secretion in the *Park2*-depleted INS-1 β-cells. INS-1 β-cells were transfected with the indicated concentration (μM) of the mixture of *Park2* siRNA (*siPark2*) and nonspecific scrambled siRNA (*siControl*) and then incubated for 48 h. Non-transfected wild-type (WT) cells and mock-transfected (Mock) cells were used as controls. Each *Park2* knockdown experiment was repeated five times. (A) Dose-dependent knockdown of *Park2* by siRNA. The protein levels of parkin and β-actin were analyzed by Western blotting, and normalized to β-actin. $^{**}p < 0.01$ and $^{***}p < 0.001$. (B) Dose-dependent reduction of glucose-stimulated insulin secretion by knockdown of *Park2*. Quantity of insulin secretion from 2×10^5 INS-1 β-cells for 2 h in response to 0.2 mM low glucose (LG) or 16.7 mM high glucose (HG) was measured with insulin RIA kit. *0.5 μM, 1.0 μM, 2.0 μM si*Park2* vs. si*Control* in LG, $^{\#}$0.5 μM, 1.0 μM, 2.0 μM si*Park2* vs. si*Control* in HG, one symbol $p < 0.05$, two symbols $p < 0.01$, and three symbols $p < 0.001$. (C) Dose-dependent reduction of *Ins1* mRNA level by knockdown of *Park2*. Real-time RT-PCR was performed with the gene specific primers for *Ins1* gene (insulin), and *Gusb* and *Gapdh* genes (relative controls). By normalizing to *Gusb* or *Gapdh*, results are expressed as fold change over control. $^{**}p < 0.01$, $^{\#\#}p < 0.01$ vs. 2.0 μM si*Control*. (D) Dose-dependent reduction of intracellular insulin content by knockdown of *Park2*. Quantity of insulin was measured using insulin RIA kit. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. 2.0 μM si*Control*.

et al., 2007). Therefore, we investigated whether *Park2* knockdown INS-1 cells showed any alterations in ROS production and mitochondrial membrane potential (mΔψ). FACS analysis revealed an increase in ROS production and a decrease in mΔψ in the *Park2*-depleted INS-1 β-cells (Fig. 4C and D). In contrast, overexpression of the *Park2* gene in INS-1 cells did not cause any increases in GSIS, intracellular ATP, or insulin expression levels (data not shown). This might reflect the presence of a sufficient amount of parkin in β-cells, as shown in Fig. 2. Taken together, these results indicated that parkin plays an important role in maintaining mitochondrial ATP generation by the β-cells, which are responsible for insulin production and secretion.

3.5. Alteration in mitochondrial morphology by knockdown of Park2 in rat INS-1 β-cells

The above experimental results indicated that reduced *Park2* gene expression affected insulin expression and secretion possibly due to alteration in mitochondrial ATP generation in pancreatic β-cells. Considering the important role of mitochondrial dynamics in β-cell function (Stiles and Shirihai, 2012), we investigated whether there were alterations in mitochondrial morphology in the *Park2*-depleted INS-1 β-cells. At first, mitochondria in the control or *Park2* siRNA-treated INS-1 β-cells were stained with MitoTracker® Red CMXRos and then mitochondrial morphology was analyzed by confocal microscopy. Increased proportion of fragmented mitochondria was observed in the *Park2*-depleted INS-1 β-cells than in control cells (Fig. 5A). Since the mitochondria-shaping proteins Mfn1, Mfn2, and Drp1 are known to be the substrates of parkin (Gegg et al., 2010; Wang et al., 2011), we investigated the expression level of the mitochondria-shaping proteins and found that Drp1 protein level was increased in the *Park2*-depleted INS-1 β-cells, but no changes in the Mfn1, Mfn2, Opa1, and Fis1 protein levels were observed (Fig. 5B).

It has been reported that α-synuclein inhibits insulin secretion in β-cells (Geng et al., 2011). Parkin ubiquitinates α-synuclein (Haass and Kahle, 2001) and rescues α-synuclein-induced mitochondrial fission (Kamp et al., 2010). We further investigated whether the α-synuclein level is changed in the *Park2*-depleted INS-1 β-cells and found a significant increase in the α-synuclein level (Fig. 5B). Since UCP2 negatively regulates insulin secretion (Zhang et al., 2001), we investigated the UCP2 expression level and found increased UCP2 expression levels in the *Park2*-depleted INS-1 β-cells (Fig. 5B). Taken together, these results indicated that parkin plays an important role in maintaining intact mitochondrial morphology of pancreatic β-cells that are closely related to insulin production and secretion.

