Identification of the matricellular protein Fibulin-5 as a target molecule of glucokinase-mediated calcineurin/NFAT signaling in pancreatic islets

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Glucokinase-mediated glucose signaling induces insulin secretion, proliferation, and apoptosis in pancreatic β-cells. However, the precise molecular mechanisms underlying these processes are not clearly understood. Here, we demonstrated that glucokinase activation using a glucokinase activator (GKA) significantly upregulated the expression of Fibulin-5 (Fbln5), a matricellular protein involved in matrix-cell signaling, in isolated mouse islets. The islet Fbln5 expression was induced by ambient glucose in a time- and dose-dependent manner and further enhanced by high-fat diet or the deletion of insulin receptor substrate 2 (IRS-2), whereas the GKA-induced increase in Fbln5 expression was diminished in Irs-2-deficient islets. GKA-induced Fbln5 upregulation in the islets was blunted by a glucokinase inhibitor, KATP channel opener, Ca2+ channel blocker and calcineurin inhibitor, while it was augmented by harmine, a dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) 1A inhibitor. Although deletion of Fbln5 in mice had no significant effects on the glucose tolerance or β-cell functions, adenovirus-mediated Fbln5 overexpression increased glucose-stimulated insulin secretion in INS-1 rat insulinoma cells. Since the islet Fbln5 expression is regulated through a glucokinase/KATP channel/calcineurin/nuclear factor of activated T cells (NFAT) pathway crucial for the maintenance of β-cell functions, further investigation of Fbln5 functions in the islets is warranted.

Glucose metabolism plays an important role in normal β-cell functions such as insulin production and insulin secretion, and also in β-cell growth and survival1-5. Glucose signaling in the pancreatic β-cells has also been shown to be involved in β-cell proliferation in both humans and rodents3-6. Glucokinase, a member of the hexokinase family, is the predominant enzyme catalyzing the phosphorylation of glucose in the pancreatic β-cells and the liver. Glucokinase acts as a glucose sensor for insulin secretion from the pancreatic β-cells7 and is required for the effects of glucose signaling on β-cell proliferation8. Heterozygous inactivating mutations of glucokinase cause type 2 maturity onset diabetes of the young (MODY2), and homozygous or compound heterozygous inactivating glucokinase mutations cause a more severe phenotype known as permanent neonatal diabetes mellitus (PNDM), which manifests at birth9. On the other hand, heterozygous activating glucokinase mutations cause persistent hyperinsulinemic hypoglycemia (PHHI)10, associated with increased β-cell mass and β-cell proliferation11. We have shown previously that glucokinase activation ameliorates endoplasmic reticulum (ER) stress-mediated apoptosis of the pancreatic β-cells12, while another report revealed that genetic activation of β-cell glucokinase causes cell apoptosis associated with DNA double-strand breaks and activation of the tumor suppressor protein p5313. Thus, glucokinase appears to play important roles in β-cell function, replication, and survival. These findings inspired the development of a therapeutic strategy for diabetes by targeting glucokinase. Glucokinase activators (GKAs) increase the glucose affinity and maximum velocity (Vmax) of glucokinase, leading to enhanced

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Fibulin-5 (Fbln5; also known as EVEC or DANCE), a matricellular protein, is essential for elastic fiber assembly. Fbln5 is secreted by various cell types, including vascular smooth muscle cells (SMCs), fibroblasts and endothelial cells. Fbln5 expression is usually downregulated after birth, but reactivated upon tissue injury. Fbln5 has several non-elastogenic functions, for example, regulation of proteases via its integrin-binding domain. Fbln5 has also been shown to bind to the α5β1 fibronectin receptor and the β1 integrin. Indeed, Fbln5 plays critical roles in cell proliferation, migration and invasion of certain tumors and smooth muscle cells. Mice lacking in Fbln5 exhibit systemic elastic fiber defects, including loose skin, tortuous aorta, emphysematous lungs, and genital prolapse. However, the precise nature of the involvement of Fbln5 in metabolism remains unknown.

In this study, we found that treatment with a GKA induced Fbln5 gene expression in mouse pancreatic islets. Although it has been reported that interaction of the islets with some specific extracellular matrix molecules is important for islet/β-cell survival, the precise expression levels and roles of these molecules in the pancreatic islets and β-cell functions remain obscure. In this study, we focused on the regulation of Fbln5 expression in the pancreatic β-cells.

**Results**

**Glucokinase activation induced Fbln5 expression in the pancreatic islets.** At first, we identified by gene expression microarray analysis (GSE41248), that stimulation of mouse pancreatic islets with a GKA for 24 hours induced Fbln5 expression in the islets (12.6-fold enhanced expression as compared to that in the vehicle control, p = 0.0043). To validate this upregulation of Fbln5 expression by treatment with a GKA in mouse pancreatic islets, we investigated Fbln5 mRNA expression in isolated islets from C57BL/6J mice. Consistent with the results of the microarray analysis, the Fbln5 mRNA expression in the isolated islets was significantly increased, in a time-dependent manner, by treatment with a GKA (Fig. 1a). Ambient glucose also induced Fbln5 expression in the islets in a concentration-dependent manner (Fig. 1b).

