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SHORT REPORT

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Senp2 expression was induced by chronic glucose stimulation in INS1 cells, and it was required for the associated induction of *Ccnd1* and *Mafa*

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ABSTRACT

Post-translational modification by bonding of small ubiquitin-like modifier (SUMO) peptides influences various cellular functions, and is regulated by SUMO-specific proteases (SENPs). Several proteins have been suggested to have diverse impact on insulin synthesis and secretion through SUMO modification in β cells. However, the role of SUMO modification in β cell mass has not been established. Here, we examined the changes in expression of *Senp* in INS1 cells and pancreatic islets under diabetes-relevant stress conditions and associated changes in β cell mass. Treatment with 25 mM glucose for 72 h induced *Senp2* mRNA expression but not that of *Senp1* in INS1 cells. Immunohistochemical staining with anti-SENP2 antibody on human pancreas sections revealed that SENP2 was localized in the nucleus. Moreover, in a patient with type 2 diabetes, SENP2 levels were enhanced, especially in the cytoplasm. Senp2 cytoplasmic levels were also increased in islet cells in obese diabetic mice. Cell number peaked earlier in INS1 cells cultured in high-glucose conditions compared to those cultured in control media. This finding was associated with increased *Ccnd1* mRNA expression in high-glucose conditions, and siRNA-mediated *Senp2* suppression abrogated it. *Mafa* expression, unlike *Pdx1*, was also dependent on *Senp2* expression during high-glucose conditions. In conclusion, Senp2 may play a role in β cell mass in response to chronic high-glucose through Cyclin D1 and Mafa.

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cell mass; diabetes; hyperglycemia; pancreatic islets; SENP2

Introduction

Enhancement of insulin secretion through increase of insulin-producing cells is an important strategy for the treatment of diabetes mellitus (DM), and there has been active research in this field, on the use of pharmacologic treatment, regenerative medicine, and transplantation of insulin-producing cells.[1-3]

Small ubiquitin-like modifier (SUMO) is a family of ubiquitin-like peptides consisting of 97 amino acids. Sumoylation is a post-translational SUMO modification which involves a series of enzymatic reactions. An isopeptide bond is formed between the C-terminal Gly residue of SUMO and a Lys residue in the substrate. Sumoylation can result in 3 general consequences for the modified protein: interference with an interaction between the target and its partner, enhanced binding with an interacting partner, or a conformational change. As a result, these modifications can alter the localization, activity or stability of the target protein.[4,5]

In pancreatic β cells, *Insulin* transcription has been reported to be negatively regulated by sumoylation of ICA512 and Mafa,[6-8] but enhanced by sumoylation of Pdx1.[9] Beta cell excitation and insulin secretion was found to be inhibited by sumoylation of voltage-dependent potassium channel, synaptotagmin VII, and Glucagon-like peptide (GLP)-1 receptor.[10-12] However, sumolyated glucokinase was observed to be stabilized and activated in insulin-secreting cells.[13] These data suggest that various molecules can affect insulin synthesis and secretion through sumoylation.

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Sumoylation has been found to be involved in cell cycle, senescence, and apoptosis in some cells,[14,15] especially in response to cellular stress.[5] Currently, the effects of sumoylation on β cell mass remain poorly understood. Glucolipotoxicity and pro-inflammatory cytokines promote sumoylation-dependent stability of P53, inhibiting β cell proliferation.[16] In contrast, sumoylation can protect against Interleukin-1β-induced apoptosis in INS-1 832/13 cells and human islets.[17] Therefore, the net effects of sumoylation machinery on β cell mass are not established.

Because there are a large number of sumoylation targets, and sumoylation is a highly dynamic process that is reversible by SUMO-specific proteases (SENPs), any single SUMO target would not be sufficient to explain the overall effects of sumoylation in cells.[5] There are 6 types of SENPs in mammalian cells (SENP1–3 and SENP5–7).[18] SENP1–3 and SENP5 were evolutionally diverged from the same branch, and have been reported to be involved in cell proliferation and death. Among them, SENP1 and SENP2 are closely related to each other, and involved in both SUMO maturation and deconjugation. SENP1 has been used in previous studies for desumoylation in insulin-secreting cells,[10,11,17] but SENP2 has yet to be examined. SENP3 and SENP5 do not appear to be constitutively expressed in human islets.[19] SENP6 and SENP7 are paralogs, and SENP7 expression has been found in human islets. However, SENP7 exhibits very low efficiency in processing full-length SUMO proteins to their mature forms.[5] Therefore, we studied the changes in SENP1 and SENP2 expression in insulin-producing cells under diabetes-relevant stress conditions and how these changes affect β cell mass.

