(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 August 2014 (28.08.2014)

(10) International Publication Number
WO 2014/128257 A1

(51) International Patent Classification:
A61K 38/57 (2006.01) *A61P 3/10* (2006.01)

(21) International Application Number:
PCT/EP2014/053419

(22) International Filing Date:
21 February 2014 (21.02.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

13305204.3	22 February 2013 (22.02.2013)	EP
61/767,948	22 February 2013 (22.02.2013)	US
13306284.4	20 September 2013 (20.09.2013)	EP
61/880,240	20 September 2013 (20.09.2013)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2014/128257 A1

(54) **Title**: SERPINS: METHODS OF THERAPEUTIC BETA-CELL REGENERATION AND FUNCTION

(57) **Abstract**: Compositions and methods of use are provided for improving β cell function and promoting pancreatic β cell proliferation *in vitro*, *in vivo* and *ex vivo*. The active agents of the pending invention comprise Serpin family peptides (e.g., SerpinB1), functional and structural analogs of Serpin family peptides and nucleic acids encoding Serpin family peptides, as well as active fragments thereof.

SERPINS: METHODS OF THERAPEUTIC BETA-CELL REGENERATION AND FUNCTION

This invention was made with Government support under grant number RO1 DK 607536 awarded by the National Institutes of Health. The Government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to compositions and methods of use for improving β cell function and promoting pancreatic β cell proliferation *in vitro*, *in vivo* and *ex vivo*. The active agents of the pending invention comprise Serpin family peptides (e.g., SerpinB1), functional and structural analogs of Serpin family peptides and nucleic acids encoding Serpin family peptides, as well as active fragments thereof.

BACKGROUND OF THE INVENTION

Diabetes has reached epidemic proportions in both developed and developing countries, and the cost of treating individuals with complications resulting from uncontrolled hyperglycemia is a major economic burden in the world. A promising but still unrealized goal of efforts to improve diabetes therapy is the identification of novel factors that promote pancreatic β cell (β cell) regeneration, with the long-term goal of increasing functional β cell mass in patients with either type 1 or type 2 diabetes. Reduced functional β cell mass is a central feature in both forms of the disease and in diabetes associated with obesity (Muoio, D.M., and Newgard, C.B. (2008). Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat. Rev. Mol. Cell Biol. 9, 193–205). While autoimmune destruction of β cells is the major cause of β cell loss in type 1 diabetes, a failure of β cells to compensate for ambient insulin resistance leads to uncontrolled hyperglycemia in type 2 diabetes.

Thus, what is needed are compositions and methods effective in promoting β cell proliferation, especially in subjects that are in need of improved β cell function.

SUMMARY OF THE INVENTION

Lending encouragement to therapeutic strategies aimed at enhancing β cell mass, decades of research indicate that β cells possess the capacity to compensate for both physiological (pregnancy) and pathological (obesity) insulin resistance (Ogilvie, R.F. (1933). The islands of langerhans in 19 cases of obesity. J. Pathol. Bacteriol. 37, 473–481; Van Assche, F.A., *et al.*, (1978). A morphological study of the endocrine pancreas in human pregnancy. Br. J. Obstet. Gynaecol. 85, 818–820). Although β cell growth in both humans and rodents has been documented to occur through self-duplication of preexisting β cells (Dor, Y., *et al.*, (2004). Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429, 41–46; Meier, J.J., *et al.*, (2008). Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 57, 1584–1594; Teta, M., *et al.*, (2007). Growth and regeneration of adult beta cells does not involve specialized progenitors. Dev. Cell 12, 817–826), albeit at low levels, the source of putative growth factor(s) mediating this process, especially in the context of insulin resistance, remains unknown. Among possible systemic regulators of β cell mass, gut-derived incretins such as glucagon-like peptide-1 (GLP-1), glucose-dependent insulin-tropic polypeptide (GIP) (Renner, S., *et al.*, (2010). Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. Diabetes 59, 1228–1238). Glucose intolerance and reduced proliferation of pancreatic beta-cells in transgenic pigs with impaired glucose-dependent insulinotropic polypeptide function. Diabetes 59, 1228–1238; Saxena, R., *et al.*; GIANT consortium; MAGIC investigators. (2010). Genetic variation in GIPR influences the glucose and insulin responses to an oral glucose challenge. Nat. Genet. 42, 142–148), adipocyte-derived adipokines including leptin (Morioka, T., *et al.*, (2007). Disruption of leptin receptor expression in the pancreas directly affects beta cell growth and function in mice. J. Clin. Invest. 117, 2860–2868) and adiponectin (Holland, W.L., *et al.*, (2011). Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. Nat. Med. 17, 55–63), muscle-derived myokines such as IL-6 (Ellingsgaard, H., *et al.*, (2008). Interleukin-6 regulates pancreatic alpha-cell mass expansion. Proc. Natl. Acad. Sci. USA 105, 13163–13168; Suzuki, T., *et al.*, (2011). Interleukin-6 enhances glucose-stimulated insulin secretion from pancreatic beta-cells: potential involvement of the PLC-IP3-dependent pathway. Diabetes 60, 537–547), macrophage-derived cytokines including IL-1b, IFNγ, and TNF-a (Wang, C., *et al.*, (2010). Cytokines in the Progression of Pancreatic b-Cell Dysfunction. Int. J. Endocrinol. 2010, 515136), bone derived osteocalcin (Ferron, M., *et al.*, (2008). Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. Proc. Natl. Acad. Sci. USA 105, 5266–5270), thyroid-derived T3/T4 hormones (Jörns, A., *et al.*, (2010). Beta cell mass regulation in the rat pancreas through glucocorticoids and thyroid hormones. Pancreas 39, 1167–1172; Verga Falzacappa, C., *et al.*, (2010). The thyroid hormone T3 improves function and survival of rat pancreatic islets during in vitro culture. Islets 2, 96–103), platelet-derived growth factor (PDGF) (Chen, H., *et al.*, (2011). PDGF signaling controls age-dependent proliferation in pancreatic b-cells. Nature 478, 349–355), serotonin (Kim, H., *et al.*, (2010). Serotonin regulates pancreatic beta cell mass during pregnancy. Nat. Med. 16, 804–808), and FGF21 (Wente, W., *et al.*, (2006). Fibroblast growth factor-21 improves pancreatic beta-cell function and survival by activation of extracellular signal-regulated kinase 1/2 and Akt signaling pathways. Diabetes 55, 2470–2478) have each been implicated. However, the lack of significant and consistent alterations in these known factors in the peripheral blood that can fully account for the β cell proliferation in the insulin-resistant LIRKO (Liver Insulin Receptor Knockout) mouse model (see, Table 1) prompted us to explore the presence of an as yet unidentified factor that is derived from an insulin-resistant liver.

Table 1: Assays of circulating growth factors, hormones, cytokines and chemokines in young vs. old Control and LIRKO mice

	Control (3Mo)	LIRKO (3Mo)	*p*	Control (12Mo)	LIRKO (12Mo)	*p*
Growth factors						
IGF1 (ng/mL)	211.9 ±18.9	68.9 ±16.6	0.0002	525.1 ±35.6	194.4 ±18.6	2.91E-06
HGF (ng/mL)	5.3 ±0.41	4 ±0.69	0.145	2.1 ±0.3	4.3 ±0.8	0.03
EGF (ng/mL)	54.75 ±17.33	79.8 ±23.73	0.4	11.7 ±3.1	14.5 ±6.1	0.7
PDGFAA (ng/mL)	0.142 ±0.05	0.163 ±0.05	0.88	3.4 ±0.4	3.6 ±0.4	0.7
PDGFBB (ng/mL)	0.073 ±0.02	0.17 ±0.07	0.22	9 ±1.5	9.8 ±1.2	0.6
VEGF (pg/mL)	2.1 ±0.2	1.3 ±0.1	0.006	1.8 ±0.3	2.1 ±0.4	0.5
FGF21 (pg/mL)	58.6 ±12.6	43.6 ±11.1	0.4	1203 ±224.6	143.2 ±29.3	0.001
Hormones						
Insulin (ng/mL)	2.3 ±0.7	11.6 ±2.4	0.01	8.2 ±1	17.8 ±4.4	0.06
Amylin (pg/mL)	103.6 ±40.9	206.8 ±51.1	0.1	358.4 ±38	900.8 ±309.6	0.1
Glucagon (pM)	25.8 ±3.4	20.9 ±4.8	0.4	13.1 ±3.9	11.5 ±3.8	0.8
Ghrelin (pg/mL)	3.5 ±0.5	2.5 ± 0.4	0.1	1.7 ±0	4.5 ±1.9	0.2
PP (pg/mL)	11.8 ±2.9	19.7 ±4	0.1	18 ±4.8	42.2 ±17.4	0.2
PYY (pg/mL)	63.1 ±12	74.8 ±13	0.5	145.9 ±38.3	86.7 ±7.6	0.2
GIP (pg/mL)	108.5 ±11.4	152.9 ±18.7	0.06	284.5 ±25	95.2 ±13.9	2.1E-05
Total GLP-1 (pg/mL)	32.1 ±5	43.6 ±11.6	0.4	59.4 ±11.4	65.9 ±17.9	0.7
Active GLP-1 (pg/mL)	23.8 ±7.4	22.5 ±6.3	0.9	25.5 ±7.6	27.7 ±9.8	0.8
Leptin (ng/mL)	12.3 ±4	8.9 ±1.7	0.4	42.9 ±6.2	27.7 ±1.7	0.04
Resistin (ng/mL)	2.4 ±0.2	2.7 ±0.2	0.3	1.3 ±0.1	1.5 ±0.1	0.2
Adiponectin (µg/mL)	10.9 ±1.4	17.5 ±2.6	0.04	21.2 ±2.3	18.8 ±1.3	0.4
Osteopontin (ng/mL)	146.2 ±8.1	157.1 ±24.6	0.7	161.3 ±16.9	232.1 ±20.5	0.01

Osteocalcin (ng/mL)	12.6 ±1.2	12.6 ±2.9	1	11.8 ±2.2	12.9 ±2.7	0.8
Gastrin (pg/mL)	39.4 ±2.3	43.1 ±4.6	0.5	45.7 ±6.2	40.9 ±5.9	0.6
Cytokines & Chemokines						
IL-1a (pg/mL)	12.4 ±2.7	14.3 ±6.4	0.8	9.4 ±3.6	21.2 ±8.4	0.2
IL-1b (pg/mL)	6.6 ±2.9	3.2 ±0.6	0.3	3.3 ±0.4	5.8 ±2.5	0.3
IL-2 (pg/mL)	1.4 ±0.1	1.2 ±0.05	0.1	1.3 ±0.3	1.5 ±0.2	0.6
IL-3 (pg/mL)	1.4 ±0.07	1.3 ±0.1	0.6	1.4 ±0	2.36 ±1	0.3
IL-4 (pg/mL)	0.7 ±0.04	0.9 ±0.2	0.4	0.8 ±0.35	0.5 ±0.04	0.4
IL-5 (pg/mL)	7.3 ±1.8	8.3 ±2.1	0.7	2.7 ±1.2	1.9 ±0.9	0.6
IL-6 (pg/mL)	3.4 ±1.4	2.8 ±1.6	0.7	27.8 ±3.4	19.4 ±2.3	0.06
IL-7 (pg/mL)	1.4 ±0.2	3.5 ±1.8	0.2	17.5 ±9.1	15 ±9.8	0.8
IL-9 (pg/mL)	13.8 ±4.2	9.1 ±2.9	0.4	3.5 ±1	7.9 ±4.3	0.3
IL-10 (pg/mL)	11.8 ±1.4	9.7 ±1.6	0.3	11.2 ±2.5	12.8 ±3.5	0.7
IL-12(P40) (pg/mL)	16.7 ±2.5	10.2 ±2.5	0.08	6.3 ±2.2	4.3 ±1	0.4
IL-12 (p70) (pg/mL)	10.5 ±2.6	9.3 ±2.9	0.7	10.2 ±7.3	6.3 ±2.1	0.6
IL-13 (pg/mL)	103.4 ±12.7	84.6 ±14.6	0.3	83.8 ±12.7	134.1 ±33.2	0.2
IL-15 (pg/mL)	7.7 ±2.2	12.5. ±7	0.5	35.7 ±18	48 ±29.4	0.7
IL-17 (pg/mL)	1.3 ±0.2	0.9 ±0.3	0.2	2.5 ±1	1.4 ±0.3	0.3
IFN-g (pg/mL)	2.9 ±0.6	2 ±0.2	0.2	3 ±2.1	1.1 ±0.2	0.4
TNF-a (pg/mL)	2.4 ±0.5	2 ±0.5	0.5	2.9 ±0.08	4.7 ±1.6	0.3
PAI-1 (ng/mL)	1.4 ±0.2	1.3 ±0.1	0.5	1.6 ±0.3	1.3 ±0.3	0.3
G-CSF (pg/mL)	217.6 ±40.7	120.6 ±25	0.06	243.8 ±51.9	169.1 ±41.5	0.3
GM-CSF (pg/mL)	12.7 ±3.4	11.2 ±4.3	0.8	ND	ND	ND
M-CSF (pg/mL)	10.3 ±3.4	4.4 ±1.2	0.1	5.2 ±3	5.3 ±2.5	1
KC (pg/mL)	84.1 ±24.7	91.8 ±11.8	0.8	47.2 ±10.3	37.4 ±8.7	0.5
IP-10 (pg/mL)	92.8 ±10.6	68.9 ±9.5	0.1	178.8 ±20.5	167.3 ±15	0.6
Eotaxin (pg/mL)	344.6 ±23.4	342.4 ±39.4	1	295.8 ±19.1	337.1 ±42	0.4

MCP-1 (pg/mL)	9.9 ±1.8	5.4 ±0.8	0.04	3.9 ±0.4	5.8 ±2.4	0.5
MIP-1a (pg/mL)	16.3 ±4	11 ±3.9	0.3	11.6 ±2.8	7.9 ±3.6	0.4
MIP-1b (pg/mL)	24.8 ±4.7	13.6 ±4	0.08	17.6 ±5.2	14.3 ± 5.1	0.6
MIP-2 (pg/mL)	6.07 ±1.5	6.07 ±1.5	0.9	ND	ND	ND
MIG (pg/mL)	131.3 ±18.6	129 ±18.7	0.9	156.3 ±28.3	108.5 ±17.1	0.2
RANTES (pg/mL)	13.9 ±1.7	10.3 ±3	0.3	5.5 ±1.4	11.9 ±2.1	0.03
LIX (ng/mL)	0.9 ±0.3	0.7 ±0.2	0.7	0.3 ±0.1	0.4 ±0.1	0.7

Integrative organ crosstalk regulates key aspects of energy homeostasis, and its dysregulation may underlie metabolic disorders such as obesity and diabetes. To test the hypothesis that crosstalk between the liver and pancreatic islets modulates β cell growth in response to insulin resistance, we used the liver-specific insulin receptor knockout (LIRKO) mouse, a unique model that exhibits dramatic islet hyperplasia. Using complementary *in vivo* parabiosis and transplantation assays, as well as *in vitro* islet culture approaches, we demonstrate that humoral, nonneural, non-cell-autonomous factor(s) induces β cell proliferation in LIRKO mice.