4. Discussion

The data presented here for Korean cohort, taken together with the previous association results (Duggirala et al., 2001; Leak et al., 2008; Lindsay et al., 2001; Wongseree et al., 2009), provide additional evidence that the *PARK2* gene is a susceptibility gene for quantitative glycemic traits and/or T2D in various ethnic populations. We found two SNPs that were significantly associated with fasting glucose and insulin secretion in response to glucose stimulation in the male KARE subjects (Table 2). However, no

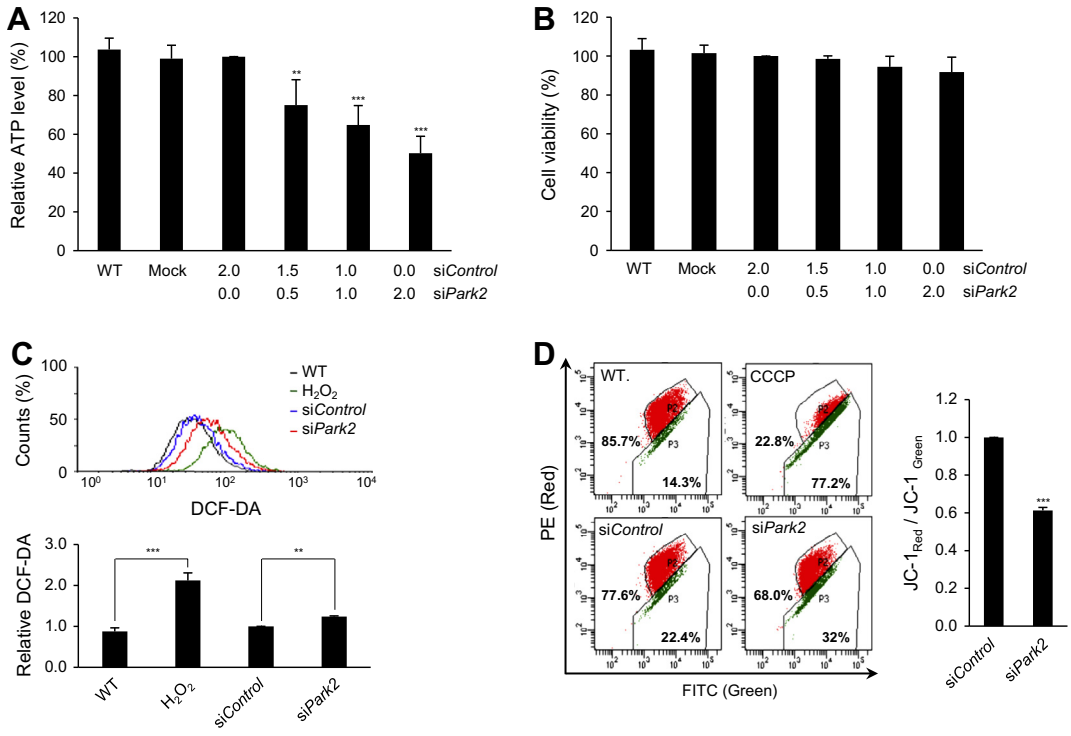

Fig. 4. Alteration in mitochondrial ATP generation in the *Park2*-depleted INS-1 β-cells. (A and B) INS-1 β-cells were transfected with the indicated concentration (μM) of the mixture of *Park2* siRNA (si*Park2*) and nonspecific scrambled siRNA (si*Control*) and then incubated for 48 h. Non-transfected wild-type (WT) cells and mock-transfected (Mock) cells were used as controls. Each *Park2* knockdown experiment was repeated three times. (A) Dose-dependent reduction of intracellular ATP level by knockdown of *Park2*. ATP levels were measured using ATP detection assay kit. Results are expressed as fold change over control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 2.0 μM si*Control*. (B) INS-1 cell viability by knockdown of *Park2*. Cell viability was determined by the Ez-Cytox assay. Results are expressed as fold change over control. (C and D) INS-1 β-cells were transfected with either 2.0 μM *Park2* siRNA (si*Park2*) or 2.0 μM nonspecific scrambled siRNA (si*Control*) and then incubated for 48 h. (C) Increase in ROS production by the knockdown of *Park2*. Untreated (WT) and H_2O_2-treated INS-1 β-cells were used as negative and positive controls, respectively, for ROS analysis. $^{**}p < 0.01$ and $^{***}p < 0.001$. (D) Reduction of mitochondrial membrane potential by knockdown of *Park2*. Dot blots of JC-1 red fluorescence (PE) and JC-1 green fluorescence (FITC) are indicated. Untreated (WT) and CCCP-treated INS-1 β-cells were used as negative and positive controls, respectively, for FACS analysis using JC-1. The ratio of JC-1 aggregate (P2) to JC-1 monomer (P3) intensity is shown. Results are expressed as fold change over control. $^{***}p < 0.001$ vs. si*Control*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

