SerpinB1 Promotes Pancreatic β Cell Proliferation

Graphical Abstract

Liver insulin resistance → SerpinB1 → Adaptive islet hyperplasia

Other sources → ?

Highlights
- Elevated serpinB1 correlates with β cell proliferation in insulin resistance
- SerpinB1 promotes β cell proliferation in multiple species
- SerpinB1 deficiency leads to maladaptive β cell proliferation in insulin resistance
- SerpinB1 inhibits elastase and activates growth/survival factor signaling pathways

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In Brief
Factors that promote compensatory β cell response to insulin resistance, a common feature in mammals, have been elusive. El Ouaamari et al. identify SerpinB1 as a hepatocyte-secretory protease inhibitor regulating β cell proliferation in humans, mice, and zebrafish. SerpinB1 acts by modulating canonical growth and survival signaling pathways.

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SerpinB1 Promotes Pancreatic β Cell Proliferation

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SUMMARY

Although compensatory islet hyperplasia in response to insulin resistance is a recognized feature in diabetes, the factor(s) that promote β cell proliferation have been elusive. We previously reported that the liver is a source for such factors in the liver insulin receptor knockout (LIRKO) mouse, an insulin resistance model that manifests islet hyperplasia. Using proteomics we show that serpinB1, a protease inhibitor, which is abundant in the hepatocyte secretome and sera derived from LIRKO mice, is the liver-derived secretory protein that regulates β cell proliferation in humans, mice, and zebrafish. Small-molecule compounds, that partially mimic serpinB1 effects of inhibiting elastase activity, enhanced proliferation of β cells, and mice lacking serpinB1 exhibit attenuated β cell compensation in response to insulin resistance. Finally, SerpinB1 treatment of islets modulated proteins in growth/survival pathways. Together, these data implicate serpinB1 as an endogenous protein that can potentially be harnessed to enhance functional β cell mass in patients with diabetes.

INTRODUCTION

While the etiopathogenesis of type 1 and type 2 diabetes is different (Bertard, 2012; Muoio and Newgard, 2008), a paucity of functional β cell mass is a central feature in both diseases (Butler et al., 2003; Henquin and Rahier, 2011; Lysy et al., 2013). Currently there is considerable interest in developing safe approaches to replenish bioactive insulin in patients with diabetes by deriving insulin-producing cells from pluripotent cells (D’Amour et al., 2006; Kroon et al., 2008; Pagliuca et al., 2014; Rezania et al., 2014) or promoting proliferation of pre-existing β cells (Dor et al., 2004; El Ouamami et al., 2013; Yi et al., 2013). While the former approach continues to evolve, several groups have focused on identifying growth factors, hormones, and/or signaling proteins to promote β cell proliferation (cited in El Ouamami et al., 2013 and Dirice et al., 2014). Compared to rodents, adult human β cells are more angiogenic to proliferation and have been suggested to turnover very slowly, with the β cell mass reaching a peak by early adulthood (Butler et al., 2003; Gregg et al., 2012; Kassem et al., 2000). Attempts to enhance human β cell proliferation have also been hampered by poor knowledge of the signaling pathways that promote cell-cycle progression (Bernal-Mizrachi et al., 2014; Kulkarni et al., 2012; Stewart et al., 2015). While two recent studies have reported the identification of a small molecule, harmine (Wang et al., 2015), and denosumab, a drug approved for the treatment of osteoporosis (Kondegowda et al., 2015) to increase human β cell proliferation, the identification of endogenous circulating factors that have the ability to replenish insulin-secreting cells is attractive for therapeutic purposes. We previously reported (Flier et al., 2001) that compensatory β cell growth in response to insulin resistance is mediated, in part, by liver-derived circulating factors in the liver-specific insulin receptor knockout (LIRKO) mouse, a model that exhibits significant hyperplasia of islets without compromising β cell secretory responses to metabolic or hormonal stimuli (El Ouamami et al., 2013). Here we report the identification of serpinB1 as a liver-derived secretory protein that promotes proliferation of human, mouse, and zebrafish β cells.
RESULTS

Identification of SerpinB1 as a Hepatocyte-Derived Circulating Protein in LIRKO Mice

To identify the putative β cell trophic factor in the LIRKO model, we performed mass spectrometry (MS)-based proteomics analyses of liver, liver explant-conditioned media (LECM), hepatocyte-conditioned media (HCM), and plasma from control or LIRKO animals (Figure 1A). Data analysis pointed to serpinB1 as the top significantly upregulated protein in all samples with substantial increases in liver (~3.3-fold), LECM (~3.7-fold), HCM (~54-fold), and plasma (~3.3-fold) (Figure 1B; red bars indicate serpinB1). To validate the proteomics data, we examined liver expression and circulating levels of serpinB1 in the LIRKO mouse. RT-PCR and western blotting experiments using cross-reactive antibody to human SerpinB1 revealed that serpinB1 mRNA (LIRKO 2.4 ± 0.6 versus control 0.6 ± 0.1; p < 0.05, n = 6) and protein levels (LIRKO 5.1 ± 0.9 versus control 1.1 ± 0.06; p < 0.05, n = 4–5) were elevated by ~5-fold in 12-week-old LIRKO mice compared to age-matched controls (Figures 1C–1E). Western blot analyses showed increased levels of serpinB1 in LIRKO-LECM (Figure 1F). SerpinA1 (also called α1-antitrypsin), which has partially overlapping biochemical activity, was not increased in LECM of LIRKO mice (Figure 1G).