Furthermore, we have discovered that a hepatocyte-derived factor(s) stimulates mouse and human β cell proliferation in *ex vivo* assays, independent of ambient glucose and insulin levels. These data implicate the liver as a critical source of β cell growth factor(s) in insulin-resistant states.

Serine peptidase inhibitor B1 (SerpinB1) is a 42 kD protein known to regulate the activity of the neutrophil proteases, elastase, cathrpsin G, proteinase-3, chymase, chymotrypsin and kallikrein-3. Thus, the role of SerpinB1 is presumably assigned to cellular proteolysis. In humans, SerpinB1 is also known as Leukocyte Elastase Inihibitor (LEI) or Monocyte/Neutrophil Elastase Inhibitor (M/NEI) and is encoded by a single gene *Serpinb1.* Four murine homologs have been cloned, including *Serpinb1a*, *Serpinb1b, Serpinb1c and Serpinb1d* and *Serpinb1a* is the ortholog of human *Serpinb1* (Benarafa et al., 2002). In the present invention SerpinB1 was identified as a top candidate β-cell growth factor by Affymetrix analysis and Proteomics screening using serum, LCM and HCM samples from the LIRKO mouse. This finding is supported by data from our recent study pointing to a serum factor as a potential pro-proliferative candidate. Furthermore, we confirmed with ELISA assays that the circulating levels of SerpinB1 were upregulated in LIRKO serum. To directly test the effects of SerpinB1 in β-cell proliferation Sivelestat and GW311616A (SerpinB1 functional analogs; i.e., pharmacological mimickers of SerpinB1 activity) and Serpin B1 were used. A direct effect of Sivelestat, GW311616A and SerpinB1 was shown in the promotion of β-cell proliferation *in vivo* and *in vitro* in a dose-dependent manner.

Earlier observations were made regarding the possible “indirect” role of SerpinB1 in β-cell physiology. SerpinB1 mRNA level was demonstrated to be commonly increased in regenerating pancreas of mice administrated with Exendin-4 or subjected to partial pancreatectomy. These data suggested SerpinB1 activity correlated with β-cell proliferation induced by Exendin-4 and after partial removal of pancreas (see, De León, et al., Identification of transcriptional targets during pancreatic growth after partial pancreatectomy and exendin-4 treatment. *Physiol Genomics.* 2006, 24:133-143). The importance of SerpinB1 in β-cell biology was also described using a chip on chip approach. It was further reported that the β-cell transcription factor pdx-1 binds to the proximal promoter of SerpinB1 locus, suggesting a possible role for SerpinB1 in mediating pdx-1 effects in pancreatic β-cells (see, Sachdeva, et al., Pdx1 (MODY4) regulates pancreatic beta cell susceptibility to ER stress. *PNAS.* 2009, 106(45):19090-19095). However, these workers did not show the involvement of SerpinB1 in β cell proliferation and do not provide the suggestion or motivation for investigating this direction.

The invention relates to further embodiments, which are outlined as follows:

In one embodiment, the present invention relates to a Serpin peptide or active fragment, or an analog thereof for use as medicament.

In another embodiment, said Serpin peptide is SerpinB1.

In yet another embodiment, said Serpin analog is a known functional analog.

In yet another embodiment, said Serpin analog is a known structural analog.

In a further embodiment, the subject has diabetes.

In yet a further embodiment, the subject is at risk of developing diabetes.

In another aspect of the present invention, it relates to a Serpin peptide or active fragment, or an analog thereof for use in a method of improving the β cell function in a subject.

In another embodiment, said Serpin peptide is SerpinB1.

In yet another embodiment, said Serpin analog is a known functional analog.

In a further embodiment, said Serpin analog is a known structural analog.

In even a further embodiment, the subject has diabetes.

In yet even a further embodiment, the subject is at risk of developing diabetes.

In another aspect of the present invention, it relates to a Serpin peptide or active fragment, or an analog thereof for use in a method of promoting pancreatic β cell proliferation in a subject.

In another embodiment, said Serpin peptide is SerpinB1.

In yet another embodiment, said population of pancreatic β cells are *in vivo*.

In another embodiment, said population of pancreatic β cells are *in vitro*.

In a further embodiment, said Serpin analog is a known functional analog.

In yet a further embodiment, said Serpin analog is a known structural analog.

In yet a further embodiment, increased pancreatic β cell proliferation *in vivo* is indicated by detecting increased glycemic control in the subject.

In another aspect of the present invention, it relates to an expression construct encoding a Serpin peptide or active fragment, or analog thereof for use in a method of improving the β cell function in a subject.

In one embodiment, said encoded Serpin peptide is SerpinB1.

In another embodiment, said encoded Serpin peptide analog is a known functional analog.

In yet another embodiment, said encoded Serpin peptide analog is a known structural analog.

In a further embodiment, the subject has diabetes.

In yet a further embodiment, the subject is at risk of developing diabetes.

In yet a further embodiment it relates to said use of a Serpin peptide or active fragment, or an analog thereof for manufacturing of a medicament for improving the β cell function in a subject.

Another aspect of the present invention relates to the before mentioned use, wherein said Serpin peptide is SerpinB1.

Yet another aspect of the present invention relates to the before mentioned use, wherein said Serpin analog is a known functional analog.

Yet another aspect of the present invention relates to the before mentioned use, wherein said Serpin analog is a known structural analog.

Yet another aspect of the present invention relates to the before mentioned use, wherein the subject has diabetes.

Yet another aspect of the present invention relates to the before mentioned use, wherein the subject is at risk of developing diabetes.

Another aspect of the present invention relates to the use of a Serpin peptide or active fragment, or an analog thereof for manufacturing of a medicament for promoting pancreatic β cell proliferation in a subject.

In one embodiment, it relates to said use, wherein said Serpin peptide is SerpinB1.

In another embodiment, it relates to said use, wherein said population of pancreatic β cells are *in vivo*.

In yet another embodiment it relates to said use, wherein said population of pancreatic β cells are *in vitro*.

In a further embodiment, it relates to said use, wherein said Serpin analog is a known functional analog.

In a further embodiment, it relates to said use, wherein said Serpin analog is a known structural analog.

In a further embodiment, it relates to said use, wherein increased pancreatic β cell proliferation *in vivo* is indicated by detecting increased glycemic control in the subject.

Another aspect of the present invention relates to the use of an expression construct encoding a Serpin peptide or active fragment, or analog thereof for manufacturing of a medicament for improving the β cell function in a subject.

In one embodiment, it relates to said use, wherein said encoded Serpin peptide is SerpinB1.

In another embodiment, it relates to said use, wherein said encoded Serpin peptide analog is a known functional analog.

In yet another embodiment it relates to said use, wherein said encoded Serpin peptide analog is a known structural analog.

In yet another embodiment it relates to said use, wherein the subject has diabetes.

In yet another embodiment, it relates to said use, wherein the subject is at risk of developing diabetes.

Another aspect of the present invention relates to a Serpin peptide or active fragment, or analog thereof for use in a method of improving β cell function in a subject, said method comprising:

a) providing i) a subject in need of improved β cell function, and ii) a therapeutic amount of a Serpin peptide or active fragment, or an analog thereof, in a pharmacological carrier;

b) administering or causing to be administered to said subject the Serpin peptide or analog thereby improving β cell function in the subject.

In one embodiment, said Serpin peptide is SerpinB1.

In another embodiment, said Serpin analog is a known functional analog or a known structural analog.

In yet another embodiment, the subject has diabetes or is at risk of developing diabetes.

Another aspect of the present invention relates to a Serpin peptide or active fragment, or analog thereof for use in a method of promoting pancreatic β cell proliferation, said method comprising:

a) providing i) a population of pancreatic β cells, and ii) a Serpin peptide or active fragment thereof, or an analog thereof, in a pharmacological carrier;

b) contacting said population of pancreatic β cells with said Serpin peptide or analog thereby promoting pancreatic β cell proliferation.

In one embodiment, said Serpin peptide is SerpinB1.

In one embodiment, said population of pancreatic β cells are *in vivo*.

In another embodiment, said population of pancreatic β cells are *in vitro*.

In one embodiment, said Serpin analog is a known functional analog or a known structural analog.

In one embodiment, increased pancreatic β cell proliferation *in vivo* is indicated by detecting increased glycemic control in the subject.

Another aspect of the present invention relates to a Serpin peptide or active fragment, or analog thereof for use in a method of improving β cell function in a subject, said method comprising:

a) providing i) a subject in need of improved β cell function, and ii) an expression construct encoding a Serpin peptide or active fragment, or a peptide analog thereof, in a pharmacological carrier;

b) administering or causing to be administered to said subject the expression construct encoding a Serpin peptide or analog thereby improving β cell function in the subject.

In one embodiment, said encoded Serpin peptide is SerpinB1.

In one embodiment, said encoded Serpin peptide analog is a known functional analog or a known structural analog.

In one embodiment, the subject has diabetes or is at risk of developing diabetes.

Another aspect of the present invention relates to a Serpin peptide or active fragment thereof for use as a diagnostic marker of insulin resistance and/or β-cell proliferation.

In one embodiment, said Serpin peptide is SerpinB1.

Yet another aspect of the present invention relates to a method of diagnosing insulin resistance and/or increased β-cell proliferation in a subject, said method comprising:

a) determining the expression level of a Serpin peptide or active fragment thereof in a sample obtained from said subject; and
b) comparing the expression level determined in step a) with the expression level of the Serpin peptide or active fragment thereof in a control sample,

wherein an increased expression level of the Serpin peptide or active fragment thereof as compared to the expression level in the control sample indicates insulin resistance and/or increased β-cell proliferation.

In one embodiment, said Serpin peptide is SerpinB1.

In one embodiment, said expression level is determined on the mRNA or protein level.

In one embodiment, said sample is blood serum or blood plasma.

In one embodiment, said control sample is obtained from a healthy individual or a group of healthy individuals.

Another aspect of the present invention relates to a Serpin-specific binding agent or a Serpin-specific nucleic acid molecule for use in a method of diagnosing insulin resistance and/or increased β-cell proliferation in a subject.

In one embodiment, said Serpin is SerpinB1.

In one embodiment, said binding agent is an antibody or an antigen-binding fragment thereof.

In one embodiment, said nucleic acid molecule is a primer or probe.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows selective β cell proliferation in LIRKO mice. Three to 4-month-old LIRKO and control mice were intraperitoneally injected with BrdU (100 mg/kg body weight) 5 hr before animals were sacrificed, and tissues were dissected, fixed and stained as indicated. (A) Pancreatic sections immunostained for insulin/BrdU/DAPI, insulin/Ki67/DAPI, insulin/TUNEL, or glucagon/BrdU/DAPI as indicated. (B) β cell mass quantification. (C and D) Quantification of BrdU+ insulin+ and Ki67+ insulin+ cells: between 2,000 and 5,000 insulin+ cells per animal were counted in control versus LIRKO pancreases, respectively. (E) Quantification of TUNEL+ insulin+ cells: between 2,000 and 5,000 insulin+ cells/mouse were counted in control versus LIRKO pancreases, respectively. (F) Quantification of BrdU+ glucagon+ cells: between 2,000 and 5,000 insulin+ cells/mouse were counted in control versus LIRKO pancreases, respectively. (G) Quantification of nuclei BrdU+ in indicated tissues: 4,000–5,000 cells/mouse were counted in each of liver, kidney, spleen, and lung, and 1,500 cells/mouse were counted in each for visceral (Visc.) and subcutaneous (Sc.) adipose tissue and skeletal muscle (Sk). (H) Representative images of proliferating cells in tissue sections stained with BrdU. Data represent mean ± SEM. *p % 0.05 and ***p % 0.001 (n = 6 in each group). See also Figure 6 and Table 2.

Figure 2 shows circulating nonneuronal nonautonomous factors drive β cell replication in the LIRKO mouse. (A) Schematic of the parabiosis experiment. See also, Figures 7, 8, and 9. (B–E) Single and parabiont models were intraperitoneally injected with BrdU (100 mg/kg body weight) 5 hr before animals were sacrificed, and pancreases were dissected and immunostained for insulin, BrdU, and DAPI. (F) Quantification of BrdU+ insulin+ cells: three sections separated by 80 mm were analyzed, and between 2,000 and 10,000 cells were counted in each group (n = 5–6 in each parabionts group). (G) Schematic of the transplantation experiment. (H) Quantification of BrdU+ insulin+ cells in islet grafts as indicated: three to six islet graft sections were analyzed and counted between 2,000 and 10,000 cells in each group (n = 3–5 in each group). Data represent mean ± SEM. *p % 0.05.

Figure 3 shows LIRKO serum induces selective β cell replication *in vivo*. Five to 6-week-old mice were injected intraperitoneally twice daily with 150 ml serum derived from 6-month-old control or LIRKO mice on days 1, 3, and 5. BrdU was injected intraperitoneally (100 mg/kg body weight) on days 2, 4, and 6. Animals were sacrificed 5 hr before the last BrdU injection, and tissues were dissected for immunostaining studies. (A) Schematic of the experimental design. (B and C) Representative images and quantification of BrdU+ insulin+ cells: two sections separated by 80 mm were analyzed, and at least 4,000 insulin+ cells were counted for each animal. (D and E) Representative images and quantification of BrdU+ glucagon+ cells: 400–600 glucagon+ cells were counted in each animal. (F and G) Representative images and quantification of TUNEL+ insulin+ cells: at least 2,000 insulin+ cells were counted in each animal. (H) Representative images and quantification of nuclei BrdU+ in indicated tissues: for each animal, 4,000–5,000 cells were counted in sections from liver, kidney, spleen, and lung, and 1,500 cells were counted in sections from visceral (Visc.) and subcutaneous (Sc.) adipose tissue and skeletal muscle. Data represent mean ± SEM. *p % 0.05 (n = 3 in each group).

Figure 4 shows LIRKO serum increases mouse and human Islet β cell replication *in vitro*. Five to 6-week-old mouse islets were stimulated with control or LIRKO serum for 48 hr. Islets were embedded in agarose and used for immunostaining studies. Culture media were assayed for glucose and insulin. WT: wild-type. (A) Schematic of the experimental protocol. See also Figures 10 and 11. (B) Representative images of mouse islets stimulated with sera derived from 3-month-old (upper panel) and 12-month-old animals (lower panel).