replication between the analyzed cohorts was detected in these two SNPs (Table 3). This discrepancy may be explained by the distinct characteristics of the cohorts, including genetic distance, subject size, and sex ratio, and environmental factors. Nevertheless, our association results indicate a significant association between *PARK2* and β-cell dysfunction at the gene level. In addition, two *PARK2* SNPs (rs555637: $p = 9.16 \times 10^{-6}$ and rs577876: $p = 1.58 \times 10^{-6}$) are recently listed in the NHLBI Family Heart Study as being associated with insulin in the dbGAP database (database of genotypes and phenotypes: http://www.ncbi.nlm.nih.gov/gap).

Complex disease traits, such as diabetes, hypertension and cancer, have many disease-related clinical phenotypes that would be highly correlated with each other. Many recent studies have shown that it is often more informative to investigate the genetic associations with the various disease-related quantitative traits, rather than merely with the binary case-control disease status, as studies of clinical quantitative traits can provide some insight into the intermediate processes in disease pathogenesis (Kim and Xing, 2009). The SNPs associated with the multiple correlated traits can be considered to be more reliable candidates for further validation than the SNPs associated with only one of the many correlated traits. Since quantitative glycemic traits and T2D status have known correlations, pairwise Pearson correlations among glycemic traits and T2D were calculated and tested in the total and sex-divided KARE subjects using the statistical software R (version 2.15) (http://www.r-project.org) (Fig. A2). The glycemic traits showed a high correlation with T2D and were also well-correlated with each other, as expected. No difference was noted in the correlation structure between data for the total subjects and the gender-divided subjects.

Several gender-specific associations of the common genetic variants with T2D have been reported; association of *ADIPOQ variants* with T2D *in men and* association of *PON1 and PPARG* variants with T2D in women (Hegele et al., 2000; Yamaguchi et al., 2007). Sexual dimorphisms in mitochondrial function and capacity in rat liver and brain tissues have been reported. The experiment for the gender-related difference in the effect of long-term high-fat-diet feeding or caloric restriction on rat liver mitochondrial function and biogenesis revealed a higher oxidative capacity in female rats than in male rats (Nadal-Casellas et al., 2010; Valle et al., 2007). Aged female rat brain showed a better mitochondrial capacity against oxidative stress than aged male brain (Guevara et al., 2011). Since significant associations produced by multiple tests were observed in men only, we carried out a heterogeneity test for sex differences. A gender-skewed association of two *PARK2* SNPs with Glu0, HbA1c, IS-1ph, IS-2ph, and T2D was observed in male subjects (Table A5). But, multiple SNPs also showed weak to moderate associations with glycemic quantitative traits in the total and female KARE subjects (Tables A2–A4). Furthermore, in the Health2 T2D cohort, the strongest association with fasting glucose was found in the total subjects (Table 3), and in the MAGIC cohort (no gender information), associations were also observed in the total subjects. Therefore, we could not conclude a gender-specific association between genetic variation in the *PARK2* gene and glycemic traits.

We confirmed the validity of our association signals by a further functional analysis of parkin in the insulin-producing rat pancreatic