Results

SENP2 *versus* SENP1 *expression in human islets and INS1 cells*

We found comparable expression of *SENP1* and *SENP2* transcripts in human islets isolated from 5 persons (Fig. 1A). When we assessed SENP2 localization on a pancreas section from a non-DM patient using immunohistochemical (IHC) staining, we found the protein localized mainly in the nuclei of both endocrine (within the dotted line) and exocrine cells (Fig. 1B, left panels) as reported previously.[18] Examination of DM patient samples revealed strong SENP2 expression not only in the nuclei but also in the cytoplasm of islets (Fig. 1B, right panels). Therefore, we next investigated which condition induces *SENP2* expression in DM. When INS1 cells were treated with palmitic acid (0.25 mM) or cultured in high-glucose (25 mM) medium for 72 h, only high-glucose induced greater *Senp2* mRNA expression compared to *Senp1* ($p < 0.01$), which was comparable to the control (Fig. 1C).

Expression of Senp2 *was increased in the islet cells of obese hyperglycemic mice*

Next, we examined Senp2 expression on pancreas sections from high-fat diet (HFD)-induced obese, non-DM mice and genetic obese DM mice, such as *ob/ob* and *db/db* mice (Fig. 2A). Like *in vitro* treatment of INS1 cells with palmitic acid, 8-week-old HFD mice did not exhibit increased Senp2 expression in the pancreata (Fig. 2B). In the case of *ob/ob* and *db/db* mice, which demonstrated persistent hyperglycemia at 13 weeks of age, *Senp2* expression in the islets was increased dramatically compared to the expression level at 5 weeks. This effect was especially prominent in the cytoplasm (Fig. 2C and 2D).

Serial changes in Senp2 *expression and cell number by high-glucose in INS1 cells*

Exploration of the time course of *Senp2* mRNA expression revealed that it was not induced until after 48 h of high-glucose treatment (Fig. 3A). This induction of *Senp2* expression was also confirmed at the protein level by western blotting (Fig. 3B). High glucose also accelerated cell numbers at 48 h with no further increase at later time points. However, the cell number peaked at 72 h under normal culture condition (Fig. 3C). The earlier peak in cell number induced by high glucose appeared to be due to enhanced cell proliferation because the *Ccnd1* (a gene for Cyclin D1) transcript initially suppressed by high-glucose was significantly increased at 48 h. This increase was maintained up to 72 h (Fig. 3D). Another factor related to β cell expansion, ribosomal protein S6 kinase 1 (S6K1) was examined because it mediates nutrient-induced islet hyperplasia and possesses candidate sites for sumoylation. Phosphorylation of S6K1 was not affected by high glucose (Fig. 3E). The cell number induced by high glucose at 72 h seemed to be influenced by cell death because there was a tendency of increase in the fraction of cleaved Caspase 3 ($p = 0.0763$), suggesting cell apoptosis (Fig. 3F).