(C) Quantification of Ki67+ insulin+ cells in (B): two sets of three serial sections separated by 80 mm were analyzed. At least 4,000–5,000 cells were counted in each experimental group (n = 5 in each group). (D) Quantification of TUNEL+ insulin+ cells in (B): at least 3,000–4,000 cells were counted in each group (n = 5 in each group). (E and F) Representative images of healthy and type 2 diabetic donor islets stimulated with control versus LIRKO serum for 24 hr. See also Table 3. (G) Quantification of Ki67+ insulin+ cells in (E) and (F): three sets of three serial sections separated by 80 mm were analyzed. At least 3,000–4,000 cells were counted in each group. See also Table 3. Data represent mean ± SEM. *p % 0.05. (See Serum Stimulation and Human Islet Studies sections in Experimental Procedures.).

Figure 5 shows hepatocyte-derived factors stimulate mouse and human Islet β cell replication *in vitro*. Five to 6-week-old mouse islets were stimulated for 24 hr with LECM or HCM obtained from control or LIRKO mice. Islets were embedded in agarose and subsequently analyzed by immunostaining. Culture media were assayed for glucose and insulin. (A) Schematic of the experimental protocol. See also Figure 11. (B) Representative images of mouse islets stimulated with LECM derived from 3-month-old (upper panel) and 12-month-old animals (lower panel). (C) Quantification of Ki67+ insulin+ cells (upper panel) and TUNEL+ insulin+ cells (lower panel): at least 3,000–5,000 cells were counted in each experimental group (n = 5 in each group). (D) Representative images of healthy human donor islets (upper panel) and type 2 diabetic donor islets (lower panel) treated for 24 hr with LECM derived from control versus LIRKO mice. See also Table 3. (E) Quantification of Ki67+ insulin+ cells in (D): between 3,000 and 4,000 cells were counted in each condition (Control LECM versus LIRKO LECM) in control islets, and at least 2,000 cells were counted in each experimental group for type 2 diabetic islets. See also Table 3. (F) Representative images of mouse islets stimulated with HCM derived from 6-month-old control versus LIRKO mice or fibroblast-conditioned media (FCM). (G) Quantification of Ki67+ insulin+ cells (upper panel) and TUNEL+ insulin+ cells (lower panel): between 4,000 and 5,000 cells were counted in each experimental group (Control HCM versus LIRKO HCM) (n = 5 in each group). (H) Representative images of type 2 diabetic donor islets stimulated with control or LIRKO HCM. See also Table 3. (I) Quantification of Ki67+ insulin+ cells in (H): at least 2,000 cells were counted in each experimental condition. See also Table 3. Data represent mean ± SEM. *p % 0.05. (See LECM Stimulation, HCM Stimulation, and Human Islet Studies sections in Experimental Procedures.)

Figure 6 shows hematoxylin and eosin staining of tissue sections from 12-month-old control and LIRKO mice. These data are related to data in Figure 1 and Table 2. It represents an H & E based histological study of tissues from the LIRKO mouse.

Figure 7 shows weekly monitoring of body weight and blood glucose in parabionts, These data are related to data shown in Figure 2A. The data indicates weight-gain in both groups of mice and shows similar blood glucose levels in parabiont models over the 16-week period.

Figure 8 shows blood glucose and insulin assays in parabiont pairs pre- and postsurgery. These data are related to data shown in Figure 2A. The data show glucose and insulin levels in parabiont models before and after a 16-week parabiosis period.

Figure 9 shows quantification of pHH3+ insulin+ cells in parabiosis experiments. These data are related to data shown in Figure 2A. These data support the observations regarding β cell proliferation in different parabiont models assessed by BrdU incorporation in Figure 2A.

Figure 10 shows quantification of Ki67+ insulin+ cells in serum-stimulated mouse islets. These data are related to data shown in Figure 4A. The time course study presented in this figure is related to the serum stimulation experiments presented in Figure 4A.

Figure 11 shows glucose and insulin assays in culture media. These data are related to data shown in Figures 4A and 5A. These data are related to *in vitro* islet culture experiments. They indicate the levels of glucose and insulin in the culture media before and after incubation of islets with serum in Figure 4A and with LECM or HCM in Figure 5A.

Figure 12 shows the stimulation of islet β cell replication *in vitro*.

Figure 13 shows an exemplary nucleotide sequence for murine SerpinB1 (SEQ ID NO: 1; the corresponding amino acid sequence is designated SEQ ID NO: 3).

Figure 14 shows an exemplary nucleotide sequence for human SerpinB1 (SEQ ID NO: 2; the corresponding amino acid sequence is designated SEQ ID NO: 10).

Figure 15 shows an SDS/PAGE analysis of hepatocyte conditioned media from control and LIRKO mice before concentration (HCM) or after concentration of Nanozeolites. Proteins were detected by silver staining. Molecular weights are shown the right.

Figure 16 (A & B) shows identification of mouse SerpinB1 by LC-MS analysis. A) shows the tryptic peptides of mouse SerpinB1 (mouse SerpinB1 is designated SEQ ID NO: 3) identified by LC/MSMS (highlighted). B) shows proteomic analysis of HCM from LIRKO mice.

Figure 17 shows SerpinB1a gene expression (box plot and bar chart intensity data for Affymetrix probe set 1416318_at specific for SerpinB1a) in liver samples from LIRKO and wild type mice.

Figure 18 shows a two-fold increase in the nu8mber of Ki67+ insulin+ cells with LIRKO serum that the replicative capacity of the LIRKO sera was reduced by 80% following heart inactivation.

Figure 19 (A – C) shows serum and LECM derived from HFD and ob/ob mice stimulate β-cell replication. Five to 6-week-old mouse islets were stimulated with serum or liver explant conditioned media (LECM) derived from chow (CD) or high fat diet (HFD), ob/ob or control (WT) males. Twenty four hours later, islets were embedded in agarose for immunostaining studies. A) Schematic of the experimental protocol. B) Islets stimulated with serum or LECM from chow or high-fat diet males were immunostained for insulin/Ki67/DAPI or glucagon/BrdU/DAPI. Quantification of insulin+Ki67+ cells. C) Islets stimulated with serum or LECM from ob/ob or control males were immunostained for insulin/Ki67/DAPI or glucagon/BrdU/DAPI. Quantification of insulin+Ki67+ cells.

Figure 20 (A – D) shows pharmacological mimickers of SerpinB1 increase islet β-cell replication *in vivo*. Five to six-week old mice were administrated with Sivelestat or GW311616A for 2 weeks. Islet β-cell and α-cell replication was assessed by immunostaining. A) Schematic of the experimental protocol of Sivelestat administration. B) Quantification of pancreatic sections harvested from animals stimulated with Sivelestat and immunostained for insulin/BrdU/DAPI or glucagon/BrdU/DAPI. C) Schematic of the experimental protocol of GW311616A delivery. D) Quantification of pancreatic sections dissected from GW311616A-treated males and immunostained for insulin/BrdU/DAPI or glucagon/BrdU/DAPI.

Figure 21 (A & B) shows SerpinB1 acts directly and promotes β-cell-specific replication. Five to six-week old male mouse islets were cultured in the presence of different molecules for 48h. Islet β-cell replication was assessed by immunostaining studies. A) Schematic of the experimental protocol. B) Representative images of human islets stimulated for 24 hours with Sivelestat or GW311616A or control conditions.

Figure 22 (A – G) shows that elastase inhibitor GW311616A enhances β-cell mass *in vivo*. Five to six-week old male mice were daily gavaged with GW311616A or vehicle (water) for 2 weeks. Mice were injected during the second week with BrdU (100 mg/kg Body weight) and sacrificed 5 hour after last injection of BrdU. Pancreases and other tissues were harvested for morphometric studies. A) Schematic of the experimental protocol of GW311616 administration. B) Pancreatic sections harvested from animals stimulated with GW311616 were immunostained for insulin/BrdU/DAPI (upper panel) or glucagon/BrdU/DAPI (lower panel). C) Quantification of β-cell mass. D) Quantification of insulin+BrdU+ cells. E) Quantification of glucagon+BrdU+ cells. F-G) Representative images and quantification of nuclei BrdU+ in liver, kidney, spleen, visceral and subcutaneous adipose tissue and skeletal muscle tissue.

Figure 23 (A – F) shows that elastase inhibitor Sivelestat enhances β-cell proliferation *in vivo*. Five to six-week old male mice were continuously infused for 2 weeks with vehicle or Sivelestat at 150 or 300 µg/kg/day. Mice were injected during the second week with BrdU (100 mg/kg Body weight) and sacrificed 5 hour after last injection of BrdU. Pancreases were harvested for morphometric studies. A-B) Representative images and quantification of nuclei BrdU+ in insulin+ cells. C-D) Representative images and quantification of nuclei pHH3+ in insulin+ cells. E-F) Representative images and quantification of nuclei BrdU+ in glucagon+ cells.

Figure 24 (A – G) shows that SerpinB1 increases β-cell mass *in vivo* secondary to enhanced β-cell proliferation. Eight to ten-week old male mice were transduced by tail vein injection with adeno-associated viruses (AAVs) encoding EGFPII or SerpinB1. Three weeks post-transduction, mice were sacrificed and blood was collected and pancreases were harvested for morphometric studies. A) Schematic of study design. B) Western blot analyses of circulating SerpinB1. C) Representative images of islet in pancreases stained with insulin (red) and DAPI (blue). D-G) Quantification of nuclei Ki67+ in insulin+ cells, β-cell mass, serum insulin levels and blood glucose.

Figure 25 (A – D) shows that SerpinB1 and elastase inhibitor chemicals enhance β-cell proliferation *in vitro*. Five to six-week old male mouse islets were cultured in the presence of different molecules for 48h. Islet β-cell replication was assessed by Ki67 immunostaining studies. A) Representative images of islets stimulated with 100 or 1000 ng/ml of human recombinant SerpinB1. B) Quantification of Ki67+insulin+ cells in islets stimulated with 1 to 1000 ng/ml of rSerpinB1 or 1000 ng/ml of ovalbumin (OVA). C) Representative images (left panel) and quantification (right panel) of Ki67+insulin+ islet cells stimulated with Sivelestat (50 µg/ml) or GW311616A (100 µg/ml). D) Representative images of human islets stimulated for 24 hours with Sivelestat or GW311616Aor control conditions.

Figure 26 (A – B) shows that SerpinB1 increases glucose-stimulated insulin secretion seven and fifteen weeks post-AAV-transduction. 8 to 10 week-old mice were transduced *in vivo* by tail vein injection with AAV encoding EGFPII or SerpinB1. Seven and fifteen weeks post-transduction, *in vivo* glucose-stimulated insulin secretion experiments were performed (dose: 3g/kg body weight).

Figure 27 shows data suggesting that SerpinB1 contributes to adaptive increased β-cell proliferation in a model of acute insulin resistance. A) 15-16 week old control or SerpinB1KO mice were anesthetized, and osmotic pumps (ALZET) containing 100 µl of either PBS or S961 (10 nmoles/week for two weeks) were implanted subcutaneously. One week after recovery, mice were injected with BrdU (100 mg/kg body weight). B) Blood glucose was monitored every 3 days. C) At the end of the experiment, pancreases were harvested and immunostained for Ki67, a marker of proliferation. D) Quantification of Ki67+ insulin+ cells.

Figure 28 summarizes the identification of SerpinB1 by Affymetrix and proteomics analyses. A) Affymetrix Genechip analysis of livers harvested from 3-month-old male control or LIRKO mice (n = 6). B) Proteomics (LC/MS) analysis of proteins extracted from hepatocyte-conditioned media from 6-month-old male control or age-matched LIRKO animals (n = 3).

Figure 29 (A – M) shows data confirming the results obtained by Affymetrix and proteomics analyses. A-C) Evaluation of SerpinB1 expression levels in LIRKO and control mice. D-F) Western blot analyses of SerpinB1 in LECM. G) Analysis of circulating SerpinB1 levels in the serum and plasma of 3- and 12-month-old LIRKO and control mice. H-M) Evaluation of the expression level of SerpinB1 in livers harvested from ob/ob and high fat fed (HFD) mice. N) Evaluation of SerpinB1 expression levels in human serum samples obtained from lean healthy individuals, obese individuals and obese individuals with non-alcoholic steato hepatitis (NASH). 2 μl of each serum sample were loaded onto a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and Western-blotted with a rabbit SerpinB1 antibody (1/1000 dilution). The secondary goat anti-rabbit antibody was used in a 1/3000 dilution. For quantification purposes, the membrane was stained with Ponceau S solution (bottom panel).

Figure 30 shows the analysis of β-cell proliferation in mice lacking elastase (ELAKO) by immunostaining and quantification of Ki67-positive β-cells.

DETAILED DESCRIPTION OF THE INVENTION

Serpins (serine protease inhibitors) are a superfamily of ~45 kDa (kD) proteins with a highly conserved tertiary structure. Serpins regulate important intracellular and extracellular proteolytic events, including apoptosis, complement activation, fibrinolysis and blood coagulation. A review of Serpins known to those of ordinary skill in the art is provided in: Benarafa, et al., Characterization of Four Murine Homologs of the Human ov-serpin Monocyte Neurophil Elastase Inhibitor MNE1 (*SERPINB1*), 2002, J. Biol Chem., 277(44):42028-42033. Other Serpins and serpin analogs are also know to those of ordinary skill in the art and reference to them can be found at, for example, www.ncbi.nlm.nih.gov/pubmed/ and other suitable databases.

Further, as used herein, the term "Serpin family" denotes a family of serine proteinase inhibitors which are similar in amino acid sequence and mechanism of inhibition, but may differ in their specificity toward proteolytic enzymes. This family includes, for example, alpha 1-antitrypsin (A1-Pi), angiotensinogen, ovalbumin, antiplasmin, alpha 1-

antichymotrypsin, thyroxine-binding protein, complement 1 inactivators, antithrombin III, heparin cofactor II, plasminogen inactivators, gene Y protein, placental plasminogen activator inhibitor, and barley Z protein. Some members of the Serpin family may be substrates rather than inhibitors of serine endopeptidases, and some serpins occur in plants where their function is not known. See, for example, US Patent Publication No. 20120195859 and references therein.

In one embodiment, the term “active fragment”, as used herein, refers to all fragments of a Serpin (poly)peptide (e.g., SerpinB1), which exhibit a β cell proliferative activity. Such β cell proliferative activity can be determined by using the assays described herein.

Exemplary (functional) analogs for use in accordance with the present invention include Sivelestat and GW311616A.

The term “cellular proliferation” and “cell proliferation” refer to an increase in the number of cells as a result of cell growth and cell division. Cell or cellular proliferation may include the inducement of cell division by resting cells or senescent cells and may include the increase in the rate of cell division of cells already undergoing cell division.

The methods described herein include the manufacture and use of pharmaceutical compositions, which include compounds identified by a method described herein as active ingredients. Also included are the pharmaceutical compositions themselves.

Pharmaceutical compositions typically include a pharmaceutically acceptable carrier. As used herein the language “pharmacological composition,” “pharmacological carrier” or "pharmaceutically acceptable carrier" includes compositions and carriers comprising one or more of, for example, saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions. Suitable pharmaceutical compositions and carriers are also defined herein to include compositions and carriers suitable for *in vitro* use, *e.g.*, for diagnostic use, research use and *ex vivo* manipulation of cells and tissues.