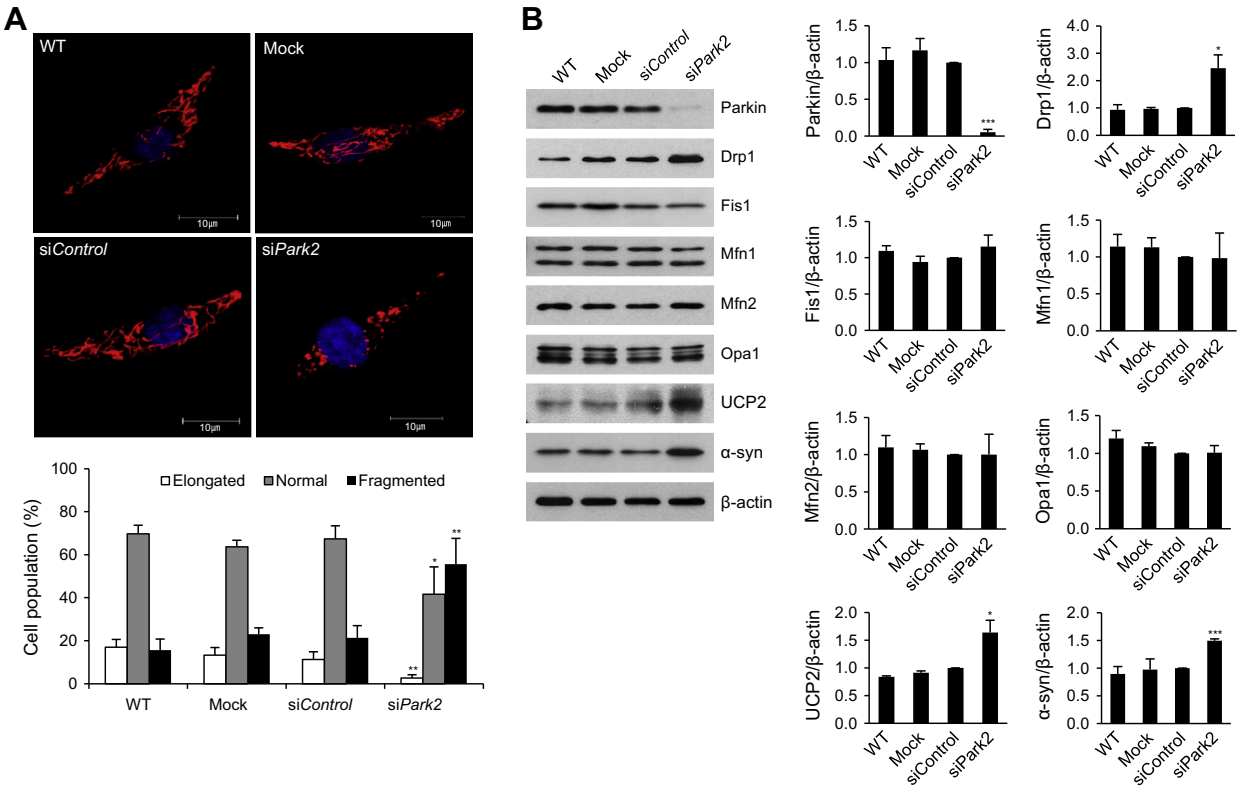

Fig. 5. Alteration in mitochondrial morphology in the *Park2*-depleted INS-1 β-cells. INS-1 β-cells were transfected with either 2.0 μM *Park2* siRNA (si*Park2*) or 2.0 μM nonspecific scrambled siRNA (si*Control*) and then incubated for 48 h. Non-transfected wild-type (WT) cells and mock-transfected (Mock) cells were used as a control. Each *Park2* knockdown experiment was repeated three times. (A) Increase in mitochondrial fragmentation by knockdown of *Park2*. Mitochondrial morphology using MitoTracker was visualized by confocal microscopy. The number of elongated, normal shaped, and fragmented mitochondria was counted in each 100 cells; $^{*}p < 0.05$, $^{**}p < 0.01$ vs. si*Control* in each elongated, normal, and fragmented mitochondria. (B) Increase in Drp1, UCP2 and α-synuclein protein levels by knockdown of *Park2*. The protein levels of parkin, mitochondria-shaping proteins (Drp1, Fis1, Mfn1, Mfn2, and Opa1), UCP2, α-synuclein, and β-actin were analyzed by Western blotting. Western blots from three independent experiments were quantified and normalized into β-actin. The quantities were determined using the Image J software (provided by the National Institute of Health) and all relative data normalized to β-actin. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. si*Control*.