We detected FBLN5 protein expression in the wild-type mouse islets, as well as in INS-1 rat insulinoma cell line (Fig. 1c and d) but not in the Fbln5−/− islets (Fig. 1c). The treatment with a GKA also increased FBLN5 protein expression levels in INS-1 cells (Fig. 1d).

Moreover, in glucokinase hetero-deficient (Gck−/−) mouse islets, GKA-stimulated Fbln5 mRNA expression levels were reduced as compared to those in the islets from wild-type mice (Fig. 1e). No difference was detected in Fbln5 mRNA expression levels between vehicle-treated Gck+/− islets and the wild-type islets (p = 0.357) (Fig. 1e).

These results suggest that Fbln5 expression is induced by glucokinase activation in the pancreatic islets. Furthermore, the GKA-induced increase in Fbln5 expression was more pronounced in the islets of mice reared on a high-fat diet for 20 weeks than in the islets of standard chow-fed mice (Fig. 1f), although there were no significant differences between the vehicle-treated islets from standard chow-fed and high-fat diet-fed mice (p = 0.24), consistent with the report that glucokinase-mediated signaling in the β-cells is activated by a high-fat diet. In contrast, in insulin receptor substrate 2 (IRS-2)-deficient (Irs-2−/−) mouse islets, basal Fbln5 expression was significantly increased compared with those of wild-type mice (Fig. 1g). However, the response of Fbln5 induction to GKA was almost abolished in Irs-2−/− mouse islets (Fig. 1g). It may also explain the more pronounced upregulation of islet Fbln5 expression in high-fat diet-fed mice than in normal chow-fed mice, as GKA is known to induce IRS-2 expression in the β-cells of mice reared on a high-fat diet. The lack of Fbln5 induction in Irs-2−/− islets suggests that IRS-2 is involved in the GKA-induced upregulation of Fbln5 expression. Moreover, we found that Fbln5 was strongly expressed in the islets of 2-week-old pre-weaning mice, the expression level decreasing by 6 or 12 weeks of age (Fig. 1h). This expression pattern of Fbln5 is consistent with the expression of the proliferation marker Ki67 in the islets (Fig. 1i).

**Glucokinase/K_{ATP} channel/calcineurin/NFAT signaling is required for glucose-mediated Fbln5 expression in islets.** We next assessed the signaling pathways underlying the GKA-induced upregulation of Fbln5 in the pancreatic islets. Treatment with D-mannoheptulose, a specific inhibitor of glucokinase, completely abolished the GKA-induced upregulation of Fbln5 in the pancreatic islets (Fig. 2a). In addition, treatment with diazoxide, a K_{ATP} channel (ATP-sensitive potassium channel) opener, also suppressed the GKA-induced elevation of Fbln5 expression in the islets (Fig. 2b).

Treatment with OSI-906, a dual insulin and IGF-1 receptor inhibitor, did not reduce the Fbln5 induction by GKA, but enhanced it (Fig. 2c). These results imply that an influx of Ca^{2+} into the β-cells via depolarization of the plasma membrane accompanied by the closure of K_{ATP} channel, and not the autocrine action of insulin, is involved in the GKA-induced upregulation of Fbln5 in the pancreatic islets. Calcineurin is activated in an intracellular Ca^{2+}-dependent manner, leading to NFAT activation by dephosphorylation and subsequent translocation of NFAT from the cytosol to the nucleus. Glucose-induced regulation of Irs-2 expression has been reported to be mediated via this Ca^{2+}/calcineurin/NFAT signaling in the pancreatic β-cells. Hence, we evaluated the effects of a Ca^{2+} channel blocker, a calcineurin inhibitor, and a DYRK1A inhibitor on the upregulation of Fbln5 in the islets treated with a GKA. Blockade of the L-type voltage-dependent Ca^{2+} channels (L-type VDCCs) with nifedipine in isolated mouse islets abrogated the GKA-induced increase in Fbln5 expression in the islets (Fig. 2d).