Pharmaceutical compositions are typically formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration.

Methods of formulating suitable pharmaceutical compositions are known to one of ordinary skill in the art, see, e.g., the books in the series Drugs and the Pharmaceutical Sciences: a Series of Textbooks and Monographs (Dekker, NY). For example, solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfate; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use can include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile or capable of being sterilized and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride, in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, PRIMOGEL® (sodium starch glycollate), or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds can be delivered in the form of an aerosol spray from a pressured container or dispenser that contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer. Such methods include those described in U.S. Pat. No. 6,468,798.

Systemic administration of a therapeutic compound as described herein can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art, or into adhesive pads, as is generally known in the art.

The pharmaceutical compositions can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

Therapeutic compounds that are or may include nucleic acids (*i.e.*, a nucleic acid encoding one or more of the Serpins or a Serpin analog of the present invention) can be administered by any method suitable for administration of nucleic acid agents, such as a DNA vaccine. These methods include gene guns, bio injectors, and skin patches as well as needle-free methods such as the micro-particle DNA vaccine technology disclosed in U.S. Pat. No. 6,194,389, and the mammalian transdermal needle-free vaccination with powder-form vaccine as disclosed in U.S. Pat. No. 6,168,587. Additionally, intranasal delivery is possible, as described in, inter alia, Hamajima et al., Clin. Immunol. Immunopathol. 88(2), 205-10 (1998). Liposomes (e.g., as described in U.S. Pat. No. 6,472,375) and microencapsulation can also be used. Biodegradable targetable microparticle delivery systems can also be used (e.g., as described in U.S. Pat. No. 6,471,996).

In one embodiment, the therapeutic compounds are prepared with carriers that will protect the therapeutic compounds against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such formulations can be prepared using standard techniques as are known to one of ordinary skill in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

The pharmaceutical compositions can be included in a kit, container, pack or dispenser together with instructions for administration.

Methods of Treatment

The methods described herein include methods for the treatment of disorders associated with impaired glucose tolerance, e.g., for the improvement of glycemic control and insulin sensitivity by promoting β cell proliferation. In some embodiments, the disorder is type 1 or type 2 diabetes. Generally, the methods include administering a therapeutically effective amount of therapeutic compound as described herein, to a subject who is in need of, or who has been determined to be in need of, such treatment.

While type 1 diabetes is characterized by an absolute insulin insufficiency due to loss of more than 80% of the β cells, type 2 diabetes is characterized by a relative insulin insufficiency driven by inadequate β cell mass in the presence of insulin resistance (i.e. an increased insulin demand). Amongst type 2 diabetes patients the relative contribution of reduced functional β cell mass and insulin resistance may vary. In particular embodiments of the present invention, the disorder is type 1 diabetes or type 2 diabetes, wherein the type 2 diabetes is characterized by a more pronounced β cell insufficiency (e.g. a more pronounced reduction of functional β cell mass) as compared to that of an average type 2 diabetes patient.

As used in this context, to "treat" means to ameliorate at least one symptom of the disorder associated with impaired glucose tolerance. Often, impaired glucose tolerance results in hyperglycemia; thus, a treatment can result in a return or approach to normoglycemia/normal insulin sensitivity. As used in this context, to “prevent diabetes,” “prevent type 1 diabetes” or "prevent type 2 DM” (i.e., type 2 diabetes mellitus), or similar, means to reduce the likelihood that a subject will develop diabetes, type 1 diabetes or type 2 DM, respectively. One of skill in the art will appreciate that a preventive treatment is not required to be 100% effective, but can instead result in a delay in the onset of T1D, T2DM, or a reduction in symptoms, e.g., an improvement in glucose tolerance.

Dosage, toxicity and therapeutic efficacy of the compounds can be determined, e.g., by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

An "effective amount," “therapeutic amount” or “sufficient amount” is an amount sufficient to effect beneficial or desired results. For example, a therapeutic amount is one that achieves the desired therapeutic effect. This amount can be the same or different from a prophylactically effective amount, which is an amount necessary to prevent onset of disease or disease symptoms. An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a composition depends on the composition selected. The compositions can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the compositions described herein can include a single treatment or a series of treatments.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

In some embodiments, the pharmaceutical composition is injected into a tissue, e.g., pancreatic tissue or liver tissue.

Serpin Nucleic Acids

The nucleic acid molecules encoding the peptides described herein (for example, the sequences of Figure 13 and 14 (SEQ ID NO: 1 and SEQ ID NO: 2), or portions thereof) can be inserted into vectors and used as expression vectors and as gene therapy vectors. Other Serpin sequences are known to those of skill in the art and can be found, for example, at www.ncbi.nlm.nih.gov/pubmed/ and similar databases. The construction of suitable, functional expression constructs and expression vectors is known to one of ordinary skill in the art. In an embodiment of the present invention, expression of the Serpin peptide is directed towards the open reading frame of the sequences given in Figures 13 and 14 (see, for example, GenBank sequence nos. NM_030666.3 and NM_025429.2). One of ordinary skill in the art will be able to detect active fragments without undue experimentation by, for example, cleavage of the peptide into fragments and testing the fragments for activity in *in vivo* and *in vitro* assays, as are exemplified below. Similarly, constructs expressing Serpin fragments can be transfected into primary and cultured cell lines suitable for responding to Serpin activity or *in vivo* model systems, as are known to those of ordinary skill in the art, some of which are exemplified below. One of ordinary skill in the art, knowledgeable of protein secondary, tertiary and quaternary structures and protein function, will be able to identify protein fragments suitable for testing.

Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see, e.g., Chen et al., PNAS 91:3054-3057 (1994)). The pharmaceutical preparation of the gene therapy *vector* can include the gene therapy *vector* in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery *vector* can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system. Further, antisense nucleic acids, short interfering RNA (siRNA), interfering RNA (RNAi) and microRNA (miRNA) can be used to regulate expression of target Serpin genes and associated regulatory peptides. Antisense technology, RNAi, siRNA and miRNA technology is known by and can be practiced by those of ordinary skill in the art.

Serpins (e.g., SerpinB1) are known to exist in the plasma making them promising candidates as a biomarker. Serpins can be used as biomarkers in view of the teachings of the present invention to, for example, monitor treatments that affect β cell proliferation and diabetes. The compositions disclosed herein can include agents that detect or bind (e.g., that detect or bind specifically) to a biomarker described herein (e.g., one or more Serpins and members of the Serpin family, as described herein). Such agents can include, but are not limited to, for example, antibodies, antibody fragments, peptides and known small molecule agents. In some instances, the compositions can be in the form of a kit. Such kits can include one or more agents that can detect or bind (e.g., that detect or bind specifically) to one or more biomarkers described herein and instructions for use.

Gene Therapy

The nucleic acids described herein, e.g., an antisense nucleic acid described herein, or a Serpin (e.g., SerpinB1) polypeptide encoding nucleic acid, can be incorporated into a gene construct to be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of an agent described herein, e.g., a Serpin (e.g., SerpinB1), or an active fragment thereof or a functional or structural analog thereof. The invention features expression vectors for *in vivo* transfection and expression of e.g., a Serpin polypeptide (e.g., SerpinB1) or an active fragment thereof or a functional or structural analog thereof, described herein. Expression constructs of such components may be administered in any biologically effective carrier, e.g., any formulation or composition capable of effectively delivering the component gene to cells *in vivo*, as are known to one of ordinary skill in the art. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (e.g., LIPOFECTIN™) or derivatized (e.g., antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out *in vivo*, as is known to one of ordinary skill in the art.

One approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral *vector* containing nucleic acid, e.g., a cDNA, encoding an alternative pathway component described herein. Infection of cells with a viral *vector* has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral *vector,* e.g., by a cDNA contained in the viral *vector,* are expressed efficiently in cells which have taken up viral *vector* nucleic acid.

Retrovirus vectors and adeno-associated virus vectors can be used as a recombinant gene delivery system for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are characterized for use in gene transfer for gene therapy purposes (for a review see, e.g., Miller, Blood 76:271-78 (1990)). A replication defective retrovirus can be packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, et al., (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14, and other standard laboratory manuals. Non-limiting examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are known to those of ordinary skill in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include *Crip, *Cre, *2 and *Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, *in vitro* and/or *in vivo* (see, for example, Eglitis, et al., Science 230:1395-1398 (1985); Danos and Mulligan, Proc. Natl. Acad. Sci. USA 85:6460-6464 (1988); Wilson, et al., Proc. Natl. Acad. Sci. USA 85:3014-3018 (1988); Armentano, et al., Proc. Natl. Acad. Sci. USA 87:6141-6145 (1990); Huber, et al., Proc. Natl. Acad. Sci. USA 88:8039-8043 (1991); Ferry, et al., Proc. Natl. Acad. Sci. USA 88:8377-8381 (1991); Chowdhury, et al., Science 254:1802-1805 (1991); van Beusechem, et al., Proc. Natl. Acad. Sci. USA 89:7640-7644 (1992); Kay, et al., Human Gene Therapy 3:641-647 (1992); Dai, et al., Proc. Natl. Acad.

Sci. USA 89:10892-10895 (1992); Hwu, et al., J. Immunol. 150:4104-4115 (1993); U.S. Pat. No. 4,868,116; U.S. Pat. No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner, et al., BioTechniques 6:616 (1988); Rosenfeld, et al., Science 252:431-434 (1991); and Rosenfeld, et al., Cell 68:143-155 (1992). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 d1324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are known to those of ordinary skill in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting non-dividing cells and can be used to infect a wide variety of cell types, including epithelial cells (Rosenfeld, et al. (1992), supra). Furthermore, the virus particle is relatively stable and amenable to purification and concentration and, as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis *in situ* where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner, et al. (1998), supra; Haj-Ahmand and Graham, J. Virol. 57:267 (1986)).

Yet another viral vector system useful for delivery of the subject gene is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka, et al., Curr. Topics in Micro. and Immunol. 158:97-129 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte, et al., Am. J. Respir. Cell. Mol. Biol. 7:349-356 (1992); Samulski, et al., J. Virol. 63:3822-3828 (1989); and McLaughlin, et al., J. Virol. 62:1963-1973 (1989)). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin, et al., Mol. Cell. Biol. 5:3251-3260 (1985) can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat, et al., Proc. Natl. Acad. Sci. USA 81:6466-6470 (1984); Tratschin, et al., Mol. Cell. Biol. 4:2072-2081 (1985); Wondisford, et al., Mol. Endocrinol. 2:32-39 (1988); Tratschin, et al., J. Virol. 51:611-619 (1984); and Flotte, et al., J. Biol. Chem. 268:3781-3790 (1993)).

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of an nucleic acid agent described herein (e.g., a Serpin (e.g., SerpinB1), an active fragment thereof or a functional or structural analog thereof polypeptide encoding nucleic acid) in the tissue of a subject. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In some embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes. Other embodiments include plasmid injection systems such as are described in Meuli, et al., J. Invest. Dermatol. 116 (1):131-135 (2001); Cohen, et al., Gene Ther 7 (22):1896-905 (2000); or Tam, et al., Gene Ther. 7 (21):1867-74 (2000).

In a representative embodiment, a gene encoding a Serpin peptide described herein can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno, et al., No Shinkei Geka 20:547-551 (1992); PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075).

In clinical settings, the gene delivery systems for the therapeutic gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g., by intravenous injection. Specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see, U.S. Pat. No. 5,328,470) or by stereotactic injection (e.g., Chen, et al., PNAS 91: 3054-3057 (1994)).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In a particular embodiment of the present invention, the expression construct encoding a Serpin peptide or active fragment, or analog thereof is an adeno-associated virus (AAV). In a further embodiment, said AAV comprises a ubiquitous CMV promoter or a liver-specific promoter, e.g., a liver-specific albumin promoter.

Cell Therapy

An agent described herein suitable for, for example, improving pancreatic β cell function or increase pancreatic β cell proliferation, e.g., a Serpin (e.g., SerpinB1), or an active fragment thereof or a functional or structural analog thereof, can also be increased in a subject by introducing into a cell, e.g., a pancreatic β cell, a nucleotide sequence that encodes a Serpin (e.g., SerpinB1), or an active fragment thereof or a functional or structural analog thereof. The nucleotide sequence can include a promoter sequence, e.g., a promoter sequence from a Serpin gene or from another gene; an enhancer sequence, e.g., 5' untranslated region (UTR), e.g., a 5' UTR, a 3' UTR; a polyadenylation site; an insulator sequence; or another sequence that modulates the expression of a Serpin (e.g., SerpinB1), or an active fragment thereof or a functional or structural analog thereof. The cell can then be introduced into the subject by methods know to one of ordinary skill in the art.

Primary and secondary cells to be genetically engineered can be obtained from a variety of tissues and include cell types which can be maintained and propagated in culture. For example, primary and secondary cells include adipose cells, fibroblasts, keratinocytes, epithelial cells (e.g., mammary epithelial cells, intestinal epithelial cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells (myoblasts) and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the genetically engineered primary or secondary cells are administered. However, primary cells may be obtained for a donor (other than the recipient). The preferred cell for the compositions and methods of the present invention is a pancreatic β cell(s) or a liver cell(s).

The term "primary cell" includes cells present in a suspension of cells isolated from a vertebrate tissue source (prior to their being plated, i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these plated cells. The term "secondary cell" or "cell strain" refers to cells at all subsequent steps in culturing. Secondary cells are cell strains which consist of secondary cells which have been passaged one or more times.

Primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected with an exogenous nucleic acid sequence which includes a nucleic acid sequence encoding a signal peptide, and/or a heterologous nucleic acid sequence, e.g., encoding a Serpin (e.g., SerpinB1), or an active fragment thereof or a functional or structural analog thereof, and produce the encoded product stably and reproducibly *in vitro* and *in vivo*, over extended periods of time (i.e., hours, days, weeks or longer). A heterologous amino acid can also be a regulatory sequence, e.g., a promoter, which causes expression, e.g., inducible expression or upregulation, of an endogenous sequence. An exogenous nucleic acid sequence can be introduced into a primary or secondary cell by homologous recombination as described, for example, in U.S. Pat. No. 5,641,670. The transfected primary or secondary cells may also include DNA encoding a selectable marker which confers a selectable phenotype upon them, facilitating their identification and isolation.

Vertebrate tissue can be obtained by standard methods such a punch biopsy or other surgical methods of obtaining a tissue source of the primary cell type of interest. For example, punch biopsy is used to obtain skin as a source of fibroblasts or keratinocytes. A mixture of primary cells is obtained from the tissue, using known methods, such as enzymatic digestion or explanting. If enzymatic digestion is used, enzymes such as collagenase, hyaluronidase, dispase, pronase, trypsin, elastase and chymotrypsin can be used.