Importantly, we observed that serpinB1 is increased in LIRKO hepatocyte lysates where neutrophil markers such as proteinase-3 (PR-3) and neutrophil elastase (NE) were not detected, therefore excluding contaminating blood cells as a significant source of serpinB1 (Figure 1H). We used recombinant human SerpinB1 (rSerpinB1) to introduce a standard curve in western blotting experiments to provide a semiquantitative measure of serpinB1 in serum samples (Figure 1I). Circulating serpinB1
was elevated in sera from 6-month-old LIRKO mice (78 ± 7.9 versus control 24.2 ± 4.2 ng equivalents/ml, p < 0.01, n = 10–12) (Figure 1J).

Serpins are a highly conserved superfamily of ∼45 kDa proteins, which are classified in 16 clades from A to P, and 36 members have been identified in humans (Silverman et al., 2001) and are known to regulate important proteolytic events. SerpinB1 is an evolutionarily conserved member of serpin clade B (Benarafa and Remold-O'Donnell, 2005) and inhibits the activity of several proteases including neutrophil elastase, cathepsin G, and proteinase-3 (Cooley et al., 2001). While serpinB1 lacks the hydrophobic signal peptide commonly harbored by secretory proteins (Remold-O'Donnell, 1993), the protein is detectable in hepatic-conditioned media and serum, suggesting that its release is mediated by an unconventional pathway (Nickel, 2010). Since previous studies reported a caspase-1-dependent mechanism of unconventional secretion (Becker et al., 2009; Chakraborty et al., 2013; Keller et al., 2008), we used human primary keratinocytes to investigate whether SerpinB1 secretion requires intact caspase-1. Consistent with previous reports (Chakraborty et al., 2013; Feldmeyer et al., 2007), irradiation of human keratinocytes with UVB light activated the inflammasome and induced release of several pro-inflammatory cytokines including IL-1β and IL-18, concomitant with caspase-1 activation. SerpinB1 is released in culture media when keratinocytes were UVB-irradiated: when caspase-1 was downregulated by a siRNA approach the SerpinB1 secretion was abolished, as was secretion of IL-1β and IL-18 (Figure S1A). Similar observations were evident when cells were treated with the caspase-1 inhibitor YVAD or pan-caspase inhibitor VAD prior to UVB treatment (Figure S1B). We also detected SerpinB1 in supernatants from cultured HepG2 cells and observed that several inflammatory molecules stimulate its release upon short-term (5 hr) or long-term (24 hr) treatment (Figure S1C). Consistent with increased levels of serpinB1 in LECM and serum from LIRKO mice, we found that caspase-1 mRNA and protein levels are increased in liver derived from LIRKO versus control groups (Figure S1D). Active caspase-1 (p20) was also highly abundant in LIRKO-LECM when compared to control conditions (Figure S1E).

To explore the clinical significance of SerpinB1 in humans, we developed an ELISA to measure plasma levels of SerpinB1 and observed that its concentration in healthy individuals ranges between 10 and 20 ng/ml (Figure S2A). Furthermore, a multivariate analysis in a cohort of 49 individuals with risk factor(s) for type 2 diabetes revealed that the range in concentration was greater, generally 4–56 ng/ml; however, interestingly, one individual with morbid obesity without diabetes (BMI = 59) exhibited extremely high levels (299 ng/ml) of circulating serpinB1. In a multivariate analysis, excluding this outlier, a positive correlation between circulating serpinB1 and insulin resistance (R² = 0.15, p = 0.026) was observed, using BMI and the composite insulin sensitivity index (CISI, Matsuda index) (Matsuda and DeFronzo, 1999) as covariates for measures of insulin sensitivity (Figure S2B). Furthermore, a search for missense variants of the corresponding gene in whole-exome sequencing data generated for 52 Joslin families with autosomal dominant diabetes showed that one of the families (for individual characteristics, see Table S1) carried a previously described variant (rs114597282, c.A269G, p.N90S) having a frequency of 1.7% among African Americans and 0.01% among Europeans in the NHLBI Exome Sequencing Project (ESP) database. The variant segregated with diabetes in this family, with all four diabetic members being heterozygous for this substitution (transmission disequilibrium test p value = 0.046) and only one non-penetrant individual being present in the youngest generation (Figure S2C). This variant is conserved among species (GERP score = 5.44) and is predicted as “probably damaging” by Polyphen (score = 0.98) and other prediction algorithms. Taken together, the significantly elevated serpinB1 in serum and hepatocyte secretome (HCM) in the LIRKO model, its presence in human sera, and its elevation in insulin-resistant states in humans, as well as the segregation of a genetic variant of serpinB1 with human diabetes prompted us to focus on this protein as a potential β cell growth factor.