Moreover, treatment with FK506, which specifically inhibits calcineurin activity, also almost completely abolished the GKA-induced increase in Fbln5 expression in the pancreatic islets (Fig. 2d). Dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs), including DYRK1A, inactivate the NFAT proteins by phosphorylating its SP-3 motif. Notably, harmine, a DYRK1A inhibitor, enhanced the Fbln5 expression induced by treatment with a GKA (Fig. 2e). The effect of harmine on the increment in Fbln5 expression in...
Figure 1. Glucokinase activation was associated with upregulation of Fbln5 gene expression in pancreatic islets. (a) Fbln5 mRNA expression levels in isolated islets from C57BL/6J mice incubated for 2, 6, 12 and 24 hours with 30 μmol/L of GKA CpdA or vehicle (DMSO) (n = 4). (b) Fbln5 mRNA expression levels in isolated islets from C57BL/6J mice (n = 4) after 24 hours’ incubation with 2.8, 5.6, 11.1 or 22.2 mmol/L of glucose. (c) Immunoblotting for FBLN5 in islets isolated from wild-type or Fbln5−/− mice. Full-length blots are presented in Supplementary Fig. 4. (d) Immunoblotting for FBLN5 in INS-1 cells treated with vehicle, 22.2 mmol/L of glucose, or 30 μmol/L of GKA CpdA for 24 hours (n = 4). Full-length blots are presented in Supplementary Fig. 4. (e) Fbln5 mRNA expression levels in islets from 12-week-old Gck+/− mice and their wild-type littermates after incubation with 30 μmol/L of GKA CpdA for 24 hours (n = 4). (f) Fbln5 gene expression levels in islets isolated from 12-week old standard chow-fed mice (SC) and 20-week of high-fat diet (HFD)-fed mice incubated with or without 30 μmol/L of GKA CpdA (vehicle; DMSO) for 24 hours (n = 4). (g) Fbln5 mRNA expression levels in islets from 12-week-old Irs-2+/− mice and their wild-type littermates after incubation with 30 μmol/L of GKA CpdA for 24 hours (n = 4). (h–i) mRNA expression levels of Fbln5 (h) or Ki67 (i) in isolated islets from 2-, 6- and 12-week-old C57BL/6J mice (n = 3–4). Data are represented as means ± SEM. *p < 0.05, **p < 0.01.
islets was blunted in the presence of FK506 (Fig. 2f). These results suggest that the transcriptional regulation of Fbln5 in the islets is mediated by glucose signaling and downstream Ca2+/calcineurin/NFAT signaling.

Fbln5−/− mice exhibited normal glucose tolerance and normal glucose-stimulated insulin secretion and β-cell proliferation evoked by GKA. To investigate the role of Fbln5 in glucose metabolism and insulin secretion, we used 8- to 12-week-old Fbln5 knockout (Fbln5−/−) mice16 to evaluate whether Fbln5 deletion may influence glucose homeostasis in vivo. Fbln5−/− mice showed normal glucose tolerance and comparable insulin secretion during an oral glucose tolerance test (Fig. 3a and b). No significant difference in glucose-stimulated insulin secretion was observed between islets isolated from Fbln5−/− mice and wild-type mice (Fig. 3c). These results imply that Fbln5 has no effect on insulin secretion in healthy young adult mice.

We next assessed the β-cell mass in the 8- to 12-week-old Fbln5−/− mice. No significant differences in the islet morphology and the β-cells area relative to the total pancreatic area were observed between the wild-type mice and Fbln5−/− mice (Fig. 3d and e). Furthermore, we evaluated the GKA-induced β-cell proliferation activity in these mice.
in \( Fbln5^{-/-} \) and wild-type islets. Treatment with GKA for 48 hours markedly increased the EdU-incorporated proliferating insulin-positive \( \beta \)-cells to a similar extent in the islets isolated from both genotypes of mice (Fig. 3f and g). On the other hand, the fluorescent intensity of insulin was significantly increased in the GKA-treated islets compared with the vehicle-treated islets in wild-type mice, but not in \( Fbln5^{-/-} \) mice (see Supplementary Fig. S1a). This result in islets from wild-type mice is consistent with the observation that glucokinase activation enhances insulin gene expression and insulin secretion in \( \beta \)-cells\(^{12,34} \). However, GKA-induced insulin secretion was not decreased in \( Fbln5^{-/-} \) islets compared with wild-type islets (see Supplementary Fig. S1b). Insulin content
Adenovirus-mediated Fbln5 overexpression increased glucose-stimulated insulin secretion and inhibited cell proliferation in INS-1 cells. Next, we evaluated the properties of Fbln5 by forced expression of Fbln5 gene expression using an adenovirus vector (Ad-Fbln5) in INS-1 cells. Following adenovirus-mediated infection of Ad-Fbln5 in INS-1 cells, overexpression of Fbln5 was confirmed by measuring the mRNA and protein expression levels (Fig. 4a and b). The cells overexpressing Fbln5 showed enhanced insulin secretion in the presence of 11.1 mmol/L glucose as compared to the control cells (1.6-fold, \( p = 0.017 \)), although basal insulin secretion was not significantly different between the Ad-Fbln5- and Ad-GFP- infected INS-1 cells (Fig. 4c). The effects of Fbln5 overexpression on the cell proliferation activity was evaluated by measuring the EdU incorporation and Ki67 expression in Ad-Fbln5-infected INS-1 cells. We found that Ad-Fbln5 almost able to ignore the GFP-signal when we adjusted the gain of signals according to the fluorescent intensity of EdU (Fig. 4d left panel). The ratio of EdU-incorporated proliferating INS-1 cells to the total count of INS-1 cells tended to be decreased in the Ad-Fbln5-infected cells as compared with that in the control cells (Fig. 4d). In addition, Ad-Fbln5-infected INS-1 cells showed significant reduction in the Ki67 expression (Fig. 4e). These results indicate that overexpression of Fbln5 enhances insulin secretion whereas decreases cell proliferation in β-cells.