The resulting primary cell mixture can be transfected directly or it can be cultured first, removed from the culture plate and resuspended before transfection is carried out. Primary cells or secondary cells are combined with exogenous nucleic acid sequence to, e.g., stably integrate into their genomes, and treated in order to accomplish transfection. As used herein, the term "transfection" includes a variety of techniques for introducing an exogenous nucleic acid into a cell including calcium phosphate or calcium chloride precipitation, microinjection, DEAE-dextrin-mediated transfection, lipofection or electroporation, all of which are routine in the art.

Transfected primary or secondary cells undergo sufficient number doubling to produce either a clonal cell strain or a heterogeneous cell strain of sufficient size to provide the therapeutic protein to an individual in effective amounts. The number of required cells in a transfected clonal heterogeneous cell strain is variable and depends on a variety of factors, including but not limited to, the use of the transfected cells, the functional level of the exogenous DNA in the transfected cells, the site of implantation of the transfected cells (for example, the number of cells that can be used is limited by the anatomical site of implantation), and the age, surface area, and clinical condition of the patient.

The transfected cells, e.g., cells produced as described herein, can be introduced into an individual to whom the product is to be delivered. Various routes of administration and various sites (e.g., renal sub capsular, subcutaneous, central nervous system (including intrathecal), intravascular, intrahepatic, intrasplanchnic, intraperitoneal (including intraomental), intramuscularly implantation) can be used. Preferred sites for introduction are the pancreas or the liver. Once implanted in individual, the transfected cells produce the product encoded by the heterologous DNA or are affected by the heterologous DNA itself. For example, an individual who suffers from disease related to impaired pancreatic β cell function is a candidate for implantation of cells producing an agent described herein, e.g., a Serpin (e.g., SerpinB1), or an active fragment thereof or a functional or structural analog or mimic thereof as described herein or known to those of ordinary skill in the art.

An immunosuppressive agent, e.g., drug, or antibody, can be administered to a subject at a dosage sufficient to achieve the desired therapeutic effect (e.g., inhibition of rejection of the cells). Dosage ranges for immunosuppressive drugs are known in the art. See, e.g., Freed, et al., N. Engl. J. Med. 327:1549 (1992); Spencer, et al., N. Engl. J. Med.

327:1541 (1992); Widner, et al., N. Engl. J. Med. 327:1556 (1992)). Dosage values may vary according to factors such as the disease state, age, sex, and weight of the individual.

EXEMPLIFICATION

Example 1

Concerted efforts in diabetes research that were aimed at identifying molecules that specifically promote β cell regeneration without adverse proliferation of cells in other tissues. To determine whether Liver Insulin Receptor Knockout (LIRKO) mice, which manifest a dramatic hyperplasia of the endocrine pancreas, exhibit increased proliferation in extrapancreatic tissues, we injected bromodeoxyuridine (BrdU; 100 mg/kg body weight) intraperitoneally in 3-month-old LIRKO mice and assessed proliferation of β cells, a cells, and cells in metabolic organs such as the liver, adipose and skeletal muscle, and in nonmetabolic tissues such as the lung, kidney, and spleen. We observed a 2-fold increase in β cell mass (LIRKO 1.32 ± 0.2 versus control 0.68 ± 0.08 mg; $p < 0.05$; $n = 6$) in LIRKO mice compared to littermate controls that was due to enhanced β cell proliferation evidenced by a 2.5-fold increase in BrdU incorporation (LIRKO 1% ± 0.08% versus control 0.4% ± 0.07% BrdU+ β cells; $p < 0.001$; $n = 6$) and Ki67 staining (LIRKO 1.34% ± 0.1% versus control 0.51% ± 0.08% Ki67+ β cells; $p < 0.001$; $n = 6$) in the LIRKOs. TUNEL staining did not reveal significant differences in the number of apoptotic β cells between groups. We also observed no difference in a cell proliferation (LIRKO 0.24% ± 0.09% versus control 0.29% ± 0.1% BrdU+ a cells; $n = 6$) (Figures 1A–1F), or in the proliferation of cells in multiple non-β cell tissues, including visceral adipose, subcutaneous adipose, muscle, kidney, liver, or spleen. Although we did observe some increase in proliferating lung cells (LIRKO 0.7% ± 0.02% versus control 0.43% ± 0.08% BrdU+ cells; $n = 6$; $p < 0.05$) (Figures 1G and 1H), histological analyses of tissues dissected from 12-month-old LIRKOs revealed no tumor-like phenotypes (Figure 6; Table 2), and the life span of the LIRKOs was similar to littermate controls. These data indicate that LIRKO mice exhibit a robust β cell -specific proliferation in response to insulin resistance.

Table 2: Histological characteristics of 12-month-old Control and LIRKO mice

	Control	LIRKO
Pancreas	mild pancreatitis	very mild pancreatitis
Liver	severe steatosis	no steatosis + focal dysplasia + hyperplastic nodules
Skeletal muscle	normal	normal
Visceral adipose	severe lymphocyte infiltration	mild lymphocyte infiltration
Subcutaneous adipose	normal	normal
Spleen	normal	normal
Kidney	normal	normal
Lung	normal	normal

Example 2

Circulating Nonneuronal Nonautonomous Factors Drive β Cell Replication in LIRKO Mice.

To directly address whether β cell proliferation in the LIRKO mouse is mediated by systemic factors, we first used a parabiosis model (Bunster, E., and Meyer, R.K. (1933). An improved method of parabiosis. Anat. Rec. 57, 339–43). Five to 6-week-old male mice were surgically joined at the shoulder and hip girdles, and successful anastomosis was confirmed within 2 weeks of joining by Evans Blue Dye injection (data not shown). Animals remained parabiosed for 16 weeks, and β cell replication was subsequently assessed by BrdU incorporation (Figure 2A). Three surgical models were generated: control/control, control/LIRKO, and LIRKO/LIRKO. All parabiont groups grew normally, with a weekly increase in their body weights, and the blood glucose of the parabiont partners was within the normal range and did not significantly differ between groups (Figures 7A–7C). After 16 weeks of parabiosis, LIRKO and control parabionts displayed similar fasting blood glucose levels and circulating insulin levels and were higher in control partners joined with LIRKOs compared to nonparabiosed controls and controls parabiosed with controls (Figures 8A–8D). As expected, BrdU incorporation revealed low β cell mitosis in control mice and a significant elevation in LIRKO animals (control 0.03% ± 0.005% versus LIRKO 0.14% ± 0.02% BrdU+ β cells; $p < 0.005$; $n = 5–6$). We also noted a low level of β cell proliferation in the control parabionts (Figures 2F and 9) compared to the single controls (Figures 1C and

1D). We believe this may be secondary to the parabiosis procedure itself and requires further investigation. BrdU incorporation was similar in pancreatic β cells of same-genotype parabionts: low in control/control (~0.03% BrdU+ β cells; n = 5–6); and high in LIRKO/LIRKO (~0.19% BrdU+ β cells; n = 5–6). Interestingly, BrdU incorporation was significantly increased in pancreatic β cells of control mice joined with LIRKO mice (control in control/LIRKO parabionts 0.09% ± 0.01% versus control in control/control parabionts [0.03% ± 0.004% and 0.03% ± 0.008%] BrdU+ β cells; p < 0.01; n = 5–6) (Figures 2B–2F). The latter observations were confirmed by immunostaining for phospho-Histone H3 (pHH3) (Figure 9). These data indicate the presence of cell nonautonomous, circulating factors produced in LIRKO mice that promote β cell replication. Previous studies have implicated neural pathways in modulating β cell proliferation in a cell-nonautonomous fashion (Imai, J., *et al.*, (2008). Regulation of pancreatic beta cell mass by neuronal signals from the liver. Science 322, 1250–1254). To evaluate a possible influence of such neural effects on β cell proliferation in the LIRKO model, we undertook transplantation studies to assess β cell replication. A total of 125 size-matched islets freshly isolated from either control or LIRKO mice were transplanted under the kidney capsule of either control or LIRKO recipients. To minimize systematic error, each recipient mouse (control or LIRKO) was transplanted with two islet grafts, one derived from control and the other derived from LIRKO donors, under the left and right kidney, respectively (Figure 2G). Sixteen weeks after transplantation, islet grafts were harvested, sectioned, and analyzed for β cell BrdU incorporation. As expected, control islets grafted into control animals exhibited minimal β cell proliferation (0.017% ± 0.017% BrdU+ β cells). Intriguingly, the same donor-derived control islets showed an ~8-fold increase in β cell replication when transplanted instead into LIRKO recipients (0.139% ± 0.03% BrdU+ β cells; p < 0.05; n = 3–5). Notably, LIRKO islets transplanted into LIRKO recipients exhibited robust β cell replication, reminiscent of the increased β cell proliferation seen in the pancreas of unmanipulated LIRKO mice, whereas this response was blunted when LIRKO islets were grafted instead into control animals (Figure 2H). Taken together, these two complementary experimental strategies provide evidence that circulating nonneural and non-cell-autonomous factors contribute to expanding β cell mass in response to insulin resistance.

Example 3

LIRKO Serum Induces Selective β Cell Replication *In Vivo*.

We next sought to evaluate the relative importance of bloodborne molecules versus cells in the induction of β cell proliferation in the LIRKO model. Five to 6-week-old male mice were injected intraperitoneally with freshly isolated serum from 6-month-old control or LIRKO mice, respectively, twice a day (150 ml per injection) on days 1, 3, and 5. The recipients were injected with BrdU (100 mg/kg body weight) once a day on days 2, 4, and 6. The pancreases were harvested on day 6 to assess b and a cell replication (Figure 3A). Control mice injected with LIRKO serum (LIRKO(s)) displayed an ~2-fold increase in their endogenous β cell, but not α cell, replication compared to littermates injected with control serum (control(s)) (Figures 3B–3E). We saw no significant difference in the number of TUNEL+ β cells (Figures 3F and 3G) between LIRKO(s) and control(s)-injected groups. Assessment of BrdU incorporation in extrapancreatic tissues, including liver, subcutaneous adipose, muscle, kidney, spleen, and lung, revealed no significant differences in proliferation between groups, whereas a mild decrease was observed in visceral adipose (Figure 3H). This *in vivo* study confirms that a circulating molecule(s), stable in serum, selectively promotes β cell proliferation in the LIRKO model.

Example 4

LIRKO Serum Increases Mouse and Human Islet β Cell Replication *In Vitro*.

To gain further insight into the mode of action of this circulating β cell growth factor, we next established an *in vitro* functional assay to directly assess the impact of LIRKO or control serum on β cell replication in isolated mouse islets. We cultured islets in media containing serum from LIRKO or control mice and then assessed β cell proliferation using Ki67 immunostaining and fluorescence microscopy. Randomly selected Ki67+ β cells in each of the groups in all experiments were confirmed by confocal microscopy (Figure 4A).

To evaluate the nature of the factor we subjected LIRKO serum to heat inactivation by exposing it to 100 ºC for 15 min. The serum treatment induced up to 80% protein loss as measured by Bradford assay and visualized on Ponceau S solution stained-acrylamide gel. While, the proliferative action of LIRKO serum was evident by a two-fold increase in the number of Ki67+ insulin+ cells, the replicative capacity of LIRKO sera was reduced by 80% following heat inactivation (Figure 18) suggesting that the active principle mediating β-cell proliferation is a protein.

We next tested the ability of 6-month-old LIRKO serum to stimulate β cell proliferation and found that a 1:10 dilution of serum derived from LIRKO mice increased β cell proliferation in primary islets at 24 and 48 hr (Figure 10). LIRKO serum from 3-month-old mice also enhanced β cell proliferation in mouse islets (LIRKO(s) 1.58% ± 0.3% versus control(s) 0.52% ± 0.1% Ki67+ β cells; $p < 0.05$; n = 6). Moreover, mouse islets cultured in 12-month-old LIRKO serum showed a greater number of replicating β cells compared to islets incubated with age-matched control serum (LIRKO(s) 1.3% ± 0.5% versus control(s) 0.7% ± 0.2% Ki67+ β cells; p = 0.3; n = 4–6) (Figures 4B and 4C); this increase lost its statistical significance probably due to an elevated insulin resistance in aging controls that itself contributed to β cell proliferation (Kulkarni, R.N., *et al.*, (2003). Impact of genetic background on development of hyperinsulinemia and diabetes in insulin receptor/insulin receptor substrate-1 double heterozygous mice. Diabetes 52, 1528–1534; Mori, M.A., *et al.*, (2010). A systems biology approach identifies inflammatory abnormalities between mouse strains prior to development of metabolic disease. Diabetes 59, 2960–2971). TUNEL staining showed no significant difference in β cell apoptosis in islets cultured in LIRKO(s) versus control(s) (Figure 4D). Preliminary data indicate that the ability of the LIRKO serum to stimulate β cell proliferation is reduced when subjected to heat inactivation, suggesting that the putative circulating factor may be a protein (data not shown). To examine whether the proliferating effect of LIRKO serum is conserved across species, we next cultured human islets from nine healthy and two diabetic donors (for donor characteristics, see Table 3) in serum isolated from 12- to 18-month-old male LIRKO or control mice. Similar to the effects on mouse β cells, serum from LIRKO mice enhanced human islet β cell proliferation, albeit at a level lower than that reported in a recent study by Rieck, S., *et al.*, (2012).