SerpinB1 and Its Partial Mimics Promote Proliferation of Pancreatic β Cells in Multiple Species

To address whether serpinB1 promotes β cell proliferation, we cultured mouse islets in the presence of recombinant human serpinB1 or ovalbumin and evaluated proliferation by Ki67 immunofluorescence staining. Ovalbumin, encoded by serpinb14, was chosen as control because it is a serpin closely related in structure to serpinB1 but lacks pro tease inhibitory activity (Benarafa and Remold-O'Donnell, 2005). Ovalbumin-treated mouse islets displayed low β cell proliferation comparable to non-treated islets; rSerpinB1-treated islets exhibited a dose-dependent effect, and a 2-fold increase in the percentage of Ki67+ insulin+ cells was observed at the dose of 1 µg/ml (Figures 2A and 2B). We next tested whether small-molecule pharmacological agents that inhibit elastinolytic proteases, and thus partially mimic serpinB1 activity, GW311616A (Macdonald et al., 2001), and sivelestat (Kawabata et al., 1991), would affect β cell proliferation. Treatment of islets freshly isolated from male C57Bl/6 mice with GW311616A or sivelestat increased β cell proliferation (Figures 2C and 2D). To further explore the role of serpinB1 in vivo, we administered 7- to 8-week-old C57Bl/6 male mice with GW311616A, a partial mimic of serpinB1, by oral gavage (2 mg/kg/day for 2 weeks). Morphometric analyses showed that GW311616A treatment enhanced β cell mass (Figures 2E and 2F) by increasing β cell mass, but not α cell, proliferation as assessed by BrdU incorporation (Figures 2G and 2H). The lack of proliferation in extra-pancreatic tissues including liver, skeletal muscle, visceral and subcutaneous adipose tissues, spleen, and kidney (Figures S3A and S3B) suggests that GW311616A promotes selective β cell proliferation. The proliferative action of SerpinB1 was also evident in human β cells using islets obtained from 7 cadaveric organ donors (for donor characteristics, see Table S2). Quantification of Ki67+ insulin+ cells revealed that the number of proliferating β cells increased in islets cultured in serpinB1-containing media (Figures 2I and 2J). The percent of proliferating human β cells is in a range similar to those reported previously (El Ouazzami et al., 2013; Jiao et al., 2014; Rieck et al., 2012; Ritti et al., 2012; Walpita et al., 2012). Similar to its effect on mouse islets, sivelestat also increased proliferation of human β cells (Figures 2K–2M; for donor characteristics, see Table S3). To test whether sivelestat induces human β cell proliferation in vivo, we transplanted human islets (obtained from the Integrated Islet Distribution Program, IIDP) under the kidney capsule of 10-week-old male C57Bl/6 mice with GW311616A or sivelestat increased β cell proliferation (Figures 2C and 2D).
non-obese diabetic-severe combined immunodeficiency-IL2\textsuperscript{γc}null (NSG) mice (Greiner et al., 2011). At 10 days post-transplantation, osmotic pumps loaded with sivelestat (300 mg/kg/day) or vehicle were implanted into the mice and allowed to infuse for 14 days. Mice were provided BrdU in drinking water (80 mg/ml) during the 14-day treatment period and received intraperitoneal injections of BrdU (100 mg/kg body weight) on days 8, 11, and 14 post-transplantation. At 5 hr after the last