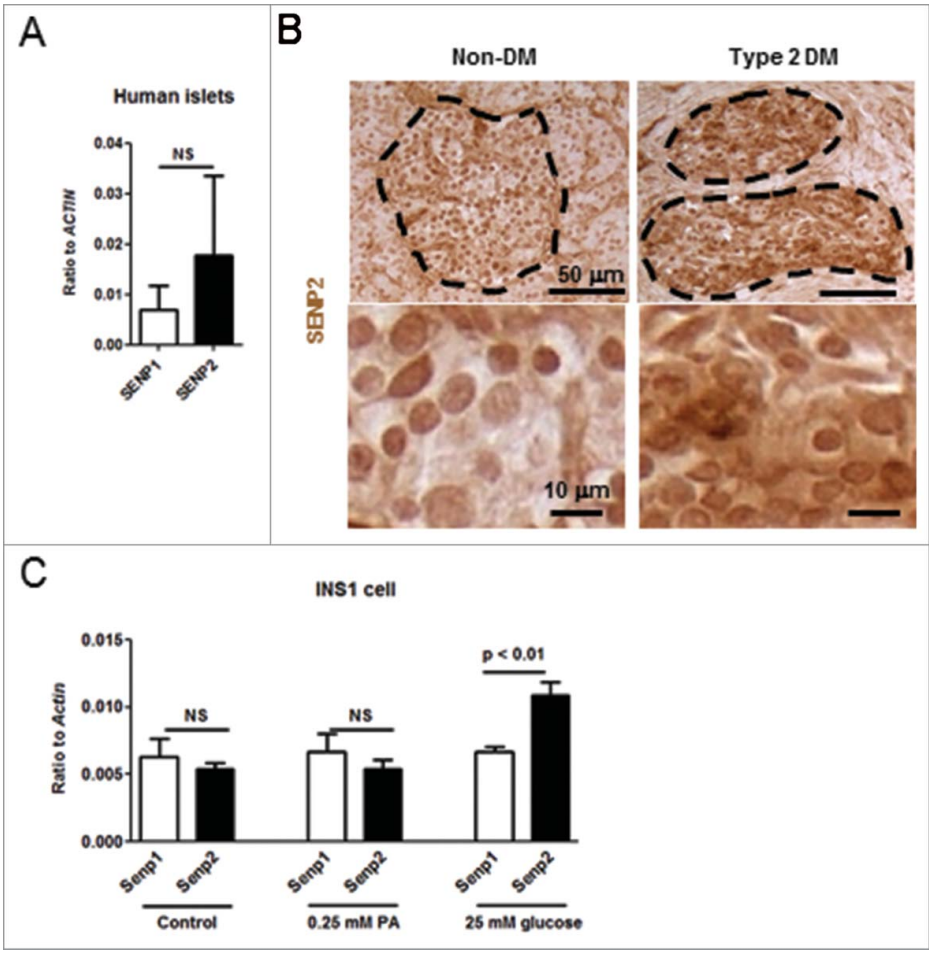

Figure 1. Expression of *Senp2* compared to *Senp1*. (A) Human islets were isolated and stabilized in the RPMI containing 10% FBS overnight. The mRNA expression of *SENP1* and *SENP2* was measured by quantitative RT-PCR and presented as ratios to *ACTIN* (n = 5). (B) IHC staining for SENP2 (brown) was performed on human pancreas harvested from a non-DM and a type 2 DM patients. Islets are indicated by dotted lines (upper panels) and presented on higher magnification (lower panels). (C) INS1 cells were cultured in RPMI containing 10% FBS, and either 0.25 mM palmitic acid or 25 mM high glucose was added for 72 h. The expression of *Senp1* and *Senp2* mRNA was measured by quantitative RT-PCR, and was presented as ratios to *Actin* expression (n = 5). Student's t-test was used for the comparisons between *Senp1* and *Senp2*. DM, diabetes mellitus; NS, no significant difference; PA, palmitic acid.

Suppression of Senp2 *expression under high-glucose conditions down-regulated cell mass and expression of* Ccnd1 *and* Mafa

When siRNA targeting *Senp2* (si*Senp2*) was transfected into cells before high-glucose treatment, *Senp2* expression decreased by 40∼50% (Fig. 4A and 4B). Because the mRNA expression level of *Senp2* was relatively low in control media, and there was no prominent induction of *Senp2* by high glucose at 48 h, suppression of *Senp2* expression using si*Senp2* was not substantial (Fig. 4A). At 48 h, enhanced expression of *Ccnd1* by high glucose was suppressed significantly by si*Senp2*, and *Mafa* expression was suppressed by si*Senp2* at 72 h. However, suppressed *Pdx1* and *Insulin* expression by high glucose was not affected by si*Senp2*. Culturing cells under high-glucose conditions for 48 h reduced cell number slightly by down-regulating *Senp2* expression ($p = 0.0617$), suggesting that maintenance of cell mass during initial high-glucose treatment required adequate expression of *Senp2* (Fig. 4C). *Ccnd1*-associated cell proliferation may play a role during this time period (Fig. 4A). However, at 72 h, *Senp2* down-regulation did not affect the cell number. At this time, cell death, as well as cell proliferation, contributed to the cell mass (Fig. 3C and 3F), indicating that *Senp2* may not be involved in cell expansion during this time period. Rather, *Senp2* may be involved in cell maturation *via Mafa* (Fig. 4B).