Table 3: Islet-donor characteristics

Donor	Gender	Ethnicity/Race	Age (years)	BMI	Diabetic donor status	Experiment
1	Male	White	55	20.1	No	stimulation with serum
2	Male	White	23	25.6	No	stimulation with serum
3	Female	White	18	26.4	No	stimulation with serum
4	Male	Hispanic/Latino	25	29.3	No	stimulation with serum
5	Male	Hispanic/Latino	50	26.5	No	stimulation with serum
6	Unkown	Unkown	64	30	No	stimulation with serum
7	Female	African american	41	42	No	stimulation with serum
8	Male	White	54	19.5	No	stimulation with serum
9	Male	African american	20	31.3	No	stimulation with serum
10	Male	Unknown	53	31	T2D	stimulation with serum
11	Female	White	38	37.8	T2D on metformin	stimulation with serum
12	Unknown	Unknown	65	31	No	stimulation with LECM
13	Unknown	Unknown	54	34	T2D	stimulation with LECM
14	Male	White	52	50	T2D	stimulation with HCM

Overexpression of hepatocyte nuclear factor-4a initiates cell cycle entry, but is not sufficient to promote β cell expansion in human islets. Mol. Endocrinol. 26, 1590–1602). Importantly, LIRKO serum was also effective in promoting proliferation of islet β cells from patients with type 2 diabetes (Figures 4E–4G). Thus, the β cell mitogen(s) present in the circulation of LIRKO mice shows conserved activity toward mouse and human islets, including islets from patients with type 2 diabetes. Glucose and insulin have been reported to promote β cell growth (Assmann, A., *et al.*, (2009). Growth factor control of pancreatic islet regeneration and function. Pediatr. Diabetes 10, 14–32; Assmann, A., *et al.*, (2009b). Glucose effects on beta-cell growth and survival require activation of insulin receptors and insulin receptor substrate 2. Mol. Cell. Biol. 29, 3219–3228; Bonner-Weir, S., *et al.*, (1989). Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion. Diabetes 38, 49–53) and are potential candidates in the LIRKO model, which manifests glucose intolerance and hyperinsulinemia (Michael, M.D., *et al.*, (2000). Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol. Cell 6, 87–97). However, our observations suggest that glucose is not a dominant factor in the LIRKO mouse for several reasons. First, control mice parabiosed to LIRKOs for 16 weeks demonstrate up to a 7-fold increase in proliferation despite normal blood glucose levels (~120 mg/dl) during the parabiosis period (Figures 7A and 7C). Second, serum used to examine the effects on β cell proliferation (see Figure 4A) was derived from either normoglycemic 3-month-old or hypoglycemic 12-month-old animals (data not shown). Finally, to further exclude a role for glucose, we cultured islets in a constant concentration of 5.5 mM glucose in experiments with serum from LIRKO or control mice (serum:culture media at 1:10 dilution) and observed an increase in proliferation in β cells only in the former group. Furthermore, the glucose levels in culture media at the beginning and at the end of islet incubation were similar in both groups (Figure 11A). We believe that insulin may be permissive but unlikely to account for the high level of β cell proliferation in our model because the levels of insulin in the diluted serum (Figure 11B) used in *in vitro* studies (see Figure 4) are significantly lower compared to the levels found in the circulation in LIRKO mice (11.63 ± 2.4 ng/ml [3-month-old LIRKOs] versus 2.59 ± 1 ng/ml [diluted serum in culture media] and 17.8 ± 4.4 ng/ml [12-month-old LIRKOs] versus 1.4 ± 1.3 ng/ml [diluted serum in culture media]) (Table 1; Michael, M.D., *et al.*, (2000). Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. Mol. Cell 6, 87–97). Together, these data support the presence of a glucose- and insulin-independent liver-derived factor that promotes the expansion of β cell mass.

Example 5

Hepatocyte-Derived Factors Stimulate Mouse and Human Islet β Cell Replication *In Vitro*.

The common embryonic origin of the liver and the pancreas (Zaret, K.S. (2008). Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nat. Rev. Genet. 9, 329–340) coupled with the robust β cell proliferation response to tissue-specific insulin resistance in the liver compared to the virtual lack of a compensatory response when insulin resistance was restricted to muscle (Brüning, J.C., *et al.*, (1998). A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. Mol. Cell 2, 559–569), adipose (Blüher, M., *et al.*, (2002). Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. Dev. Cell 3, 25–38), or brain (Brüning, J.C., *et al.*, (2000). Role of brain insulin receptor in control of body weight and reproduction. Science 289, 2122–2125) prompted us to hypothesize that the liver serves as a source of β cell growth factor(s) in response to metabolic insults such as insulin resistance. To test this hypothesis, we collected conditioned media from liver explant cultures (LECM) from either 3- or 12-month old LIRKO or control animals and evaluated their effects on β cell proliferation in mouse islets (Figure 5A). Ki67- positive β cells were significantly elevated in islets cultured in LECM from either 3- or 12- month-old LIRKO mice, compared to cells cultured in LECM derived from age-matched controls (Figure 5B). Interestingly, whereas mouse islets cultured in control LECM derived from 3- and 12- month-old animals displayed similar levels of proliferation, the levels were 2-fold higher in cultures containing 12-month-old LIRKO-LECM compared to 3-month-old control LECM (Figure 5C). This age-dependent effect of LIRKO-LECM is consistent with the age-dependent increase in β cell proliferation in LIRKO mice (Okada, T., *et al.*, (2007). Insulin receptors in beta-cells are critical for islet compensatory growth response to insulin resistance. Proc. Natl. Acad. Sci. USA 104, 8977–8982). Similarly, β cells in islets obtained from healthy human controls and patients with type 2 diabetes (for donor characteristics, see Table 3) cultured in LECM derived from LIRKO animals exhibited increased proliferation compared to islets from the same donors cultured in control LECM (Figures 5D and 5E). The liver contains multiple cell types, including hepatocytes, Kupffer cells, and endothelial cells (Si-Tayeb, K., *et al.*, (2010). Organogenesis and development of the liver. Dev. Cell 18, 175–189). To determine whether the growth factor activity in LIRKO serum is a product of hepatocytes or nonhepatic cells, we used conditioned media from cultures of primary hepatocytes (HCM), isolated from control or LIRKO mice in an *in vitro* β cell proliferation assay. Primary mouse islets cultured in LIRKO HCM exhibited markedly increased β cell proliferation compared to islets stimulated with control HCM (control HCM, 0.13% ± 0.03% versus LIRKO HCM, 0.64% ± 0.12%; $p < 0.05$; $n = 5$). The number of TUNEL+ β cells was similar in both conditions (Figures 5F and 5G). Furthermore, the proliferative effect of LIRKO HCM was also evident when human islets obtained from a patient with type 2 diabetes (for donor characteristics, see Table 3) were exposed to LIRKO HCM compared to control HCM (Figures 5H and 5I). Thus, insulin-resistant hepatocytes produce a β cell growth-promoting factor(s) that enhances proliferation of mouse and human β cells. Although numerous signaling pathways impacting β cell growth have been documented (Kulkarni, R.N., *et al.*, (2012). Human β-cell proliferation and intracellular signaling: driving in the dark without a road map. Diabetes 61, 2205–2213), specific blood-borne molecules that trigger β cell replication directly in response to insulin resistance have, to our knowledge, not been reported. The absence of a consistent increase in one or more growth factors in the serum of the LIRKOs (Table 1) supports the notion that additional unidentified factors are necessary to promote the full magnitude of proliferation observed in the LIRKO model. In summary, we provide evidence that a conserved systemic hepatocyte-derived growth factor(s) promotes β cell proliferation in mouse and human islets, supporting a liver-to-pancreas axis in the adaptive β cell growth response to insulin resistance.

Example 6

A role for SerpinB1 and related family members on mouse and human β-cell proliferation.

Pancreatic β-cell dysfunction underlies the development of both type1 and type 2 diabetes. Although the natural history of both forms diabetes is different, reduced functional β-cell mass is a common hallmark in both diseases. Regenerative approaches represent an attractive strategy to increase the number of functional β-cells. In this context, we recently reported (El Ouaamari, et al, *Cell Reports*, 2013, 3:1-10) the existence possible of liver-derived systemic factors capable of stimulating β-cell proliferation in Liver Insulin Receptor Knockout mouse (LIRKO), a unique model of islet hyperplasia and increased β-cell mass caused by insulin resistance.

Using comprehensive Affymetrix and Proteomics approaches we have now identified the superfamily of Serpin proteins as factors of β-cell growth. Among the family members, SerpinB1 was identified as the being a consistently up-regulated hepatocyte-derived systemic β-cell trophic factor.

Identification of SERPINB1 as a new potential beta cell growth factor from LIRKO mouse - Method: Proteomic analysis of hepatocyte conditioned media.

Pre-enrichment on nanozeolites

Nanozeolite LTL nanoparticles were obtained from NanoScape AG, Germany. Adsorption of proteins on the surface of Nanozeolite LTL was carried out for 90 min at 4°C by incubation of proteins from hepatocyte conditioned media (0.1 mg/ml) and nanoparticles (0.1 mg/ml) in suspension in PBS. After centrifugal separation at 16000g during 20 min, proteins bound to nanoparticles are washed twice in 0.1M ammonium carbonate buffer, ph 8.0.

Protein samples were resolved by SDS-PAGE on NuPAGE® Novex® 4-12% Bis-Tris gels using the NuPAGE® MES SDS Running Buffer according to the manufacturer's instructions (Invitrogen, Grand Island, NY) stained using the SilverQuest™ silver staining kit from Invitrogen.

Proteolytic digestion

The proteins captured on nanozeolites were reduced in the presence of 10 mM dithiothreitol, 0.05% AALS (Anionic Acid Labile Surfactants from Protea Biosciences) in 50 mM ammonium carbonate buffer, pH 8.0 at 56°C for 30 min and then alkylated by adding 20 mM iodoacetamide for 30 min at room temperature in the dark.

After the reduction and alkylation steps, bound proteins were digested with LysC (1/50 w/w), 4hrs at 37°c and then with trypsin (1/50 w/w) for 18 hr at 37°C.

After centrifugation, protein digests were collected, and AALS hydrolyzed with 1% TFA at 37 °C min. Finally enzymatic digests were subjected to MS analysis.

LC-MS analysis

LC-MS (Liquid Chromatograph Mass Spectrometer) experiments were performed on NanoAcquity UPLC (Waters, Milford, MA) connected to a hybrid LTQ (Linear Trap Quadropole) Orbitrap Velos™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a nanoelectrospray source. Protein digests were loaded onto a nanoAcquity UPLC Trap column (Symmetry C18, 5µm, 180 µm x 20 mm, Waters) and washed with 0.2% formic acid at 20 µL/min for 5 min. Peptides were then eluted on a C18 reverse-phase nanoAcquity column (BEH130 C18, 1.7 µm, 75 µm x 250 mm, Waters) with a linear gradient of 7-30% solvent B ($H_2O/CH_3CN/HCOOH$, 10:90:0.2, by vol.) for 120 min, 30-90% solvent B for 20 min, and 90% solvent B for 5 min, at a flow rate of 250 nL/min.

The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 300-1700) were acquired in the Orbitrap with a resolution of 60,000 at m/z: 400. The AGC (automated gain control) was set to 1×10^6 with a maximum injection time of 500 ms. The most intense ions (up to 20) were then isolated for fragmentation in the LTQ linear ion trap using a normalized collision energy of 28% at the default activation q of 0.25 with an AGC settings of 2×10^4 and a maximum injection time of 200 ms. The dynamic exclusion time window was set to 150 s. Samples were injected in triplicate.

LC-MS/MS data processing

LC-MS/MS data, acquired using the Xcalibur software (version 2.07, Thermo-Fisher Scientific), were processed using a Visual Basic program software developed using XRawfile libraries (distributed by Thermo-Fisher Scientific). Similar programs are known to and can be developed by one of ordinary skill in the art. Three different files were generated by this program: the first one corresponds to a MS/MS peak list (MGF file) which will be used for database searching. This MGF file contains the exact parent mass and the retention time (RT) associated with each LTQ-MS/MS spectrum. The exact parent mass is the ^{12}C isotope ion mass of the most intense isotopic pattern detected on the high resolution Orbitrap MS parallel scan and included in the LTQ-MS/MS selection window. The RT is issued from the LTQ-MS/MS scan. The second file is a MS/MS log file which reports, for each acquired MS/MS, the scan number, the ^{12}C isotope exact mass, the RT and the parent filter (LTQ selection window). The third file corresponds to the conversion of the high resolution MS raw data file into a “csv” format file which will be used for quantitative analysis.

Database searching

Database searches were done using our internal MASCOT server (version 2.1, matrix Science; www.matrixscience.com/) using the Swiss-Prot human database containing 402,482 entries. The search parameters used for post-translational modifications were a fixed modification of +57.02146 Da on cysteine residues (carboxyamidomethylation) and dynamic modifications of +15.99491 on methionine residues (oxidation), of +42.010565 on protein N-terminal residues (N-terminal acetylation) and -17.026549 on N-terminal glutamine residues (N-Pyroglu). The precursor mass tolerance was set to 5 ppm and the fragment ion tolerance was set to 0.5 Da. The number of missed cleavage sites for trypsin was set to 2. Mascot result files (".dat" files) were imported into Scaffold software (www.proteomesoftware.com/). Queries were also used for XTandem parallel Database Search. The compiled results of both database searches were exported.

Quantitative analysis and Statistical Analysis

Quantitative differential analysis of proteins was realized using a label free analysis with an in-house DIFFTAL (DIFferential Fourier Transform Analysis) software algorithm. DIFFTAL Algorithm Overview. DIFFTAL is a set of software tools developed in Sanofi under MatLab environment (www.mathworks.com) for label-free differential analysis of complex proteomic mixtures dedicated to data recorded with high resolution MSMS instruments.

DIFFTAL runs in 6 main steps. These steps consist of the following: (1) Feature detection, (2) MS matching, (3) MS/MS annotations, (4) MS/MS matching, (5) Peptide quantification report and (6) Protein relative quantifications.

Step 1: Feature detection. Each LC/MS file is treated independently for feature detection. The signal apparition is detected scan by scan by analyzing the evolution of the average signal of 3 consecutive scans. Feature detection is achieved using the peptide isotopic patterns calculated with "Averagine" algorithm. At the end of the process, a matrix of the features detected in the 3D space (m/z (mass/charge), RT (retention time) and intensity) is stored. This matrix contains links to retrieve the corresponding processed signals, which are stored in a temporary data bank.

Step 2: MS matching. All LC-MS data are matched together using a progressive alignment procedure. First, the most intense detected features are matched in agreement with m/z and RT precision windows defined by the user. Then, all peptides are used to compute a specific RT alignment model. A definitive RT window is calculated according to the dispersion observed between real and calculated RTs. Finally, every remaining unmatched m/z is checked by going back to the processed signal stored during the feature detection step. This last point allows a very confident determination of the unmatched feature class.

Step 3: MS/MS annotations. This step corresponds to the data bank search previously reported in the “Database Searching” paragraph.

Step 4: MS/MS matching. MSMS Spectrum reports exported from Scaffold are matched with the matrix of detected features using the corresponding acquisition MS/MS log files (see LC-MS/MS data processing). This matching requires starting and ending time points of each feature. Indeed, the RT feature is the time at the maximum intensity of the observed MS signal, whereas the MS/MS spectrum is recorded at any time during the peptide elution. In case of ambiguity, the comparison between the exact isotopic profile calculated from the MS/MS sequence and the detected signal at the feature RT is used for sorting. Another routine has been also introduced in the software that quantifies only the MS/MS identified peptides according to the following scheme: the time profiles of the 2 major isotopes of each identified peptide are computed in a small time window where the MS/MS spectrum was recorded. Only the co-eluted signals of these 2 isotopes are analyzed to determine the peptide RT. The 3 scans averaged signal centered at this time is then compared with the full theoretical peptide isotopic pattern. This additional quantification is compared to the first one to generate a final result report. The convergence of these two quantification routines is used to improve the quantification confidence and identification coverage.

Step 5: Peptide Quantification report: Peptide quantification is calculated from the statistical analysis of the previous matrix. Statistical analyses were realized with DanteR program, an R based software written by Tom Taverner (Thomas.Taverner@pnl.gov and Ashoka Polpitiya for the U.S. Department of Energy (PNNL, Richland, WA, USA: on the World Wide Web (www): omics.pnl.gov/software). The median intensity value of the detected feature population is used to normalize the 3 replicate injections of the same sample. Only peptides detected at least 2 times (over replicates) are kept and an average intensity value per sample is calculated for each peptide. A threshold value representing the minimum detectable signal level is used instead of quantification for non-detected peptide.