β-cells. We manipulated the *Park2* gene expression and provide the first experimental evidence to support an important role of parkin in maintenance of β-cell function, including insulin production and secretion. Decreased ATP synthesis has been implicated in β-cell dysfunction (Anello et al., 2005; Newsholme et al., 2012) and, as expected, *Park2*-depleted INS-1 β-cells showed decreased cellular ATP levels (Fig. 4A). Dissipation of ROS and maintenance of mΔψ are important in cellular ATP generation, and mitochondrial dysfunction due to ROS or disruption of the mΔψ will lead reduce insulin expression and secretion (Patti and Corvera, 2010). In our study, depletion of *Park2* in the INS-1 cells resulted in an increase in ROS levels and a decrease in mΔψ (Fig. 4C and D). UCP2 is activated by ROS, which leads to reduced ATP synthesis and lower ATP content in β-cells (Ma et al., 2012). ATP synthesis and the ATP/ADP ratio are negatively regulated by UCP2 expression level, and UCP2 upregulation therefore inhibits insulin secretion (Zhang et al., 2001). Our results showed increased UCP2 protein expression in the *Park2*-depleted INS-1 cells (Fig. 5B). This may have resulted from the accumulation of depolarized mitochondria, as indicated by the reduced mΔψ in the *Park2*-depleted INS-1 cells (Fig. 4D). Furthermore, ROS handling and mΔψ maintenance are also important in insulin secretion (Ma et al., 2012; Patti and Corvera, 2010).

Several substrates of parkin are known to be located in the mitochondria. The mitochondria-shaping proteins Drp1, Mfn1, and Mfn2 are well-known substrates of parkin (Gegg et al., 2010; Wang et al., 2011). Altered mitochondrial morphological dynamics have been reported to contribute to impaired insulin secretion in diabetic states (Shenouda et al., 2011). Analysis of mitochondrial morphology by confocal microscopy revealed an increased proportion of fragmented mitochondria in the *Park2*-depleted INS-1 cells when compared to control cells (Fig. 5A). We also found that the levels of mitochondrial fission-regulating Drp1 protein were increased in the *Park2*-depleted INS-1 cells, while no changes were observed in the Fis1, Mfn1, Mfn2, and Opa1 protein levels (Fig. 5B). These findings suggest that increased mitochondrial fragmentation in the *Park2* knock-down cells may be due to the upregulation of Drp1 in response to the downregulation of parkin.

Importantly, α-synuclein is another mitochondrial substrate of parkin (Haass and Kahle, 2001). The α-synuclein reduces ATP synthesis and mΔψ by inducing the complex I dysfunction (Chinta et al., 2010) and inhibits insulin secretion in β-cells by binding to the K_{ATP} channel at insulin-secretory granules (Geng et al., 2011). We found a significant increase in the α-synuclein level in the *Park2*-depleted cells (Fig. 5B). Our results suggest that the reduced ATP production in the *Park2*-depleted INS-1 cells may be a result of the accumulation of α-synuclein in the mitochondria. Further, as α-synuclein-induced mitochondrial fission is rescued by parkin (Kamp et al., 2010), our data showed that fragmented mitochondria in the *Park2*-depleted cells may be due to the accumulation of α-synuclein in mitochondria as well as the increase of Drp1 level (Fig. 5B).

The *PARK2* gene has known mutations associated with certain cases of familial PD and has also been suggested as a candidate susceptibility gene for T2D by several association studies. Interestingly, diabetes is also associated with an increased risk of PD onset (Xu et al., 2011), which suggests that shared specific mechanisms

may underlie the pathogenesis of both diseases. Recently, dysregulation of insulin function in the brain has been implicated in the pathogenesis of neurodegenerative diseases such as PD (Craft and Watson, 2004). Since parkin plays a crucial role in mitophagy (Jin and Youle, 2012), we propose that the impaired mitochondrial QC caused by downregulation of the *PARK2* gene contributes to the β-cell dysfunction, just as the impaired mitochondrial QC caused by *PARK2* gene mutation contributes to neuronal dysfunction.

In summary, the association analysis revealed that genetic variants in the *PARK2* gene were significantly associated with fasting glucose and insulin secretion in Korean KARE cohort. Our results also demonstrated an important role of parkin in the regulation of insulin production and GSIS caused by mitochondrial alterations in β-cells. This is the first report to demonstrate a role of the *PARK2* gene in β-cell physiology, based on experimental functional analysis. This study may provide insights into the genetic basis of T2D and its relationship to the maintenance of mitochondrial integrity.

Acknowledgements

This work was supported by the National Research Foundation of Korea(NRF) grant funded by the Korean government (2009-0093189, 2012-051426, and 2012R1A1A2009459).

We thank the Korea Centre for Disease Control for providing the clinical information and genotype data (4845-301, 4851-302) obtained from the Consortium for Large Scale Genome Wide Association Study III (2011-E73004-00) and the Korea National Institute of Health Study (2012-N73002-00, 4845-302-210-13), the Republic of Korea. Data on glycemic traits have been contributed by MAGIC investigators and have been downloaded from www.magicinvestigators.org.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mce.2013.09.031.

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