Discussion

In this study, we showed the glucose-signaling-induced transcriptional regulation of Fbln5 in pancreatic islets, which is mediated by glucose metabolism via glucokinase and downstream Ca\(^{2+}\)/calcineurin/NFAT signaling pathway (Fig. 5).

Because β-cells are exposed to high ambient glucose concentrations under the diabetic condition, glucokinase, which acts as a glucose sensor, transmits the impact of the hyperglycemia to the β-cells. In the pancreatic islets, glucokinase is mainly expressed in the β-cells, with very low levels of expression observed in the α-cells (unpublished data). We confirmed GKA-induced increase in FBLN5 expression in INS-1 cells. Immunohistochemical analysis of INS-1 cells also supported that FBLN5 is expressed in the β-cells. However, further investigation using more specific antibody is needed to clarify the localization of FBLN5 since we observed FBLN5 signal not only in cytoplasm but also in the nucleus in INS-1 cells. FBLN5 immunofluorescence from paraffin-embedded tissue was mainly detected in non-β-cells tissue in the islets. FBLN5 is reported to be deposited on microfibrils during the development of mature elastic fiber. Co-staining FBLN5 with CD34 in islets from paraffin-embedded specimens indicated that FBLN5 is strongly expressed in endothelial cells or small vessels in islets, consistent with the previous reports, which showed FBLN5 secretion from vascular smooth muscle cells or endothelial cells. FBLN5 was also detectable in β-cells in the non-paraffinized cultured islets. It is therefore possible that FBLN5 in β-cells was lost or masked in the process of paraffin embedding or deparaffinization. Since dual inhibition of insulin and IGF-1 receptor with OSI-906 did not abrogate GKA-induced Fbln5 upregulation in the islets, it is unlikely that this upregulation is mediated by an autocrine action of insulin on the insulin receptor. Because FBLN5 is a secreted protein, islet-derived FBLN5 might be deposited outside of the islets and play a role in non-islet tissue functions.

Our data showed that GKA-induced upregulation of Fbln5 was more pronounced in islets isolated from obese mice reared on a high-fat diet than in the islets of control mice fed normal chow. This could be explained by the involvement of glucokinase in the compensatory β-cell hyperplasia induced by a high-fat diet. Consistent with this notion, we found that Irs-2 deletion increased basal Fbln5 expression and attenuated the GKA-induced upregulation of Fbln5 in the isolated islets. Chronic hyperglycemia in Irs-2-/- mouse may cause the elevation in Fbln5 in the islets at the basal state. Other factors that are related to insulin resistance with Irs-2 deletion in mice can possibly be involved in this basal elevation. Glucose-induced transcriptional regulation of Irs-2 gene expression in the β-cells is mediated by the Ca\(^{2+}\)/calcineurin/NFAT pathway, which is involved in β-cell proliferation in mice and humans. In addition, the Dyrk1A inhibitor has been demonstrated to enhance β-cell proliferation in mice. It is also reported that GKA-induced increase of the mRNA expressions of Nfatc1 and its downstream genes are involved in β-cell maturation and β-cell proliferation in neonatal islets. A recent study showed that glucose-induced mouse pancreatic β-cell proliferation is mediated via IRS-2, MTOR, and cyclin D2, but not by the insulin receptor. We also found that the Fbln5 expression was higher in the islets harvested from pre-weaning mice, which showed robust β-cell proliferation as confirmed by the high Ki67 expression. Fbln5 is strongly expressed during embryogenesis and plays a role in tissue remodeling. Therefore, Fbln5 could be a predictor for compensatory β-cell proliferation and remodeling of β-cell mass induced by activation of IRS-2 expression.

How does NFAT signaling regulate the transcriptional activity of Fbln5? Fbln5 expression is positively regulated via transforming growth factor-β (TGF-β) in fibroblasts or epithelial cells. Calcineurin inhibitors induce the TGF-β receptor-triggered signaling cascade in the mesangial cells or kidney. Hypoxia-inducible factor-1α (HIF-1α) can stabilize and accumulate in β-cells under hypoxic conditions, which is involved in β-cell proliferation and adaptive β-cell hypertrophy. HIF-1α has been shown to bind with NFAT4 and repress NFAT4 activity, which suggests that HIF-1α might regulate Fbln5 expression through NFAT4. Considering the results of our study, Fbln5 might also be regulated by HIF-1α in β-cells under hypoxic conditions.
(HIF-1α) is also a Fbln5-inducible factor in the endothelial cells. HIF-1α expression is also reportedly regulated through calcineurin activity or dephosphorylation of RACK1 in mast cells. We have identified NFAT consensus sequences in the 5′-upstream region of the mouse Fbln5 gene at: −698 to −693 (AGGAAA), +386 to +391 (TGGAAA), +428 to +433 (TGGAAA), +591 to +596 (TGGAAA), and 4 other sites from the first transcription initiation site. Further analysis, including of the TGF-β and HIF-1α pathways, are needed to clarify the precise mechanism of Fbln5 transcription via NFAT in pancreatic islets.