Figure 2. SerpinB1 and Its Partial Mimics Promote Proliferation of Mouse and Human Pancreatic \(\beta\) Cells
(A) Representative images of mouse islets treated with ovalbumin or SerpinB1 and co-immunostained for Ki67, insulin, and DAPI.
(B) Quantification of Ki67\textsuperscript{+} insulin\textsuperscript{+} cells (in A). Data represent mean ± SEM; \(*p \leq 0.01\) (n = 6–12 per group).
(C) Representative images and quantitation of insulin\textsuperscript{+} Ki67\textsuperscript{+} cells of islets isolated from wild-type male mice and cultured for 48 hr in the presence of 100 \(\mu\)g/ml of either sivelestat or GW311616A.
(D) Quantification of insulin\textsuperscript{+} Ki67\textsuperscript{+} cells of sivelestat-treated islets (in C). Data represent mean ± SEM; \(p \leq 0.05\) (n = 3 per group for GW311616A studies and n = 6 per group for sivelestat studies). 5- to 6-week-old wild-type male mice were treated with GW311616A for 2 weeks. Islet \(\beta\) cell and \(\alpha\) cell proliferation was assessed by immunostaining.
(E) Pancreatic sections co-immunostained for BrdU and insulin and DAPI (two left panels) or co-immunostained for glucagon and BrdU and DAPI (two right panels).
(F) Quantification of \(\beta\) cell mass (in E). Data represent mean ± SEM; \(p \leq 0.05\) (n = 4–5 per group).
(G) Quantification of insulin\textsuperscript{+} BrdU\textsuperscript{+} cells (in E). Data represent mean ± SEM; \(p \leq 0.01\) (n = 4–5 per group).
(H) Quantification of glucagon\textsuperscript{+} BrdU\textsuperscript{+} cells (in E). Data represent mean ± SEM; \(p \leq 0.01\) (n = 4–5 per group).
(I) Representative images of human islets treated with ovalbumin or SerpinB1 and co-immunostained for Ki67, insulin, and DAPI.
(J) Quantification of Ki67\textsuperscript{+} insulin\textsuperscript{+} cells (in I). Data represent mean ± SEM; \(p \leq 0.05\), **\(p \leq 0.01\) (n = 7 per group). For details of the human donors please see Table S2.
(K) Representative images of human islets treated with vehicle or sivelestat and co-immunostained for Ki67, insulin, and DAPI.
(L and M) Quantification of Ki67\textsuperscript{+} insulin\textsuperscript{+} cells (in K). For details of human donors (Donor #1, L; Donor #2, M) please see Table S3.
(N) Experimental workflow for transplantation studies to explore the effects of sivelestat on human \(\beta\) cell proliferation in vivo.
(O) Representative images of human islet grafts retrieved from mice treated with sivelestat or vehicle and co-immunostained for BrdU, insulin, and DAPI.
(P) Quantification of BrdU\textsuperscript{+} insulin\textsuperscript{+} cells (in O).
(Q) Representative images of endogenous pancreases harvested from mice treated with sivelestat or vehicle and co-immunostained for BrdU, insulin, and DAPI.
(R) Quantification of BrdU\textsuperscript{+} insulin\textsuperscript{+} cells (in Q). For details of human donors please see Table S3. Data represent mean ± SEM; \(p \leq 0.05\), **\(p \leq 0.01\) (n = 5–6 per group for retrieved human islet grafts and n = 3 for endogenous pancreases). Arrows indicate proliferating cells.
BrdU injection islet grafts and endogenous pancreases were harvested to assess β cell proliferation (Figure 2N). As assessed by co-immunostaining with anti-insulin and anti-BrdU antibodies, human islet grafts retrieved from mice treated with sivelstat exhibited higher β cell proliferation compared to vehicle-treated controls (Figures 2O and 2P). In parallel, islet β cell proliferation was also increased in endogenous pancreases harvested from NSG mice infused with sivelstat (Figures 2O and 2R). The in vivo effect of sivelstat on β cell proliferation was also evident in C57Bl/6 mice (Figures S4A and S4B).

Next, to determine whether the potentiation of β cell proliferation by serpinb1 is conserved across species, we examined serpinb1-overexpressing zebrafish larvae (Figure 3A). Whereas the human clade B serpin loci encode 13 proteins (serpinB1–13) with distinct functions, the corresponding locus in zebrafish is substantially simpler: it includes a distinct serpinb1 orthologous gene with a strikingly conserved reactive center loop, suggesting conserved function (Benarafa and Remold-O'Donnell, 2005). The overexpressing larvae were generated by cloning serpinb1 downstream of a ubiquitous promoter (beta-actin), i.e., generating widespread mosaic overexpression of serpinb1 (see details in Supplemental Experimental Procedures). The same cloning procedure was performed for the controls H2A-mCherry and serpina7 (another member of the zebrafish Serpin family).

We started by determining serpinb1’s effect on β cell regeneration using different transgenic zebrafish larvae expressing nitroreductase (NTR)—an enzyme that converts metronidazole to a cytotoxic product—under the control of the insulin promoter; incubating these larvae in metronidazole results in the specific ablation of their β cells (Andersson et al., 2012). Each construct was injected, together with mRNA encoding transposase, into 1-cell-stage Tg(ins:-flag-NTR);Tg(ins:Kaede) embryos, giving rise to zebrafish larvae in which the β cells are visualized by the GFP Kaede. From 3 to 4 days post fertilization (dpf), we used metronidazole to ablate the β cells of mosaically overexpressing larvae and control larvae, and at 6 dpf we examined whether overexpression of any of the proteins had increased β cell regeneration. Overexpression of serpinb1 strikingly increased regeneration of the β cell mass by 50%, whereas none of the controls had a significant effect (Figures 3B–3F). To determine serpinb1’s effect on β cell proliferation, we examined the incorporation of EdU, when compared to control larvae (Figures 3K and 3L). Serpinb1 did not significantly increase the total number of β cells, but it significantly increased the number of β cells that incorporated EdU (Figures 3M and 3N). Together, these data provide evidence for serpinb1 as a phylogenetically conserved protein that stimulates β cell proliferation in multiple species including zebrafish, mouse, and man.