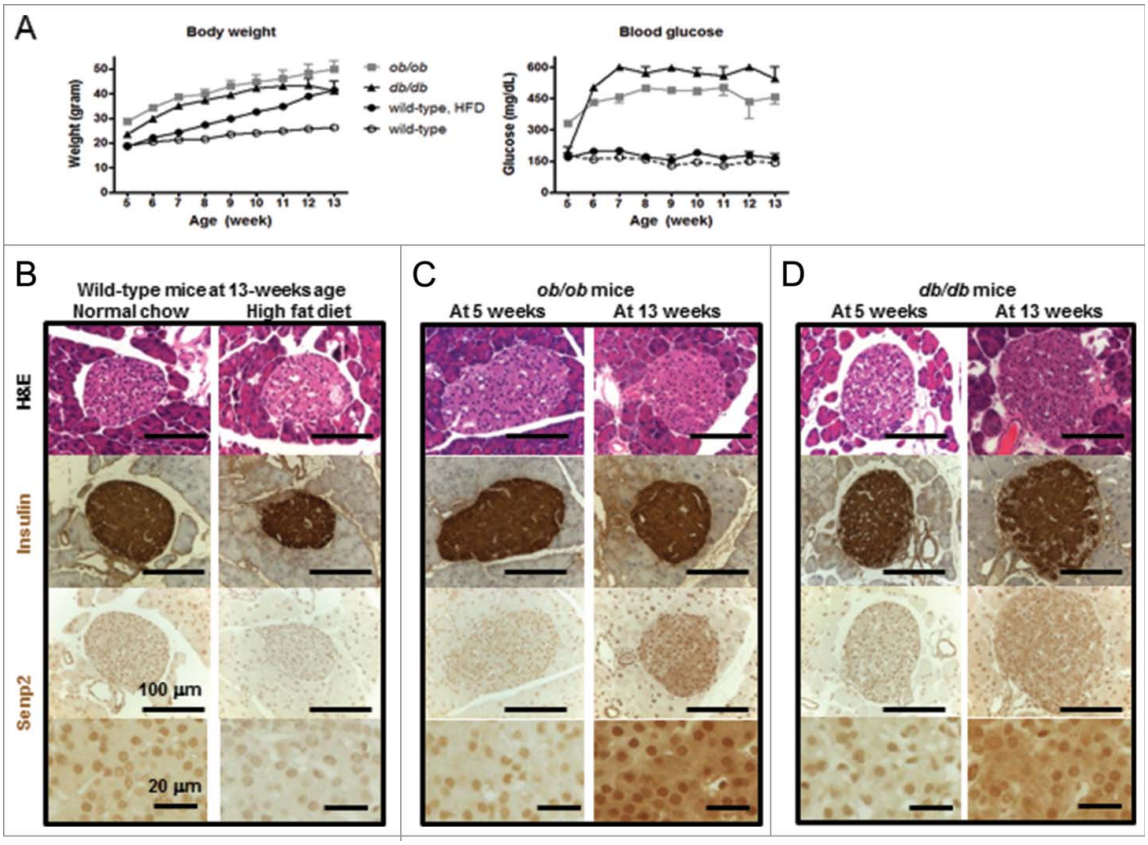

Figure 2. Expression of *Senp2* in the islets of hyperglycemic mice. (A) Male C57BL/6 wild-type mice were fed with normal chow or HFD for 8 weeks, with monitoring of blood glucose and body weight every week. Male *ob/ob*, and *db/db* mice at the same age were monitored, too. Numbers of animals were 2 in the each group. (B-D) H&E stain sections and IHC staining (brown color) for Insulin and Senp2 of pancreas from each group. H&E, hematoxylin and eosin; HFD, high fat diet; IHC, immunohistochemical.

Overexpression of SENP2 *during high-glucose conditions enhanced cell mass but downregulated* Insulin *expression*

Overexpression of human *SENP2* (h*SENP2*) using adenovirus harboring an expression system for human *SENP2* (Ad-h*SENP2*) was observed after 48 h (Fig. 5A and 5B). Human *SENP2* overexpression did not affect the expression of endogenous *Senp2, Ccnd1*, and *Mafa*. However, unlike the control media, *Insulin* expression was down-regulated by Ad-h*SENP2* in cells cultured in high-glucose media (Fig. 5B). Although overexpression of *SENP2* did not affect *Ccnd1* and *Mafa*, it increased the cell number significantly regardless of high-glucose conditions (Fig. 5C).

Discussion

Although SENP1 and SENP2 are known to have similar functions in both maturation and deconjugation of SUMO,[5] only SENP1 has been examined in previous studies in the context of desumoylation in insulin-secreting cells.[10,11,17] According to our study using INS1 cells and human tissues, SENP2 appears to regulate the physiologic response to chronic metabolic stress in insulin-secreting cells (Fig. 1), while SENP1 (but not SENP2) is redox-dependent.[20,21] In the case of muscle cells, SENP2 (but not SENP1) regulated fatty acid metabolism through desumoylation of peroxisome proliferator–activated receptors.[22,23] Unlike C2C12 myotubes from these studies, palmitic acid and HFD did not affect *Senp2* expression in insulin-secreting cells (Fig. 1C and 2B). Thus, different stimulations are required for inducing different SENPs according to cell types. Here, we conclude that chronic high glucose is responsible for inducing SENP2 expression in insulin-producing cells.

Induction of Senp2 expression by high-glucose concentration in insulin-producing cells would be highly complex because there are multiple sumoylation targets. In addition, Senp2 regulates sumoylation by both maturation and deconjugation of SUMO peptides. Recently, the global effects of