Loss of systemic Fbln5 expression had no significant effects on the insulin secretion from the pancreatic islets or β-cell proliferation/expansion in young adult mice, suggesting that Fbln5 does not seem to be involved in β-cell function.
development or functions at this stage of life. However, the effects of Fbln5 on pancreatic β-cell functions under diabetic- or insulin-resistant conditions remain unclear. In addition, we showed that Fbln5 expression is abundant in the islets from fetal or pre-weaning mice. Testing juvenile mice, therefore, is required for further investigation into the physiological role of Fbln5 in the context of developmental process. Fbln5 overexpression in INS-1 cells revealed that Fbln5 could positively regulate glucose-stimulated insulin secretion from the pancreatic β-cells. By contrast, Fbln5 overexpression possibly suppress cell proliferation in the INS-1 cells. In fact, Fbln5 overexpression decreased Ki67 expression in INS-1 cells, although Fbln5 and Ki67 expression were increased in proliferating juvenile islets. Fbln5 is reported to promote cell proliferation or tumor growth in mouse 3T3-L1 fibroblasts or human HT1080 fibrosarcoma cells, mouse pancreatic ductal adenocarcinoma, and human gastric cancer MGC-803 cells. On the other hand, several previous studies have demonstrated inhibition of cell proliferation by Fbln5 overexpression in mouse vascular smooth muscle cells, human breast cancer cells, mink lu Mv1Lu epithelial cells, primary human saphenous vein endothelial cells, and rat retinal pigment epithelial cells. Thus, further investigation of the pathway that mediate Fbln5 action on β-cell proliferation is required. These effects of Fbln5 on β-cell functions and β-cell proliferation might be explained by the distinct proliferative and functional state of the β-cells. A previous study showed that a high rate of insulin production suppressed β-cell proliferation because of increased ER stress, in a cell-autonomous manner. On the other hand, genes involved in β-cell functions were suppressed when proliferation-related genes were upregulated in replicating β-cells.

There is a report in the literature which suggests that another matricellular protein, SPARC, which is expressed in stromal cells within the islets, can regulate β-cell growth and survival by inhibiting growth factor responses. Thus, the interactions between Fbln5 and pancreatic β-cell functions, which are still poorly understood, may represent novel molecular mechanisms involved in glucose metabolism and provide new insights for the treatment in diabetes.

Figure 5. A schematic representation of glucokinase-mediated Fbln5 expression in pancreatic islets. Fbln5 gene expression was induced by glucokinase activation through ambient high glucose concentrations or GKA in pancreatic islets. Depolarization of the membrane accompanied by the closure of K<sub>ATP</sub> channels, Ca<sup>2+</sup> influx and calcineurin activation are required for this Fbln5 upregulation. A DYRK1A inhibitor, harmine, enhanced Fbln5 expression in the islets induced by glucokinase activation, possibly via NFAT signaling. DYRK1A; dual-specificity tyrosine phosphorylation-regulated kinase 1 A, GKA; glucokinase activator, K<sub>ATP</sub> channel; ATP-sensitive potassium channel, NFAT; nuclear factor of activated T cells, VDCC; voltage-dependent Ca<sup>2+</sup> channels.
In summary, we demonstrated that expression of the matricellular protein Fbln5 is upregulated by high ambient glucose concentrations in the pancreatic islets through glucokinase-dependent glucose and downstream Ca\(^{2+}\)/calcineurin/NFAT signaling. Further study of the regulation of islet Fbln5 expression is warranted, especially in relation to glucose signaling and proliferation of β-cells.

**Methods**

**Animals and Animal Care.** All the animal procedures were performed in accordance with the guidelines of the Animal Care Committee of Yokohama City University. The protocol was approved by the Yokohama City University Institutional Animal Care and Use Committee (IACUC) ( Permit Number: F-A-16-026). C57BL/6J mice were purchased from Jackson. We backcrossed Fbln5 knockout (Fbln5\(^{-/-}\)) mice with C57BL/6J mice more than 10 times. Both Fbln5\(^{-/-}\) mice and wild-type littermates were fed a standard chow (MF, Oriental Yeast, Tokyo, Japan) or a high-fat diet (Clea Japan, Tokyo, Japan). All the experiments were conducted on male littermates. Animal housing rooms were maintained at a constant room temperature (25°C) and a 12-hour light (7:00 a.m.)/dark (7:00 p.m.) cycle.

**Adenovirus.** Fbln5-overexpressing recombinant adenovirus (Ad-Fbln5) and GFP-expressing control adenovirus (Ad-GFP) were used for the experiments at a multiplicity of infection of 50 viruses per cell. In brief, the FLAG-tagged full-length rat Fbln5 was inserted in an adenoviral vector (pACCMVpLpA(-) loxP-SSP). Viruses were generated by transfection into the Human Embryo Kidney 293 (HEK293) cell line.

**Islet isolation and culture.** Isolation of islets from mice was conducted using collagenase, as described in a previous report. The isolated islets were cultured in RPMI 1640 medium (Wako Pure Chemical Industries) containing 5.6 mmol/L glucose supplemented with 10% FCS, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. The islets were treated with 30 μmol/L of GKA Cpd A, 50 μmol/L of nifedipine, 10 μmol/L of FK506, 10 μmol/L of D-mannohexulose (Toronto Research Chemicals), 200 μmol/L of diazoxide (Wako Pure Chemical Industries), 200 nmol/mL of OSI-906 (Selleck chemicals). All the reagents were added concomitantly to the medium in each experiment.