SerpinB1 Deficiency Leads to Maladaptive β Cell Proliferation in Insulin-Resistant States

To assess the in vivo relevance of serpinB1 in the adaptive β cell response to insulin-resistant states, we challenged control or serpinb1a-deficient (serpinB1KO) mice with stimuli that caused acute or chronic insulin resistance. To evaluate the response to acute insulin resistance, we adopted two approaches: first, we treated 16-week-old control male mice with the insulin receptor antagonist S961 (10 nmol/wk) for 2 weeks (Figure S5A) and observed progressive hyperglycemia in the mice (Figure S5B) as previously described (Yi et al., 2013). SerpinB1KO mice treated with S961 peptide showed elevated random-fed blood glucose that was higher when compared to S961-treated controls. No differences were observed in blood glucose levels between PBS-treated control and serpinB1KO mice (Figure S5B). Quantitation of proliferation by co-immunostaining BrdU+ insulin+ cells revealed an ~10-fold increase in S961-infused mice when compared to respective PBS-treated controls (Figures S5C and S5D). Importantly, while PBS-infused serpinB1KO mice showed a low level of proliferating β cells similar to PBS-infused controls, S961-treated serpinB1KO mice showed a detectable, but attenuated, response; the number of BrdU+ insulin+ cells was ~40% fewer compared with S961-treated control mice (Figures S5C and S5D). The reduction in adaptive β cell proliferation was supported by an attenuated increase in the number of β cells that co-stained positive for phospho-histone H3 (pH3), an additional marker of cell proliferation (Figure S5E).

In a second model, we fed 16-week-old control and serpinB1KO mice with 60% kcal high-fat diet (HFD) for 10 weeks and analyzed β cell proliferation by BrdU incorporation and immunofluorescence staining. An ~50% reduction in the number of BrdU+ insulin+ cells in serpinB1KO-HFD mice compared to age-matched control-HFD mice (Figures S5F and S5G) suggested impaired compensatory β cell proliferation; this was confirmed by staining for two additional proliferation markers including pH3 (Figures S5H and S5I) and Ki67 (Figures S5J and S5K). However, we did not observe significant alterations in β cell mass in serpinB1KO as compared to control mice in either the S961 or the short-term HFD models, suggesting that additional factors likely contribute to increasing the β cell mass in these short-term insulin resistance models. In a third model, we explored whether serpinb1 is critical for long-term β cell response by subjecting 8-week-old control and serpinB1KO mice to low- or high-fat diets (LFD or HFD) for 30 weeks, which led to chronic insulin resistance as shown by hyperinsulinemia in both groups (control, LFD: 1.5 ± 0.2 versus HFD: 9.6 ± 1.5 ng/ml; p < 0.05; serpinB1KO, LFD: 1.8 ± 0.2 versus HFD: 3.7 ± 0.7 ng/ml; p < 0.05; n = 4–5). As expected, control mice challenged with HFD, compared to the corresponding LFD cohort, showed enhanced β cell proliferation and mass. In contrast, mice lacking serpinb1 challenged with a similar HFD showed significantly lower increases in β cell proliferation and mass (Figures 4A–4E). Taken together, these data suggest that the effects of serpinb1 for β cell compensatory hyperplasia are more apparent in chronic insulin resistance.

SerpinB1 Activates Proteins in the Growth Factor Signaling Pathway

To demonstrate whether protease inhibitory activity is critical for enhancement of β cell proliferation by SerpinB1, we tested...
Figure 3. Overexpression of Serpinb1 in Zebrafish Enhances β Cell Regeneration and Proliferation

(A) Schematic of experimental plan.

(B–E) Representative images at 6 dpf of Tg(ins:kaede);Tg(ins:CFP-NTR) transgenic larvae that had not been injected (control), or were injected at the 1-cell stage with transposase mRNA + bactin:H2A-mCherry, bactin:serpina7, or bactin:serpinb1; were subjected to β cell ablation by metronidazole during 3–4 dpf; and were subsequently allowed to regenerate for 2 days.

(F) Quantification of β cell regeneration at 6 dpf in control (n = 87), bactin:H2A-mCherry-overexpressing (n = 61), bactin:serpina7-overexpressing (n = 46), and bactin:serpinb1-overexpressing (n = 36) Tg(ins:kaede);Tg(ins:CFP-NTR) larvae.

(G–J) Control (n = 27) and bactin:serpinb1-overexpressing (n = 18) Tg(ins:H2B-GFP);Tg(ins:Flag-NTR) transgenics were treated with metronidazole from 3 to 4 dpf to ablate the β cells and subsequently incubated with EdU during regeneration from 4 to 6 dpf. Representative confocal images at 6 dpf of control (G) and bactin:serpinb1-overexpressing (H) larvae showing β cells in green and the β cells that had incorporated EdU in yellow (green and red overlap; arrowheads).

(I) Quantification of the total number of β cells at 6 dpf. (J) Quantification of β cells that incorporated EdU during β cell regeneration from 4 to 6 dpf.