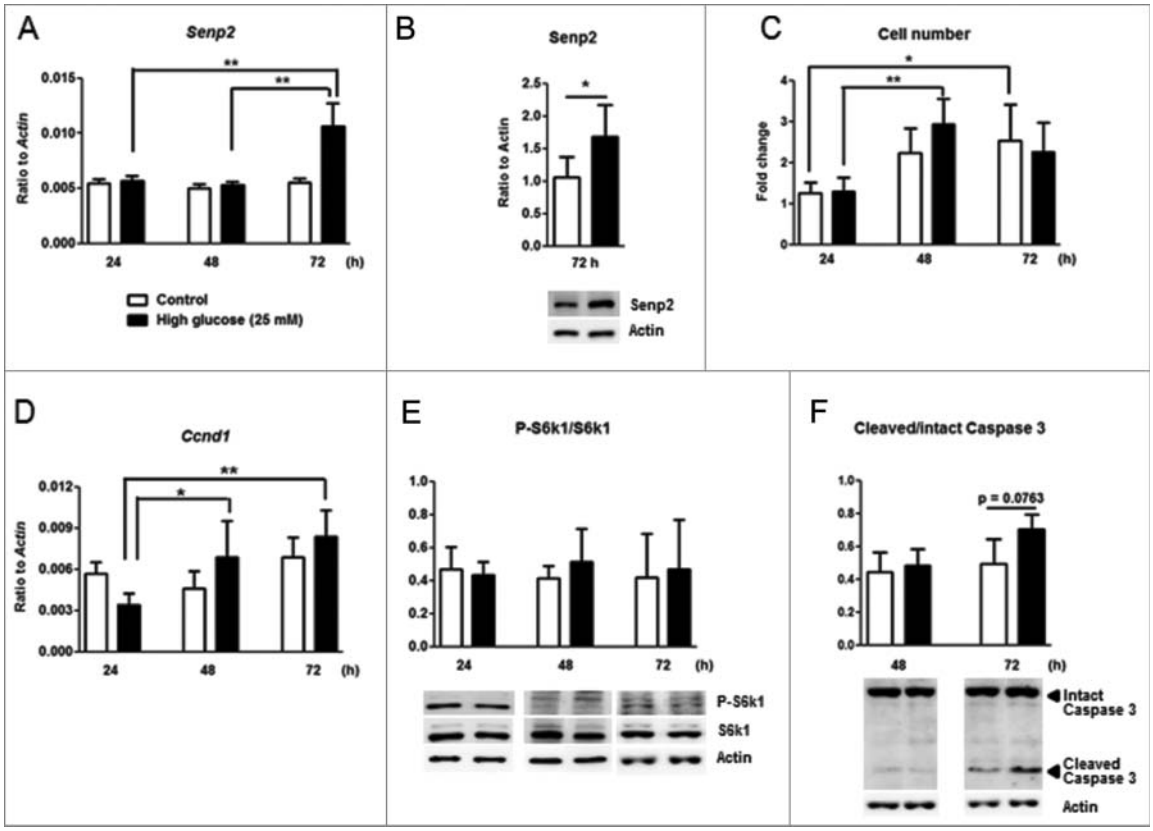

Figure 3. Expression of *Senp2* and cell number according to the duration of high glucose in INS1 cells. INS1 cells were incubated in the control media (RPMI containing 10% FBS and 11 mM glucose) and high-glucose (25 mM) media for the indicated time, and the sequential changes were studied. (A) *Senp2* mRNA was measured by quantitative RT-PCR, and was presented as ratios to *Actin* expression (n = 3). (B) Senp2 protein levels were measured by the western blotting, and presented as ratios to Actin expression (n = 4). (C) Cell number was presented as a fold of seeded cell number (n = 3). (D) Expression of *Ccnd1* transcript was measured by quantitative RT-PCR (n = 3). (E) S6K1 phosphorylation was detected by the western blotting and presented as ratios to total S6K1 expression (n = 3). (F) Caspase 3 cleavage was examined by the western blotting and presented as ratios to intact Caspase 3 (n = 4). Open bars, control media with 11 mM of glucose; solid bars, high-glucose (25 mM) media. Representative protein gel blots are below the graphs for B, E & F. In A, C, D and E, 2-way repeated measures ANOVA and Bonferroni posttests were performed. In B and F, paired t-test was performed. *, $p < 0.05$; **, $p < 0.01$; NS, no significant difference.

Senp1, rather than sumoylation of specific targets, were examined in insulin exocytosis.[21,24] We hypothesized that induction of *Senp2* both in the nucleus and cytoplasm during hyperglycemia plays a role in islet mass[25] because *db/db* and *ob/ob* mice exhibit active β cell hyperplasia and enhanced β cell mass around 13 weeks of age,[26-28] when *Senp2* is significantly induced (Fig. 2C and 2D).

There are several SUMO candidates related with β cell mass. Cyclin D1[29] expression was reported to be suppressed by Sam68 sumoylation.[15] Thus, we examined mRNA expression of *Ccnd1*. According to the data from time course study of *Senp2* expression and si*Senp2* transfection, the increase in *Ccnd1* at 48 h required the maintenance of *Senp2* expression (Fig. 3A, 3D, and 4A), and was concomitant with changes in cell number (Fig. 3C and 4C). Although overexpression of h*SENP2* increased cell numbers at 48 h (Fig. 5C), it did not enhance *Ccnd1* induction (Fig. 5B). In other words, the Cyclin D1–associated cell expansion in response to a high-glucose concentration at 48 h required constitutive *Senp2* expression, however, induction of *Ccnd1* was not necessary for the increase in cell number promoted by h*SENP2* overexpression. Although not examined here, cell death could be regulated by h*SENP2* overexpression.

The activity of the β cell transcription factors Mafa and Pdx1 was also dependent on sumoylation status,[8,9] and they may contribute to Senp2-regulated β cell mass. In particular, when high-glucose stimulation persisted up to 72 h, a slight but significant induction of *Mafa* occurred with prominent up-regulation of *Senp2*. Moreover, the *Mafa* induction was abrogated by si*Senp2* transfection (Fig. 4B). Mafa is a key transcription factor in the