**Oral glucose tolerance test.** All the mice were denied access to food for 14–16 hours before the oral glucose tolerance test (OGTT) and then orally loaded with glucose at 1.5 mg/g body weight. Blood glucose levels and serum insulin levels were determined using Glustest Neo Super (Sanwa Chemical Co. Kanagawa, Japan) and an insulin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan), respectively.

**Glucose-stimulated insulin secretion in isolated islets and INS-1 cells.** Ten islets isolated from Fbln5\(^{-/-}\) mice and wild-type mice were incubated at 37°C for 1.5 hours in Krebs-Ringer bicarbonate buffer containing 2.8, 11.1 or 22.2 mmol/L of glucose. When examining the effect of Fbln5 deficiency on GKA-induced insulin secretion, islets were incubated at 37°C for 1.5 hours in Krebs-Ringer bicarbonate buffer containing 2.8 mmol/L glucose with or without 30 μmol/L of GKA Cpd A, or 11.1 mmol/L glucose without GKA Cpd A. For measuring insulin content, islets were extracted with acid ethanol. INS-1 cells were infected with adenovirus (Ad-GFP or Ad-Fbln5) and cultured for 48 hours. Subsequently, the cells were incubated at 37°C for 2 hours in Krebs-Ringer bicarbonate buffer containing 2.8, 11.1 or 22.2 mmol/L of glucose. Then, the insulin concentration in the assay buffer and insulin content was measured with an insulin ELISA kit.

**Cell culture.** INS-1 (832/13) cells were cultured in RPMI 1640 containing 10 mmol/L of HEPES, 11.1 mmol/L of glucose, 10% FBS, 1 mmol/L of sodium pyruvate, 2 mmol/L of L-glutamine, 50 μmol/L of 2-mercaptoethanol, 100 units/mL of penicillin and 100 μg/mL of streptomycin. The cells were maintained at 37°C in humidified air containing 5% CO\(_2\). Before the experiments, the INS-1 cells were starved by incubation in RPMI1640 medium containing 2.8 mmol/L of glucose, 100 units/mL of penicillin, and 100 μg/mL of streptomycin for 16 hours.

**Histological analysis.** Pancreatic tissue sections from embryonic day15 and 8-week-old Fbln5\(^{-/-}\) mice and wild-type mice were analyzed after formalin fixation and paraffin embedding. For non-paraffinized tissue staining, isolated islets from 8-week-old wild-type mice attached to 0.1%-gelatin-coated coverslips (Falcon) were analyzed after fixation with paraformaldehyde. Pancreatic islets isolated from 8-week-old wild-type mice were analyzed after fixation without paraformaldehyde. The sections or attached islets on coverslips were immunostained with antibody directed against insulin (Santa Cruz Biotechnology), glucagon (Abcam), somatostatin (GeneTex), CD34 (Santa Cruz Biotechnology), or rabbit polyclonal anti-fibulin-5 (BSYN 1923; 1:100). FBLN5 signal was enhanced by tyramide signal amplification, using a TSA Fluorescein System (Perkin Elmer, NEL741001KT), in paraffin-embedding sections. Biotinylated secondary antibodies, a VECTASTAIN Elite ABC Kit, and a DAB Substrate Kit (Vector Laboratories) were used to examine the sections using bright-field microscopy to determine the β-cell mass, and Alexa Fluor 488-, 555- and 647-conjugated secondary antibodies (Invitrogen) were used for the fluorescence microscopy. Images were acquired using a BZ-9000 microscope (Keyence) or the FluoView FV1000-D confocal laser scanning microscope (Olympus). The proportion of the area of the pancreatic tissue occupied by β-cells was calculated using BIOREVO software (Keyence), as described previously. The fluorescence levels of insulin in GKA-treated wild-type and Fbln5\(^{-/-}\) islets were determined using ImageJ software. All images, which were acquired under the same condition, were converted to gray scale. Then, we randomly selected 5 regions of separate islets in each group and measured fluorescence levels. The fluorescent intensity was normalized by the mean background fluorescence levels.
Proliferation Assay. Isolated islets from Fbln5−/− mice and wild-type mice were incubated with a modified thymidine analog. EdU (5-ethyl-2'-deoxyuridine; Click-iT EdU, Invitrogen Cat. No. C10637) in the presence or absence of 30 µmol/L of GKA. After the treatment for 48 hours, the islets were fixed and sections were prepared of the embedded islets in 1% agarose-gel. INS-1 cells were infected with an adeno-virus vector (Ad-GFP or Ad-Fbln5) and cultured for 48 hours. Then, the cells were incubated with 10 µM of EdU for 3 hours and fixed. EdU incorporation and detection were performed as described in the manufacturer’s protocol. The images were taken using the FluowView FV1000-D confocal laser scanning microscope. We counted EdU-positive proliferative cells after adjusting the gain of fluorescence. At that condition there were no significant changes in the fluorescent intensity between GFP-positive cells and GFP-negative cells.