(K–N) To determine whether Serpinb1 affects β cell proliferation during regular development, we treated control (n = 25) and bactin:serpinb1-overexpressing Tg(ins:H2B-GFP) (n = 21) transgenic larvae with EdU from 4 to 6 dpf. Representative confocal images at 6 dpf of control (K) and bactin:serpinb1-overexpressing (L) larvae showing β cells in green and the β cells that had incorporated EdU in yellow (green and red overlap; arrowhead). (M) Quantification of the total number of β cells at 6 dpf. (N) Quantification of β cells that incorporated EdU from 4 to 6 dpf. Data shown are the mean ± SEM; ****p < 0.0001, **p < 0.01, *p < 0.05. Arrows indicate proliferating cells.
different commercially available SerpinB1 recombinant proteins that bear a tag sequence at the N or C terminus. As reported previously, insect cell-derived SerpinB1, which is identical to the native protein (Cooley et al., 1998), forms a covalent complex (approximately 66 kD) with each of its target proteases (Cooley et al., 2001); this is shown for human neutrophil elastase (NE) and porcine pancreatic elastase (PE) (Figure 5A). Second, peptidase assays demonstrated that insect cell-derived untagged SerpinB1 from two independent preparations dose-dependently decreased activity of these proteases (shown for NE); however, the commercial proteins that are tagged at the N terminus (GeneCopoeia or OriGene) or C terminus (OriGene) only minimally inhibited peptidase activity of NE (Figure 5B). GeneCopoeia N-tagged SerpinB1 and OriGene C-tagged serpinB1 formed small amounts of complex, which was maximal with <0.1 or 0.3 molar equivalents NE, respectively, consistent with low inhibition in the peptidase assay; the OriGene C-tagged serpinB1 was also partially degraded (Figure 5C). For OriGene N-tagged serpinB1, no complex was detected on incubation with 0.3 molar equivalents of NE, and the recombinant serpin was completely degraded by NE; proteolytic degradation of the serpin by NE was confirmed by inactivating NE with DFP (diisopropyl fluorophosphate) (Amrein and Stossel, 1980) before use (Figure 5C). Insect cell-derived untagged serpinB1 was nearly quantitatively converted to complex or was further converted to the post-complex species, and importantly, no active 26 kDa NE band remained (Figure 5C). GeneCopoeia serpinB1, which lacks the ability to form a complex with neutrophil elastase and is unable to reduce peptidase activity, did not stimulate β cell proliferation as opposed to untagged serpinB1 (Figure 5D). These findings suggest that the ability to inhibit protease is a requirement that is necessary for the β cell proliferation-enhancing action of SerpinB1.

To gain initial insights into the signaling pathways mediating β cell proliferation in response to SerpinB1, we considered a phosphoproteomics approach. Protein phosphorylation has been

Figure 4. SerpinB1 Deficiency Leads to Maladaptive β Cell Proliferation in Insulin-Resistant States
8-week-old control or serpinb1a−/− (serpinB1KO) male mice were challenged with low-fat diet (LFD) or HFD for 30 weeks. 5 hr before sacrificing, mice were injected with BrdU (100 mg/kg body weight).
(A) Representative images of pancreases co-immunostained for BrdU and insulin and DAPI (left panel). Representative images of pancreases co-immunostained for Ki67 and insulin and DAPI (middle panel). Representative images of pancreases co-immunostained for pHH3 and insulin and DAPI (right panel).
(B) Quantification of BrdU⁺ insulin⁺ cells (in A).
(C) Quantification of Ki67⁺ insulin⁺ cells (in A).
(D) Quantification of pHH3⁺ insulin⁺ cells (in A).
(E) Measurement of β cell mass. Data represent mean ± SEM; *p ≤ 0.05 (n = 4–6 per group). Immunostaining for BrdU and Ki67 markers, shown in (A), were performed on consecutive sections. Arrows indicate proliferating cells.
long accepted as a major currency in signal transduction pathways, and cell proliferation is known to be regulated by signaling modules that include the MAP kinase pathways. Further, measurement of phosphorylation dynamics represents a more direct way to identify potential pathways and regulatory targets compared to other techniques, such as gene expression profiling. Briefly, isolated islets from C57Bl/6 male mice were cultured for 10, 30, or 120 min in the presence of 1 mg/ml of ovalbumin or SerpinB1. Subsequently, islets were subjected to phosphopeptide enrichment and LC-MS/MS analysis (Mertins et al., 2014) (Figure 6A). As shown by the heatmap (Figure 6B), a 10 min treatment with SerpinB1 had a minimal effect on the islet phosphoproteome. However, islets incubated with SerpinB1 for 30 or 120 min exhibited an enhanced phosphorylation of ~250 proteins with at least 2-fold change when compared to islets cultured with ovalbumin (for additional details, see data submitted to ProteomXchange with accession number PXD003182). The modulation of several phosphoproteins identified at 30 min was sustained 2 hr after SerpinB1 treatment. Ingenuity pathway analysis (IPA) revealed that SerpinB1 activated key proteins in the growth factor (insulin/IGF-1) signaling cascade. In early events (within 10 min), SerpinB1 stimulated MAPK3 phosphorylation, a kinase previously implicated in the proliferation of β cells (Hayes et al., 2013). Treatment for 30 or 120 min was characterized by activation of several proteins in the insulin/IGF-1 signaling cascade including IRS-2 (Kubota et al., 2004; Withers et al., 1998) and GSK3 (Liu et al., 2010). Finally, phosphoproteomics analyses also revealed increased phosphorylation of several proteins regulating cell survival and function, including protein kinase cAMP-dependent regulatory subunits (PRKAR1A, PRKAR1B, and PRKAR2B) (Hussain et al., 2006; Jhala et al., 2003) and phosphodiesterase 3B (PDE3B) (Händahl et al., 2002). In independent experiments, we confirmed, by western blots, the altered phosphorylation of MAPK, PRKAR2B, and GSK3 subunits in response to treatment with SerpinB1 (Figures 6C–6E; lower panels show quantification). Incubation of freshly isolated islets with serpinB1 did not significantly affect glucose-stimulated secretion compared to...
controls, suggesting that effects of serpinB1 on islet β-cell proliferation do not adversely impact insulin secretion under the conditions tested (Figure 6F). Together, these data suggest that SerpinB1 enhances proliferation/survival by modulating proteins in the growth factor signaling pathway.