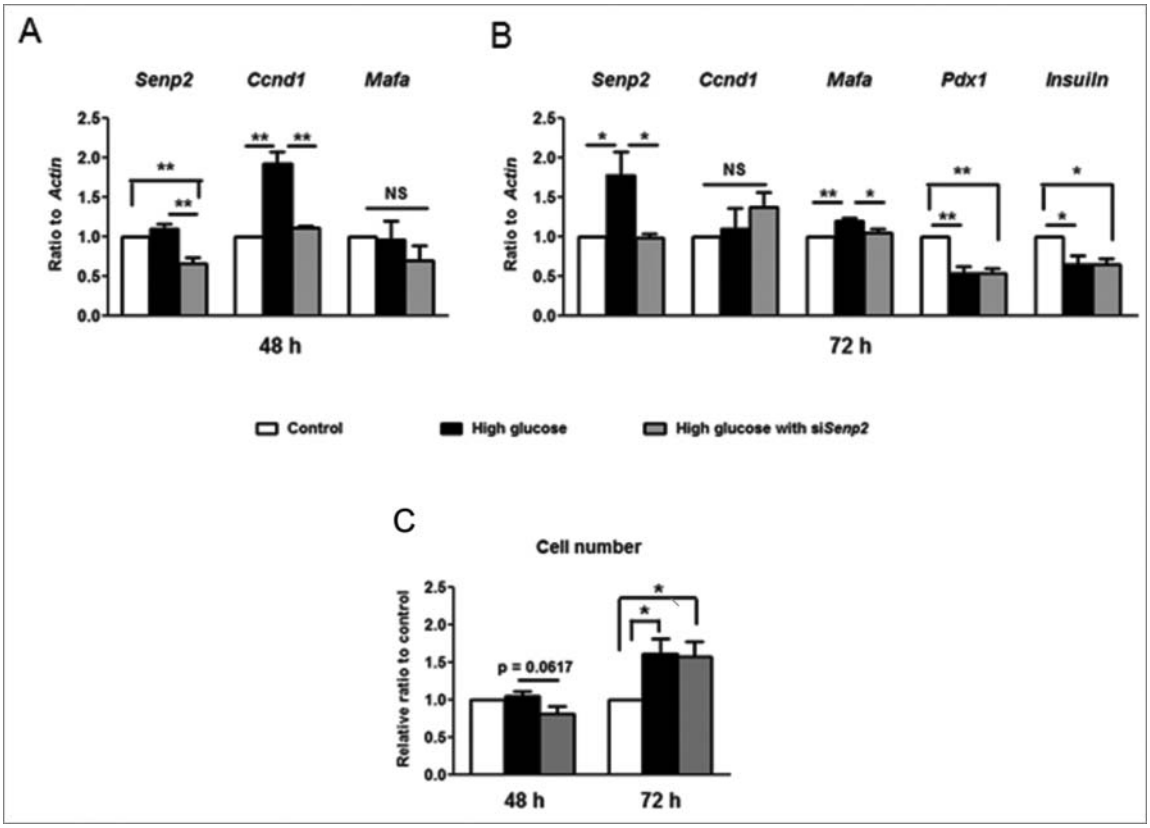

Figure 4. Suppression of *Senp2* during high glucose and associated changes in *Ccnd1, Mafa* and cell number. INS1 cells at 80% confluence were transfected with siNS and si*Senp2*. After overnight incubation, the media was changed with and without high glucose (25 mM) for 48 and 72 h (n = 4). The ratios of each transcript to *Actin* measured by quantitative RT-PCR were presented as relative values compared to the control at 48 h (A) and at 72 h (B). (C) Cell number was compared to the control at 48 h and at 72 h. One-way ANOVA and Tukey's Multiple Comparison Test were used. *, $p < 0.05$; **, $p < 0.01$; NS, no significant difference.

islet that is highly regulated by glucose levels. Mafa levels are reported to reduce upon development of hyperglycemia in *db/db* mice, and human MAFA expression is markedly decreased in β cells of type 2 DM.[30] Mafa also regulates postnatal β cell mass in rodents.[31] Preserving Mafa expression in diabetic islet β cells improves glycemic control through inhibition of β cell death and augmentation of *Insulin* transcription.[32] Therefore, there is a possibility that glucose-regulated Senp2 effects on β cell mass may be mediated, at least in part, by Mafa.

We found that overexpression of h*SENP2* under high-glucose condition inhibited *Insulin* transcription (Fig. 5B). Therefore, although Senp2 was required for β cell mass (Fig. 4C and 5C), excess Senp2 might accelerate high-glucose-induced suppression of *Insulin* transcription. However, there is also a possibility that the supra-physiologic levels of SENP2 protein generated by overexpression led to the removal of SUMO peptides from more targets than usual, perhaps non-specifically. This possibility might have confounded the results.

As mentioned before, another determinant for cell mass is the regulation of cell death. We found a statistically non-significant increase in cleaved Caspase 3 after 72 h of high-glucose conditions ($p = 0.0763$, Fig. 3F) that might affect the downward in cell number during that time ($p = 0.0633$ between 48 and 72 h of high glucose by paired t-test, Fig. 3C). *Senp2* induction and the regulation of sumoylation status of apoptosis-related pathways may contribute to the marginal increase in cleaved Caspase 3, as reported previously.[17] Therefore, both cell proliferation and cell death should be further evaluated to determine the role of SENP2 induction during hyperglycemia in β cell mass.