Real-time PCR. Total RNA was isolated from the pancreatic islets using an RNase-free DNase and RNasey Kit (Qiagen, Valencia, CA). cDNA was prepared using High Capacity cDNA Reverse-Transcription Kits (Applied Biosystems). Quantitative PCR was performed by using TaqMan Gene Expression Assays (7900 Real-Time PCR System; Applied Biosystems) with the THUNDERBIRD qPCR Master Mix (TOYOBO). All the probes were purchased from Applied Biosystems (mouse β-Ki67, Mm00488601_m1, mouse β-actin, Mm00607939_s1, mouse Ki67, Mm01278617_m1, rat Fbln5, Rn00667969_m1, rat β-actin, Rn00667969_m1, rat Ki67; Rn01451446_m1). Data were normalized to the expression level of β-actin.

Immunoblotting. For immunoblotting, isolated mouse islets and INS-1 cells were lysed in ice-cold RIPA buffer with protease and phosphatase inhibitor cocktail. The islets and cell extracts were subjected to immunoblotting. The primary antibodies used were rabbit anti-FBLN5 (BSY1923) at the dilution of 1:100, or Anti-Fibulín-5 Antibody (Millipore) at the dilution of 1:5000, and β-actin (Sigma-Aldrich). Densitometry was performed using Image J software.

Statistical analysis. All the data are expressed as the means ± SEM, and were analyzed using the Student’s t test or ANOVA. Differences between two groups were analyzed by Student’s t test (Figs 1a, 3b-c and e, 4, Supplementary Fig. S1b-c). For comparisons among more than two groups, we used the one-way ANOVA followed by the Tukey HSD post hoc test (Figs 1b and d–i, 2a and d, 3g, Supplementary Fig. S1a). When the data had unequal variance, we used Welch’s one-way ANOVA followed by the Games-Howell post hoc test (Fig. 2b,c,e,f). Differences were considered significant if the p value was <0.05 (*) or <0.01 (**).
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22. Yue, W. et al. Fibrin-5 suppresses lung cancer invasion by inhibiting matrix metalloproteinase-7 expression. Cancer research 69, 6339–6346, doi:10.1158/0008-5472.can-09-0398 (2009).

23. Lomas, A. C. et al. Fibrin-5 binds human smooth-muscle cells through alphaBeta1 and alphaBeta1 integrins, but does not support receptor activation. Biochem J 405, 417–428, doi:10.1042/BJ20070400 (2007).

24. Tang, J. C., Xie, A. Y. & Cai, X. J. [Diverse functions of fibrin-5 in tumors]. Molekularnaia biologiia 48, 875–880 (2014).

25. Schiemann, W. P., Blobe, G. C., Kalume, D. E., Pandey, A. & Lodish, H. F. Context-specific effects of fibrin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion. Fibrin-5 is induced by transforming growth factor-beta and affects protein kinase cascades. J Biol Chem 277, 27367–27377, doi:10.1074/jbc.M201418200 (2002).

26. Hirai, M. et al. Fibrin-5/DANCE has an elastogenic organizer activity that is abrogated by proteolytic cleavage in vivo. J Cell Biol 176, 1061–1071, doi:10.1083/jcb.200611026 (2007).

27. Hammar, E. et al. Extracellular matrix proteins regulate pancreatic beta-cells against apoptosis: role of short- and long-term signaling pathways. Diabetes 53, 2034–2041, doi:10.2337/db04-0304 (2004).

28. Weber, L. M., Hayda, K. N. & Anseth, K. S. Cell-matrix interactions improve beta-cell survival and insulin secretion in three-dimensional culture. Tissue engineering. Part A 14, 1959–1968, doi:10.1089/ten.tea.2007.0238 (2008).

29. Takakomo, T. et al. Crucial role of insulin receptor substrate-2 in compensatory beta-cell hyperplasia in response to high-fat diet-induced insulin resistance. Diabetes, obesity & metabolism 10(Suppl 4), 147–156, doi:10.1111/j.1463-1326.2008.00951.x (2008).

30. Rusnak, F. & Mertz, P. Calcineurin: form and function. Physiological reviews 80, 1483–1521 (2000).

31. Hogan, P. G., Chen, L., Nardone, J. & Rao, A. Transcriptional regulation by calcium, calcineurin, and NFAT. Genes & development 17, 2205–2232, doi:10.1101/gad.1102703 (2003).

32. Demozay, D., Tsunekawa, S., Briaut, I., Shah, R. & Rhodes, C. J. Specific glucose-induced control of insulin receptor substrate-2 expression is mediated via Ca2+-dependent calcineurin/NFAT signaling in primary pancreatic islet beta-cells. Diabetes 60, 2892–2902, doi:10.2337/db11-0341 (2011).

33. Gwack, Y. et al. A genome-wide Drosophila RNAi screen identifies DYRK-family kinases as regulators of NFAT. Nature 441, 646–650, doi:10.1038/nature04631 (2006).