As non-detected peptide intensities are replaced by detection threshold, a protein which is identified, for example, in the treated sample but not detected in the control sample is represented with a minimum positive fold change which is the result of the treated signal divided by the minimum detectable signal.

Step 6: Protein quantification: Finally, peptides arising from the same protein are grouped to evaluate the peptide fold change dispersion. Protein-level inferences are performed utilizing all of the available peptide abundances and a likelihood ratio test to compute p-values (Karpievitch, et al., 2009a). Significant up or down protein expression changes are sorted and plotted by p-value from hypothesis testing through the sample types and the replicate analyses.

Results: Differential proteomic analysis of control and LIRKO hepatocyte conditioned media (HCM)

Hepatocytes from control and LIRKO mice were cultured in serum free medium and supernatants collected after 18hrs.

To concentrate secreted proteins from diluted HCM and eliminate small molecules artefacts from the culture medium that do not allow LC-MS analysis, we developed a proteomic approach based on enrichment of the proteins using zeolite LTL nanocrystals as described by Cao J., et al. (Nanozeolite-driven approach for enrichment of secretory proteins in human hepatocellular carcinoma cells, Proteomics. 2009, 9, (21):4881-8) followed by enzymatic digestion of the proteins directly on nanobeads.

Before enzymatic digestion, adsorption of the proteins was controlled by SDS/PAGE (Figure 15). Both LIRKO and control supernatants showed similar and highly complex protein profiles before and after adsorption onto nanoparticles.

The resulting peptides were identified using high-performance liquid chromatography tandem mass spectrometry LC-MS/MS analysis. Proteins were identified by searching MS and MS/MS data of peptides against the UniProtKB/Swiss-Prot protein knowledgebase using the MASCOT search engine and then quantified by a label free quantitative LC-MS analysis using in-house DIFFTAL software algorithm. Relative quantification of each protein was obtained by averaging the intensity ratios of the three most intense derived peptides (or two derived peptides if only two unique peptides were identified) as described in the experimental procedure.

We realized 3 independent proteomic analyses to compare LIRKO and control HCM starting from independent hepatocyte cultures from different mice.

We identified 514, 1670 and 1280 proteins in these 3 different analysis according to the concentration and amount of proteins available.

Among these proteins, we identified 12 proteins that were up-regulated and 8 proteins that were down-regulated in the LIRKO HCM with the respective ratio above 2 or under 0.5 and p-values lower than 0.05, in all the experiments.

Mouse SerpinB1 was identified by LC-MSMS by 12 unique tryptic peptides given a protein coverage of 37% and in the 3 independent experiments, SerpinB1 was shown to be up-regulated in LIRKO hepatocyte supernatants with the respective ratio of 17.5, 11.6 and 18.4 and p-values smaller than 0.01 (Figure 16 A & B).

The up-regulation of SerpinB1 in LIRKO hepatocytes was confirmed at the RNA level by transcriptomic analysis of mouse liver explants showing that the differential observed at the protein level is due to an overexpression of the protein and not a modification of a secretory pathway in LIRKO mouse liver.

Transcriptomics analysis of liver samples from LIRKO model

Based on the above results, we next analyzed LIRKO mouse liver gene expression.

Animals and Sample Preparation:

The total number of mice used for gene expression analyses was 20 animals (3 months old [n=12 animals], and 24 months old [n=8 animals]). Liver tissue samples were excised rapidly from animals and snap-frozen in liquid nitrogen and stored at -80 degree Celsius (^{O}C).

Affymetrix GeneChip Analysis:

The general use of oligonucleotides arrays for gene expression monitoring has been described in U.S. Pat. No. 6,177,248. In our practical application, the used micro arrays (GeneChips) from Affymetrix, Santa Clara, CA. USA contain deoxynucleotide sequences that represent approximately 39,000 mouse transcripts and variants from >34,000 well characterized mouse genes (Mouse Genome 430 2.0 GeneChip). Each transcript and variant is represented by 11 different oligonucleotide probes with 25 basepairs in length. Sequences used in the design of the array were selected from GenBank, dbEST, and RefSeq. The sequence clusters were created from the UniGene database (Build 107, June 2002) and then refined by analysis and comparison with the publicly available draft assembly of the mouse genome from the Whitehead Institute Center for Genome Research (MGSC, April 2002).

150 mg of liver tissue were lysed in Qiagen RLT buffer with an UltraTurrax homogenizer. Total RNA from the tissue lysates was isolated with Qiagen RNeasy kit including proteinase K digestion, DNase digestion and an additional RNeasy cleanup step as recommended by the manufacturer (Qiagen). Integrity of RNA samples has been checked by RNA nano assay (Agilent 2100 BioAnalyzer; Agilent, Santa Clara, CA).

First and second strand cDNA synthesis were performed with 10 µg of each total RNA using SuperScript SSII RT polymerase system (Invitrogen) and a T7(dT)24 primer linking the T7 RNA polymerase promoter and oligo(deoxythymidine)24. Double strand cDNA was phenol-chloroform extracted followed by ethanol precipitation and resuspended in 12 µl RNAse-free water. Biotin-UPT and -CTP labelled cRNA was transcribed *in vitro* using Enzo BioArray High Yield RNA Transcript Labelling Kit (Enzo Diagnostics, NY, NY) and purified by RNeasy cleanup and ethanol precipitation. Aliquots of every total RNA and cRNA were monitored before and after each purification step by UV-spectrophotometry, agarose gel electrophoresis and RNA nano assay (Agilent 2100 BioAnalyzer). 15 µg cRNA samples were fragmented at 94 degree Celsius for 35 min in 40 mM Tris/acetate pH 8.1, 100 mM KOAc and 30 mM MgOAc, added to hybridisation buffer and hybridised to Affymetrix GeneChip for 16-18 hours at 45 degree Celsius and 60 rpm in a rotating hybridization oven (Hybridization Oven 640, Affymetrix). Micro arrays were washed in a fluidics station (GeneChip Fluidics Station 450, Affymetrix) and double-stained with streptavidin-phycoerythrin conjugate (Molecular Probes, Life Technologies, Grand Island, NY), anti-streptavidin antibody and again streptavidin-phycoerythrin conjugate to enhance signal intensity according to the methodologies described by Affymetrix. After washing the micro arrays were scanned with the GeneChip Scanner 3000 7G (Affymetrix), which is controlled by Affymetrix software GeneChip Operating System (GCOS) v1.4. Quality control of each chip was performed according the Affymetrix quality criteria, including mean average difference, raw intensity and 3'/5' ratio of housekeeping genes beta-actin and GAPDH.

For real time experiments, total RNAs were extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA). One µg RNA was used for a reverse transcription step using high-capacity cDNA Archive Kit (Applied Biosystems). cDNA was analyzed by ABI 7900HT system (Applied Biosystems). TBP was used as an internal control. Primers for SerpinB1: 5′-GCTGCTACAGGAGGCATTGC-3′ (forward) [SEQ ID NO: 4] and 5′-CGGATGGTCCACTGTGAATTC-3′ (reverse) [SEQ ID NO: 5], for betatrophin; 5′-CACTGTACGGAGACTACAAGTGC-3′ (forward) [SEQ ID NO: 6] and 5′-GTGGCTCTGCTTATCAGCTCG-3′ (reverse) [SEQ ID NO: 7] and for TBP; 5'-ACCCTTCACCAATGACTCCTATG-3' (forward) [SEQ ID NO: 8] and 5'-ATGATGACTGCAGCAAATCGC-3' (reverse) [SEQ ID NO: 9].

Data Analysis

Bioinformatics analysis of the Affymetrix raw data has been performed in the Array Studio software package from OmicSoft Corp. Cary, NC, USA. For this Affymetrix cel files have been first processed with Robust Multi-array Average (RMA) as normalization method and the data have been then log2 transformed. For detection of expressed genes all Affymetrix probe sets with intensity signals of <6 in at least 25% of the samples each of the LIRKO and wildtype group have been filtered out. Principal component analysis (PCA) has been applied to all samples as a quality control measure. To detect differentially expressed genes a pairwise ANOVA statistical test has been applied between the LIRKO and the wildtype control group. Criteria for determining differentially expressed genes with statistical significance were changes in expression levels higher than 2-fold and a P-value <0.05. The analysis result for Serpinb1a expression from Affymetrix probe set 1416318_at specific for Serpinb1a is shown in Figure 17. Serpinb1a gene expression was found to be significantly up-regulated in liver samples from 3 months old LIRKO mice by a factor of 3.3. Significant up-regulation of Serpinb1 in liver could be confirmed in samples of 24 months old LIRKO (see Figure 17). The identification of SerpinB1 by Affymetrix and proteomics analyses is summarized in Figure 28.

Confirmation of Affymetrix and Proteomics Data

To confirm our Affymetrix and Proteomics data, we examined the expression of SerpinB1 in the liver and evaluated circulating levels of SerpinB1 in the LIRKO mouse. We observed that SerpinB1 mRNA (LIRKO 2.4±0.6 vs. control 0.6±0.1, $p<0.05$, n=6) and protein levels (LIRKO 5.1±0.9 vs. control 1.1±0.06, $p<0.05$, n=4-5) are 5-fold higher in 3-month-old LIRKO mice compared to age-matched controls (Figure 29 A-C). Western Blot analyses showed increased levels of SerpinB1 in LECM (Figure 29 D). Importantly, SerpinB1 was increased in LIRKO hepatocyte lysates, whereas neutrophil markers, such as proteinase 3 and neutrophil elastase, were not detected, ruling out contaminating blood cells as a source of SerpinB1 (Figure 29 F). Moreover, circulating SerpinB1 was increased in sera of both 3-month-old (LIRKO 7.9±1.4 vs. control 3.6±0.3, $p<0.05$, n=5-6) and 12-month-old (LIRKO 10.6±0.9 vs. control 7.7±0.5, $p<0.05$, n=4-5) LIRKO mice. Similar data were obtained when SerpinB1 was assayed in plasma (Figure 29 G). We next evaluated the expression level of SerpinB1 in livers harvested from other models of insulin resistance: leptin-deficient (*ob/ob)* mice and high fat diet (HFD) mice. Similar to LIRKO mice, we demonstrated that mRNA (*ob/ob* 4.9±1.5 vs. control 1.3±0.2, p=0.07, n=5) and protein (*ob/ob* 3.4±0.5 vs. control 1.3±0.2, $p<0.05$, n=5) expression of liver SerpinB1 is upregulated in *ob/ob* mice (Figure 29 H-J). LECM prepared from ob/ob livers exhibited high levels of SerpinB1 as compared to age-matched control LECM (Figure 29 K). Further, SerpinB1 protein abundance was 2-fold higher (HFD 3.2±0.3 vs. control 1.6±0.3, $p<0.01$, n=6) in livers derived from HFD compared to control animals (Figure 29 M), although we did not observe a statistically significant variation in the corresponding hepatic mRNA levels (HFD 1.7±0.5 vs. control 2.2±0.5, p=0.5, n=6; Figure 29 L). The lack of an increase at the mRNA level and increased protein levels suggests SerpinB1 is regulated at the post-transcriptional level in HFD model, a feature observed with other proteins (He, et al., 2009; Vannay, et al., 2004). Together, these data strongly implicate SerpinB1 as a potential marker of insulin resistance and/or a marker associated with β-cell regeneration/proliferation.

Evaluation of SerpinB1 expression levels in human serum samples obtained from (1) lean healthy individuals, (2) obese individuals and (3) obese individuals with non-alcoholic steato hepatitis (NASH) revealed a potential increase in SerpinB1 expression in the serum of obese individuals as compared to lean individuals, while expression levels appeared lower in the obese + NASH group as compared to the other two groups (Figure 29 N).

Example 7

Effects of SerpinB1 and neutrophil elastase inhibitors on β-cell proliferation

To test whether SerpinB1 is a β-cell growth factor, we cultured freshly isolated primary mouse islets in presence of various doses of recombinant human SerpinB1 or ovalbumin (SerpinB14) and evaluated β-cell proliferation by Ki67 immunofluorescent staining (known to those of ordinary skill in the art). We observed that while mouse islets cultured in ovalbumin (1μg/ml) displayed normal low β-cell proliferation, isolated islets cultured with recombinant SerpinB1 exhibited a dose-dependent increase in Ki67+ insulin+ cells; the data reached statistical significance when islets were cultured at a concentration of 1 μg/ml of SerpinB1 compared to controls (islets cultured at a similar concentration of ovalbumin).

To evaluate whether increased SerpinB1 in liver contributes to increased endogenous β-cell replication observed in insulin resistant models, we assessed the ability of serum and media collected from culturing liver explants (Liver Explant Conditioned Media, LECM) harvested from HFD and ob/ob mice to stimulate islet β-cell proliferation *in vitro*. To this end, islets derived from 5-6 week old C57BL/6J male mice were cultured for 24 hours in media containing serum (dilution 1:10) or LECM (dilution 1:10) derived from animals fed either chow diet (CD) or high fat diet (HFD) or from ob/ob animals and respective controls, followed by evaluation of β-cell proliferation by Ki67 immunostaining and fluorescence microscopy (Figure 19 A). We observed that sera derived from HFD (HFD serum 0.85% ± 0.1% vs. CD serum 0.04% ± 0.1%, p=0.03, n=7) and ob/ob (ob/ob serum 0.69% ± 0.1% vs. WT serum 0.26% ± 0.05%, p=0.09, n=3-5) both significantly stimulated β-cell proliferation. While mouse islets cultured in ob/ob LECM as compared to WT LECM showed a significant two-fold increase in -cell replication (ob/ob LECM 0.27% ± 0.03% vs. WT LECM 0.16% ± 0.03%, p=0.04, n=4), the effects of HFD LECM also showed a greater effect but did not reach statistical significance compared to CD LECM treated islets (HFD LECM 2% ± 0.4% vs. CD LECM 1.5% ± 0.3%, p=0.39, n=6), (Figure 19 B and C). These observations provide a functional correlation between increased liver SerpinB1 and enhanced islet β-cell hyperplasia in insulin resistance (see also Figure 27).

To address whether SerpinB1 acts directly to stimulate β-cell proliferation, we cultured mouse islets in the presence of various doses of human recombinant SerpinB1 and a control (ovalbumin), and evaluated β-cell proliferation by Ki67 immunofluorescence staining (Figure 21 A). We observed that while the control (ovalbumin; 1µg/ml) treated mouse islets as expected displayed low β-cell proliferation, treatment with recombinant SerpinB1 exhibited a dose-dependent increase in Ki67+insulin+ cells and reached statistical significance at a dose of 1µg/ml (Figure 25 A & B).