In summary, we identified that the expression of *Senp2*, but not *Senp1*, is triggered by chronic high-glucose stimulation in insulin-producing cells. *Senp2* expression was not induced by either

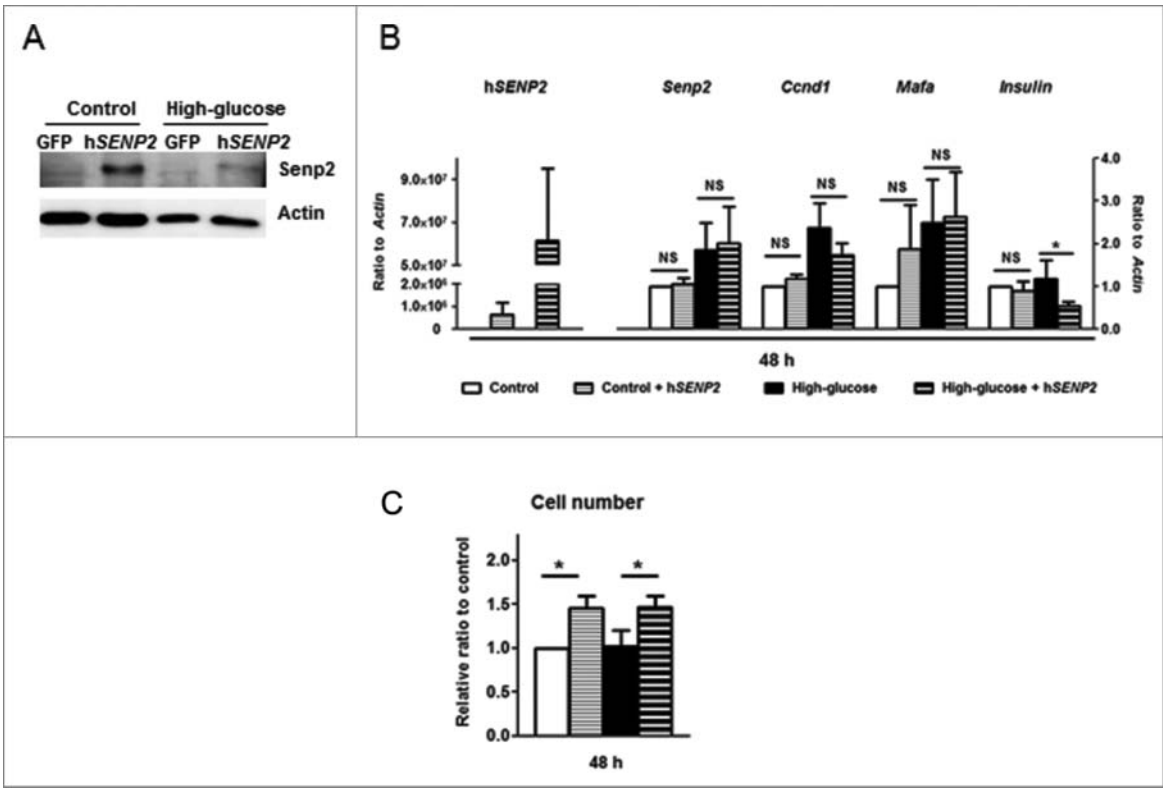

Figure 5. Overexpression of h*SENP2* and associated changes in *Ccnd1, Insulin* and cell number. INS1 cells at 80% confluence were infected with Ad-h*SENP2* and Ad-GFP. After overnight incubation, the media was changed with and without high glucose (25 mM) for 48 h (n = 8∼9). (A) A representative picture of western blotting. (B) The ratios of each transcript to *Actin* were presented as relative values compared to the control. h*SENP2* was detected only in the cells infected by Ad-h*SENP2*. (C) Cell number was compared to the control. Paired t-test and Wilcoxon matched-pairs signed rank test were used between the indications. *, $p < 0.05$; NS, no significant difference.

in vitro palmitate or an *in vivo* HFD. The increased *Senp2* level in response to high-glucose conditions was more prominent in the cytoplasm than in the nucleus of human and mouse samples. Adequate *Senp2* expression during cell mass expansion under chronic high-glucose conditions was required for the initial induction of *Ccnd1* and subsequent *Mafa* expression. However, excessive and untimely expression of *Senp2* inhibited *Insulin* expression.

Materials and methods

Animal experiments

Male C57BL/6 (wild-type) mice were purchased from Orient Bio, while C57BL/6J Ham Slc-*ob/ob* mice (*ob/ob*) and C57BLKS/J lar-Lepr^{db}/Lepr^{db} (*db/db*) mice were purchased from SLC (Shizuoka). After acclimation for 1 week, fed blood glucose levels and body weights were monitored weekly with a One Touch Ultra glucometer (Johnson & Johnson). C57BL/6 mice were fed normal chow or HFD consisting of 60% fat. All animal experiments were conducted in accordance with the institutional guidelines of the Institutional Animal Care and Use Committee of Seoul National University Hospital.

INS1 cell culture

INS1 cells were maintained in RPMI medium (Welgene, LM 011-01) containing 10% fetal bovine serum (FBS). Palmitic acid or high glucose was added after overnight stabilization following cell seeding or transfection. Palmitate (Sigma-Aldrich, p0500) was dissolved in ethanol, and mixed with fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich, P0500), to prepare medium with BSA-conjugated palmitate (1:3 molar ratio).