34. Lu, M. et al. Characterization of a novel glucokine activator in rat and mouse models. PLoS One 9, e88431, doi:10.1371/journal.pone.0088431 (2014).

35. Williamson, M. R., Shuttleworth, A., Canfield, A. E., Black, R. A. & Kiely, C. M. The role of endothelial cell attachment to elastic fibre molecules in the enhancement of monolayer formation and retention, and the inhibition of smooth muscle cell recruitment. Biomaterials 28, 5307–5318, doi:10.1016/j.biomaterials.2007.08.019 (2007).

36. Yanagisawa, H., Schlueterman, M. K. & Brekken, R. A. Fibrin-5, an integrin-binding matricellular protein: its function in development and disease. J Cell Commun Signal 3, 337–347, doi:10.1007/s12079-009-0066-3 (2009).

37. Heit, J. J. et al. Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. Nature 443, 345–349, doi:10.1038/ nature05097 (2006).

38. Goodyer, W. R. et al. Neonatal beta cell development in mice and humans is regulated by calcineurin/NFAT. Developmental cell 23, 21–34, doi:10.1016/j.devcel.2012.05.014 (2012).

39. Drrice, E. et al. Inhibition of DYRK1A Stimulates Human beta-Cell Proliferation. Diabetes 65, 1660–1671, doi:10.2337/db11-1127 (2016).

40. Wang, P. et al. A high-throughput chemical screen reveals that harmane-mediated inhibition of DYRK1A increases human pancreatic beta cell replication. Nat Med 21, 383–388, doi:10.1038/nmm.3820 (2015).

41. Stamateris, R. E. et al. Glucose Induces Mouse beta-Cell Proliferation via IRS2, MTOR, and Cyclin D2 but Not the Insulin Receptor. Diabetes 65, 981–995, doi:10.2337/db13-0529 (2016).

42. Aukol et, S. et al. Molecular mechanisms of TGF beta receptor-triggered signaling cascades rapidly induced by the calcineurin inhibitors cyclosporin A and FK506. Journal of immunology (Baltimore, Md.: 1950) 150 (1998), 2831–2845, doi:10.4049/jimmunol.150.6.2831 (1998).

43. Guadall, A. et al. Fibrin-5 is up-regulated by hypoxia in endothelial cells through a hypoxia-inducible factor-1 (HIF-1alpha)-dependent mechanism. J Biol Chem 286, 7093–7103, doi:10.1074/jbc.M110.162917 (2011).

44. Liu, Y. et al. Calcineurin promotes fibulin-5 expression in pancreatic islet cells in response to high fat and caloric intake. J Biol Chem 286, 7093–7103, doi:10.1074/jbc.M110.162917 (2011).

45. Preis, M. et al. Effects of fibulin-5 on attachment, adhesion, and proliferation of primary human endothelial cells. Biochem Biophys Res Commun 348, 1024–1033, doi:10.1016/j.bbrc.2006.07.156 (2006).

46. Li, F., Xu, H., Zeng, Y. & Yin, Z. Q. Overexpression of fibulin-5 in retinal pigment epithelial cells inhibits cell proliferation and migration and downregulates VEGF, CXC4R, and TGFβ1 expression in co-cultured choroidal endothelial cells. Current eye research 37, 540–548, doi:10.3109/02713683.2012.665861 (2012).

47. Sibhat, M. et al. Reduced Insulin Production Relieves Endoplasmic Reticulum Stress and Induces beta Cell Proliferation. Cell Metab 23, 179–193, doi:10.1016/j.cmet.2015.10.016 (2016).

48. Knochendoer, A. et al. The Genetic Program of Pancreatic beta-Cell Replication In Vivo. Diabetes 65, 2081–2093, doi:10.2337/db16-0003 (2016).

49. Ryall, C. L. et al. Novel role for matricellular proteins in the regulation of islet beta cell survival: the effect of the SPARC on survival, proliferation, and signaling. J Biol Chem 289, 30614–30624, doi:10.1074/jbc.M114.579380 (2014).

50. Shirakawa, J. et al. Protective effects of dipeptidyl peptidase-4 (DPP-4) inhibitor against increased beta cell apoptosis induced by dietary sucrose and linoleic acid in mice with diabetes. J Biol Chem 286, 25467–25476, doi:10.1074/jbc.M114.217216 (2011).

51. Hohmeier, H. E. et al. Isolation of INS-1-derived cell lines with robust APT-sensitive K+-channel-dependent and -independent glucose-stimulated insulin secretion. Diabetes 49, 424–430, doi:10.2337/diabetes.49.3.424 (2000).

52. Shirakawa, J. et al. Effects of liraglutide on beta-cell-specific glucokinase-deficient neonatal mice. Endocrinology 153, 3066–3075, doi:10.1210/en.2012-1165 (2012).

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Author Contributions
T.O., J.S., and Y.Tc designed the study. T.O. and J.S. performed the experiments, analyzed the data and wrote the manuscript. H.Y. provided us the Fbln5KO mice and contributed to the discussion. M.K., S.Y., K.T., and Y.To. assisted in the experiments. All authors gave final approval of the version to be published.

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