**DISCUSSION**

Identification of molecules that have the ability to enhance proliferation of terminally differentiated cells is a desirable goal in regenerative medicine, particularly in diabetes where β-cell numbers are reduced. Here, we identified serpinB1 as an endogenous liver-derived secretory protein that stimulates human, mouse, and zebrafish β-cell proliferation.

One interesting aspect of serpinB1 viewed as a secretory molecule is its lack of the classical hydrophobic signal peptide. Our data indicate that inflammation stimulates unconventional secretion of serpinB1 in a caspase-1-dependent manner. It is important to note, however, that the levels of several circulating cytokines in the LIRKO model are comparable to those observed in age-matched controls (El Ouaamari et al., 2013) and hence excludes systemic inflammation as a physiological factor triggering serpinB1 release in vivo. It is possible that the absence of insulin signaling in the liver interferes with caspase-1 activation and thus serpinB1 release. This notion is compatible with a previous report suggesting the suppressive role of insulin/IGF-1 in caspase-1 processing (Jung et al., 1996) and is consistent with increased levels of active caspase-1 in LIRKO-derived hepatocytes that are blind to insulin.

Since inhibition of proteases is SerpinB1’s reported biochemical function to date (Cooley et al., 2001), we postulated that the enhancing effect of SerpinB1 on β-cell proliferation involves the intermediacy of a protease. Indeed, recombinant SerpinB1 proteins lacking the ability to inhibit protease activity were unable to enhance β-cell proliferation in vitro. This observation suggests that SerpinB1 neutralizes a protease that would otherwise interfere with proliferation. In fact, the small-molecule inhibitors of elastases, GW311616A and sivelestat, directly enhanced proliferation of mouse and human insulin-producing cells. The parallel findings for GW311616A, sivelestat, and SerpinB1 make elastases strong candidates. While SerpinB1 action could be explained by its ability to modulate phosphorylation of key molecules (e.g., MAPK3, GSK3β/α, and PKA) of the insulin/IGF-1 growth/survival pathways, it is unclear how SerpinB1...
precisely regulates these pathways. One possibility is that these pathways are activated through SerpinB1-mediated protease inhibition, particularly inhibition of elastase molecules known to be expressed in pancreatic β cells (Kutlu et al., 2009). This idea is consistent with previous reports suggesting the role for neutrophil elastase in modulating proteins in the insulin/IGF-1 signaling pathway (Brustow et al., 2008; Houghton et al., 2010; Talukdar et al., 2012). Elucidation of interactions with other proteases such as proteinase-3 and cathepsin G in the β-cell and its potential role in regulating insulin sensitivity will further assist in deciphering the signaling pathways activated by SerpinB1. Alternative possibilities that require further investigation include interactions with protease-activated receptors (PARs), which are expressed in islets (J.S., A.E.O., and R.N.K., unpublished data).

Using zebrafish, we determined that serpinB1’s ability to potentiate β-cell proliferation is conserved from fish to mammals. Moreover, in zebrafish we showed that serpinB1 can potentiate β cell proliferation in vivo analogous to the in vivo effects we observed in mouse and human islets. By ablating the β cells in zebrafish, we also observed that serpinB1 can stimulate β-cell regeneration and warrants studies to examine its role during β-cell development.

In sum, the identification of SerpinB1 as a conserved endogenous secretory protein that promotes proliferation of β cells across species constitutes an important step to achieve regeneration of functional β cells. While it is likely that additional factors will be identified, the next challenge will be to explore whether one or a combination of these factors can safely, specifically, and reversibly enhance human β-cell mass with the long-term goal of restoring normoglycemia in patients with diabetes.