Human islet preparation

Human pancreatic tissues were obtained from 5 subjects (1 with DM and 4 without DM) who underwent surgery for a pancreatic mass. The study was approved

by the institutional review board of Seoul National University Hospital (IRB No. 0901-010-267), and written informed consent was obtained from all the subjects. The mean and standard error of mean of age was 55 ± 11 y old. Two subjects were male and the body mass index was 24.1 ± 2.5 kg/m^2. The HbA1C of the DM patient was 8.3 %. From the extracted pancreatic tissues, 0.4 ~3.0 g of normal-looking sections upon gross inspection was used for islet isolation. Pancreatic tissues were digested with collagenase (SERVA Electrophoresis GmbH, #17455) and islets were separated using Ficoll gradients centrifugation.[33] After washing, the human islets were hand-picked, counted and incubated in RPMI medium containing 10% FBS.

*si*Senp2 *transfection*

When INS1 cells reached 70~80% confluence, they were transfected with si*Senp2* (Santa Cruz Biotechnology, sc-72204) or a negative control small interfering RNA (siNS) (Bioneer, SN-1003) at 200 nM using Lipofectamine RNAiMAX (Thermo Fisher Scientific, #56532).

*Infection with Ad-h*SENP2

Ad-h*SENP2* prepared as previously described[23] was generously provided by Professor Kyong Soo Park (Seoul National University). INS1 cells at 70~80% confluence were transfected with adenovirus containing a construct expressing green fluorescent protein (Ad-GFP) and Ad-h*SENP2* at a multiplicity of infection of 45. Then the cells were cultured in high-glucose (25 mM) media for 48 h.

Cell counting

INS1 cells were seeded in 12-well plates. After incubation for 24~72h of high glucose in each experiment, the cells were detached with 0.25% trypsin–EDTA (Gibco, #25200-056) and harvested. Thoroughly resuspended cells were loaded into the C-Chip (INCYTO, DHC-N01-5) for manual cell counting.

Antibodies

Primary antibodies used for western blotting and IHC staining were as follows: anti-SENP2 (Santa Cruz Biotechnology, SC-67057), anti-Insulin (Sigma-Aldrich, I2018), anti-Actin (Sigma-Aldrich, A5441), anti-S6K (Cell Signaling Technology, #2708), anti-P-S6K (Cell Signaling Technology, #9234), and anti-Caspase-3 (Cell Signaling Technology, #9662) antibody.

Morphologic analyses

Human and mouse pancreata were formalin-fixed and paraffin-embedded before 4-μm serial sections were prepared. Hematoxylin and eosin (Sigma-Aldrich, S3309) staining and IHC staining were performed.

Western blotting

Total protein was extracted from the homogenized cells, subjected to SDS/PAGE, and then immunoblot analysis was performed.

RT-PCR

Total RNA was isolated from islets and INS1 cells using TRIzol reagent (Invitrogen, #15596026). Quantitative RT-PCR was performed using M-MLV Reverse transcriptase, (Promega, M1705) on the LightCycler® 96 Real-Time PCR System (Roche). The results were normalized to *Actin*. Primer sequences are as follows.

Senp1: F, GAAGTCTTTGCCTCGAAACC, R, TGTCGC CTCTGAGTTTTCTT;

Senp2: F, ACTTCCCGAGCAAAGAGAAAG, R, CGGG TGATTCGCAACTTG;

Human *SENP1*: F, TCACTGCCATGTATCTGCAT, R, CTGTTTCCCTGTGACCATCT;

Human *SENP2*: F, CTCAGGAACAGGCTGTAACA, R, CAGGACAGACAGAGTTTCCA;

Ccnd1: F, GCCTACAGCCCTGTTACCTG, R, ATTT CATCCCTACCGCTGTG;

Mafa: F, CTTCTGCCTCCGTTTTCTTG, R, CCAGT CGAGTATAGCAGGAC;

Actin: F, GAAGCTGTGCTATGTTGCTCTA, R, GGA GGAAGAGGATGCGGCA;

Human *ACTIN*: F, ACGAGACCACCTTCAACTCGA TC, R, AGGTCCTTCCTGATGTCCACGT.

Statistics

All values are expressed as the mean ± standard error of mean. The statistical methods applied are described in each figure legend. P-values less than 0.05 were considered to be statistically significant.

Abbreviations

Ad-GFP	Adenovirus containing the expression system for green fluorescent protein
Ad-h*SENP2*	Adenovirus containing the expression system for human *SENP2*
BSA	bovine serum albumin
db/db mice	C57BLKS/J lar-Lepr^{db}/Lepr^{db} mice
DM	diabetes mellitus
FBS	fetal bovine serum
GLP-1	glucagon-like peptide-1
H&E	hematoxylin and eosin
HFD	high fat diet
h*SENP2*	human *SENP2*
IHC	immunohistochemical
ob/ob mice	C57BL/6J Ham Slc-*ob/ob* mice
RT-PCR	reverse transcription polymerase chain reaction
S6K1	ribosomal protein S6 kinase 1
SENP	SUMO-specific protease
siNS	negative control small interfering RNA
si*Senp2*	small interfering RNA for *Senp2*
SUMO	small ubiquitin-like modifier.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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