**EXPERIMENTAL PROCEDURES**

**Animals**

All mice studied were 6- to 8-week-old males on the C57BL/6 background, except where indicated otherwise. Mice were housed in pathogen-free facilities and maintained in the Animal Care Facilities at Joslin Diabetes Center, Boston, MA; Foster Biomedical Research Laboratory, Brandeis University, Waltham, MA; or Boston Children’s Hospital. Studies conducted and protocols used were approved by the Institutional Animal Care and Use Committees of the Joslin Diabetes Center and/or Brandeis University and/or Boston Children’s Hospital and were in accordance with National Institute of Health guidelines. See the Supplemental Experimental Procedures for details of the animal genotypes. For short-term studies, 16-week-old serpinB1KO and age-matched wild-type male mice were challenged with HFD (Research Diet, catalog# D12450J) or HFD (Research Diet, catalog# D12492) for 30 weeks.

**LECM and HCM Preparation**

The preparation of liver explant-conditioned media (LECM) and hepatocyte-conditioned media (HCM) have been described previously (El Ouaamari et al., 2013). See Supplemental Experimental Procedures for additional information.

**LC-MS/MS-Based Proteomics**

Proteomic analyses were performed as previously described (Zhou et al., 2010). See Supplemental Experimental Procedures for additional information.

**Mouse Islet Studies**

Islets were isolated from 6- to 8-week-old male C57BL/6 mice using intra ductal collagenase technique (El Ouaamari et al., 2013). Islets were hand-picked and cultured overnight in RPMI 1640 media containing 7 mM glucose and 10% fetal bovine serum (FBS) and penicillin/streptomycin (1% v/v). After 3 hr starvation in RPMI 1640 media containing 3 mM glucose and 0.1% BSA, islets were stimulated as indicated (with recombinant protein or small molecules) for 48 hr and then embedded in agarose and paraffin, sectioned, and used for immunostaining studies as described below and in El Ouaamari et al. (2013).

**Human Islet Studies**

Human islets were obtained from the Integrated Islet Distribution Program. All studies and protocols used were approved by the Joslin Diabetes Center’s Committee on Human Studies (CHS#5-05). Upon receipt, islets were cultured overnight in Miami Media #1A (Cellgro). The islets were then starved in Final Wash/Culture Media (Cellgro) for 3 hr before being stimulated with Miami Media #1A supplemented with sivelestat or GW311616A. 24 hr later, islets were embedded in agarose and used for immunostaining studies (described below).

**Immunostaining Studies**

Pancreases and in vitro stimulated islets were analyzed by immunostaining using anti-Ki67 (BD), anti-phospho-histone H3 (pHH3) (Millipore), anti-BrdU (Dako), anti-insulin (Abcam), or anti-glucagon (Sigma-Aldrich) antibodies. Quantification of replicating β and α cells and calculation of β cell mass were performed as described previously (El Ouaamari et al., 2013).

**Phosphoproteomics Analysis**

Phosphoproteomics analyses were performed as described in the Supplemental Experimental Procedures. To validate the phosphoproteomics findings, frozen SerpinB1-treated and ovalbumin-treated islets were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris [pH 7.2], 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) containing 200 μM orthovanadate, protease, and phosphatase inhibitors (Sigma-Aldrich) (Lew et al., 2013) and subjected to western blot analyses. pMAPK3, total MAPK3, and total GSK3 antibodies are from Cell Signaling, pPRKAR2B and total PRKAR2B are from Santa Cruz. pGSK3 antibody is from Millipore.

**RT-PCR**

Total RNA was extracted and reverse transcribed, and qPCR was performed as outlined in the Supplemental Experimental Procedures.

**Statistical Analysis**

All data are presented as mean ± SEM. Data were analyzed using unpaired, two-tailed Student’s t test, ANOVA, or multivariate analyses as appropriate, and a p value < 0.05 was considered statistically significant.

**ACCESSION NUMBERS**

The accession number for the mass spectrometry phosphoproteomics dataset reported in this paper is ProteomeXchange: PXD003182.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.12.001.

**AUTHOR CONTRIBUTIONS**

A.E.O. and R.N.K. conceived of the idea, designed experiments, analyzed the data, and wrote/editied the manuscript. Individual experimental contributions are as follows: E.R.-O. and W.-J.Q. contributed equally to design of experiments, providing reagents, and writing/editing the manuscript; J.-Y.Z., M.A.G., R.D.S., and W.-J.Q. were responsible for proteomics and phosphoproteomics experiments; E.D. contributed to islet isolation and transplantation studies; N.G., J.H., J.B., J.S., D.F.D.J., S.K., S.B., G.Q., and C.W.L. provided technical assistance; H.-D.B. conducted serpinB1 secretion studies in keratinocytes; R.M. assisted with mouse experiments; L.H., J.G., and Y.G. conducted mouse experiments and ELISA and biochemical assays; A.B.G. provided...
human samples and contributed to analysis of human ELISA assays; A.D. and P.J. provided data on the serpinB1 variant; C.K. and O.A. conducted zebrafish studies. All authors read and approved the manuscript.

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