A major substrate of SerpinB1 is neutrophil elastase. To investigate whether the proliferative action of SerpinB1 is mediated by antagonizing neutrophil elastase activity, we assessed the ability of the neutrophil elastase inhibitor, Sivelestat (Sivelestat is the International Nonproprietary Name (INN) as given by the World Health Organization (WHO); the chemical name is: *N*-{2-[({4-[(2,2-dimethylpropanoyl)oxy]phenyl}sulfonyl)amino]benz-oyl}glycine), to stimulate islet β-cell proliferation *in vitro*. Using various doses, we observed that low doses (1 and 5 µg/ml; see Figure 12) do not enhance β-cell proliferation. Conversely, a substantial increase in the number of proliferating insulin+ cells was observed in islets cultured at higher doses (e.g. 10 and 50 µg/ml). Importantly, Sivelestat at a dose of 100 µg/ml increased the number of human (EndoC-βH1) Ki67 positive β-cells compared to non-treated cells.

Pharmacological molecules that mimic SerpinB1 promote β-cell replication *in vivo*

To explore whether increased β-cell replication secondary to enhanced SerpinB1 activity can be recapitulated *in vivo*, we used pharmacological compounds Sivelestat (Kawabata, et al., 1991) and GW311616A (Macdonald, et al., 2001), that are selective inhibitors of elastase, one of the major substrates of SerpinB1. Using osmotic pumps, we delivered a continuous infusion of Sivelestat for a 2-week period followed by evaluating the number of replicating β-cells by BrdU incorporation (Figure 20A). We observed a significant effect at the dose of 300µg/kg/day and importantly, the proliferation was restricted to β-cells and did not significantly alter glucagon-producing α-cells (Figure 20B and Figure 23 A & B, E & F). The effect on β-cells was also confirmed by pHH3 staining (Figure 23 C & D). In a second approach we gavaged mice with the NE inhibitor, GW311616A, at a dose of 2 mg/kg/day for 2 weeks (Figure 20C and Figure 22 A). Similar to the effects of Sivelestat we observed a significantly enhanced β-cell mass and an increased β-cell proliferation but not α-cell proliferation as assessed by BrdU incorporation (Figure 20D and Figure 22 B-E). Moreover, cell proliferation was not increased in extra-pancreatic tissues including liver, skeletal muscle, visceral and subcutaneous adipose tissue, spleen and kidney (Figure 22 F & G). We also showed that both GW311616A and Sivelestat directly increased mouse islet β-cell proliferation *in vitro* (Figure 25 C). Finally, mice lacking elastase exhibited increased β-cell proliferation as compared to age-matched animals (Figure 30). Taken together, these data indicate that SerpinB1 promotes β-cell proliferation, in part, by inhibiting elastase.

We next tested whether the effects are conserved by treating human islet β-cells with the pharmacological agents that mimic the activity of SerpinB1. Assessment of Ki67+ insulin+ cells revealed that the number of replicating insulin-producing cells is significantly higher when islets were incubated with either Sivelestat or GW311616A (Figure 21 A & B and Figure 25 D). These data indicate that SerpinB1 acts directly to promote human β-cell proliferation and indicates therapeutic implications for Sivelestat and GW311616A in treatment of α-cell and β-cell related disorders.

Example 8

Effect of SerpinB1 on proliferation of mouse and human β-cell *in vitro* and *in vivo*

To directly assess the role of SerpinB1 on β-cell replication *in vitro*, mouse hepatocytes will be infected with constructions overexpressing SerpinB1 or negative constructions for 24, 48 or 96 hours. One of ordinary skill in the art is capable of constructing suitable expression vectors. Cultured media from infected cells will be used to stimulate mouse or human primary islets and β-cell proliferation will be assessed in *in vitro* assays. To assess the impact of SerpinB1 on β-cell proliferation *in vivo* we are generating Associated-adenoviruses (AAV) driving the expression of SerpinB1 (using, for example, the sequences of Figures 13 and 14, i.e. SEQ ID NO: 1 and SEQ ID NO: 2) using a ubiquitous CMV promoter or a liver specific albumin promoter. Injection of AAV-albumin-SerpinB1 via the tail injection will allow for over-expression of the SerpinB1 in the liver. The effects of this over-expression on β-cell proliferation will be assessed 12-16 weeks after injection of the AAV.

As an initial experiment, we constructed AAVs encoding EGFPII or mouse SerpinB1 and assessed the effect of short term (3 weeks) liver overexpression of SerpinB1 *in vivo* by tail vein injection in 8 to 10 week-old mice (Figure 24 A). Concomitant with increased circulating levels of SerpinB1 (Figure 24 B), mice transduced with AAV-SerpinB1 exhibited an increased β-cell mass, increased insulin serum levels and an enhanced β-cell replication as assessed by Ki67 immunostaining (Figure 24 C-G). The islet/pancreas area was increased to a similar extent as the β-cell mass (data not shown). The liver-specific alpha-1-anti-trypsin (A1AT) promoter was used as promoter.

Next, 8 to 10 week-old mice were transduced *in vivo* by tail vein injection with AAV encoding EGFPII or SerpinB1 (Figure 26). Seven and fifteen weeks post-transduction, *in vivo* glucose-stimulated insulin secretion experiments were performed (dose: 3g/kg body weight). Mice overexpressing SerpinB1 showed enhanced glucose-stimulated insulin secretion seven and fifteen weeks post-AAV transduction (Figure 26 A and B). These results strongly suggest that long term over-expression of SerpinB1 enhances glucose-stimulated insulin secretion *in vivo*.

In a second model, mice over-expressing the AAV-SeprinB1 in the liver will be transplanted with human islets to create a “humanized mouse model”. Mice will be monitored for body weight, blood glucose for 2, 4 and 16 weeks. At the end of the experiment, islet grafts and pancreases will be harvested and analyzed for proliferation and survival of endocrine cells. This model will directly indicate whether altering the expression of SerpinB1 in the liver promotes human β-cell proliferation *in vivo* – with important implications for human therapy.

Example 9

Mechanisms underlying the actions of SerpinB1

To gain insights into the mechanisms underlying the effects of SerpinB1 we will undertake several approaches as outlined below:

a) Further examine how expression of SerpinB1 using an adeno-associated-virus (AAV-SerpinB1) in the liver will potentially impact β-cell proliferation.

b) Plasma membrane localization of SerpinB1 substrates in hepatocytes and pancreatic β-cells. We will first analyze the localization of SerpinB1 by immunostaining and western blotting of hepatocytes and β-cells to investigate whether the major substrate of SerpinB1 (neutrophil elastase) is expressed at the plasma membrane. We will also analyze expression and localization of other substrates including proteinase 3 and chymase.

c) Identification of signaling cascades downstream of SerpinB1. Mouse and human islets treated with SerpinB1 and Sivelestat for 5, 10, and 60 minutes will be subjected to proteomics analysis to identify substantial variations in key phospho-protein signaling molecules.

d) We will dissect the SerpinB1 signaling pathway(s) by creating gain-of function and loss-of function mouse models of potential candidates identified in b).

e) The role of SerpinB1 as permissive factor insulin signaling: We will examine how SerpinB1 interacts with proteins in other growth factors (e.g. insulin and insulin-like-growth factor1) to investigate whether the effects are additive or synergistic.

f) A recent study showed that mice injected with recombinant neutrophil elastase demonstrated decreased levels of IRS-1 and downstream signaling in liver (Saswata, et al, *Nature Medicine* 2012). Therefore, one plausible mechanism by which SerpinB1 and Sivelestat are acting may be directly related to their ability to limit the Neutrophil elastase-mediated IRS-1 down-regulation. In this context, we plan to analyze whether SerpinB1 act as factor enhancing the expression and activation of elements of insulin signaling including IRS-1 and downstream signaling molecules.

g) We plan to undertake studies in human islets and human β-cells to further establish the role of SerpinB1 and related family members on their ability to safely and significantly enhance β-cell proliferation with the long term goal of using this approach to enhance functional β-cell mass in humans with diabetes.

All studies discussed above have therapeutic implications.

5) Anticipated Results: We describe the identification of a new liver-derived β-cell growth factor promoting β cell proliferation in the context of insulin resistance. Preliminary data demonstrate that “SerpinB1” is crucial to promote β-cell mass. The studies in progress and planned will provide additional data to support the potential use of SerpinB1 and/or the modulation of SeprinB1 production and function, and one or more of its family members (e.g., Clade B family), as potential therapeutic agents to enhance functional β-cell mass in humans for the treatment and/or prevention of type 1 and type 2 diabetes in humans.

Experimental Procedures – not noted elsewhere herein

Animals

Mice were housed in pathogen-free facilities and maintained on a 12 hr light/dark cycle in the Animal Care Facility at Joslin Diabetes Center, Boston, and the Foster Biomedical Research Laboratory, Brandeis University, Waltham, MA. All studies conducted and protocols used were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and Brandeis University and were in accordance with NIH guidelines. LIRKO mice were generated by crossing Albumin-*Cre* to $IR^{flox/flox}$ on a mixed genetic background and were backcrossed for more than 15 generations on the C57/Bl6 background. LIRKO mice (Albumin-$Cre^{+/-}$,$IR^{flox/flox}$) and their littermate Lox controls (Albumin-$Cre^{-/-}$,$IR^{flox/flox}$) were genotyped as described previously by Okada et al. (2007, Insulin receptors in beta-cells are critical for islet compensatory growth response to insulin resistance. Proc. Natl. Acad. Sci. USA 104, 8977–8982). Blood glucose was monitored using an automated glucose monitor (Glucometer Elite; Bayer), and plasma insulin was detected by ELISA (Crystal Chem). To produce HFD animals, C57/Bl6 mice were placed on a high fat diet consisting of 45% of kcal for 12 weeks prior to experiments. Ten -week- old ob/ob mice and their age-matched controls were purchased from the Jackson Laboratory (Bar Harbor, ME).

Parabiosis

Parabiosis surgery was performed as described earlier by Eggan et al. (2006, Ovulated oocytes in adult mice derive from non-circulating germ cells. Nature 441, 1109–1114). Cross-circulation was determined 2 weeks after surgery by Evans Blue transmission (Pietramaggiori, G., *et al.*, (2009). Improved cutaneous healing in diabetic mice exposed to healthy peripheral circulation. J. Invest. Dermatol. 129, 2265–2274). Body weight and blood glucose of parabiont animals were monitored weekly. After a 16 week parabiosis period, animals were sacrificed, and pancreases were collected for morphometric analysis.

Islet Isolation and Transplantation

Islets were isolated from 9-month-old mice using the intraductal collagenase technique (Kulkarni, R.N., *et al.*, 1999); El Ouaamari, *et al.*, 2013, Cell Rep., 3(2):401-410). Altered function of insulin receptor substrate-1-deficient mouse islets and cultured beta-cell lines. J. Clin. Invest. 104, R69–R75). Islets were handpicked, concentrated in a pellet, and kept on ice until transplantation (Flier, S.N., *et al.*, (2001). Evidence for a circulating islet cell growth factor in insulin-resistant states. Proc. Natl. Acad. Sci. USA 98, 7475–7480). Surgery was performed in mice after intraperitoneal injection (15 ml/g body weight) of a 1:1 (w/v) mixture of 2,2,2-tribromoethanol and *tert*-amyl alcohol and diluted 1:50 with PBS (pH 7.4). The capsules of the kidneys were incised, and the islets were implanted near the upper pole of each kidney in 5-month-old male mice. The capsules were cauterized, and the mice were allowed to recover on a heating pad.

Growth Factors and Hormones Assays

ELISA-based assays were used to measure growth factors and hormones, including IGF-1 (catalog #MG100; R&D Systems), HGF (catalog #ab100686; Abcam), EGF (catalog #IB39411; IBL-America, Minneapolis, MN), PDGFAA (catalog #DAA00B; R&D Systems), PDGFBB (catalog #MBB00; R&D Systems, Minneapolis, MN), VEGF (Millipore, Billerica, MA), FGF21 (catalog #EZRMFGF21-26K; Millipore), Gastrin (catalog #E91224mu; USCN Life Science, Hubei 430056, PRC), Adiponectin (catalog #EZMADP-60K; Millipore), Ostepontin (catalog #MOST00; R&D Systems), and Osteocalcin (catalog #EIA4010; International). Multiplex-based assays were used to measure endocrine hormones (catalog #MENDO-75; Millipore), gut hormones (catalog #MGT-78K; Millipore), adipokines (catalog #MADPK-71K; Millipore), and Cytokines/Chemokines (catalog #MPXMCYTO-70K.lxt; Millipore).

Serum Stimulation

Sera were obtained after coagulated blood was centrifuged twice for 15 min at 8,000 rpm at 4^{O}C and stored at ~80^{O}C until use. Pancreatic islets were isolated from 5-week-old male mice by liberase and thermolysin digestion (Roche), handpicked, and cultured for 16 hr in RPMI 1640 with 7 mM glucose and 10% FBS (v/v). A total of 150 size-matched mouse islets were starved in RPMI 1640 with 0.1% BSA (v/v) containing 3 mM glucose for 3 hr and thereafter treated with RPMI 1640 with 5.5 mM glucose supplemented every 12 hr with 10% (v/v) serum obtained from 3- or 12-month-old LIRKO and control mice. Twenty-four to 48 hr later, islets were handpicked, fixed with 4% paraformaldehyde, embedded in agarose/paraffin, and sectioned for immunohistochemistry studies. To evaluate β cell replication, sections were analyzed by fluorescent microscopy subsequent to Ki67, TUNEL, and insulin immunostaining.

LECM Stimulation

Liver explant-conditioned medium (LECM) preparation was adapted from Nicoleau, et al. (2009, Endogenous hepatocyte growth factor is a niche signal for subventricular zone neural stem cell amplification and self-renewal. Stem Cells 27, 408–419). Mice were anesthetized with Avertin (240 mg/kg intraperitoneally), and 100 mg liver explants were dissected from LIRKO or control mice. Explants were washed twice in cold PBS, incubated in PBS at 37^{O}C for 30 min, and then cultured in serum-free Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose. After a 3 day incubation, LECM were collected, centrifuged, and kept at ~80^{O}C till use. Islets were initially starved for 3 hr in DMEM containing 3 mM glucose and 0.1% BSA and thereafter stimulated for 24 hr with DMEM/5.5 mM glucose media containing 10% LECM. Islet β cell proliferation and apoptosis were analyzed by fluorescent microscopy after Ki67, TUNEL, and insulin immunostaining.

HCM Stimulation

Hepatocytes were isolated from 6-month-old LIRKO and control mice by collagenase digestion via portal vein perfusion (Sun, R., *et al.*, (2005). IL-6 modulates hepatocyte proliferation via induction of HGF/p21cip1: regulation by SOCS3. Biochem. Biophys. Res. Commun. 338, 1943–1949). Mice were anesthetized with Avertin (240 mg/kg intraperitoneally), and the portal vein was cannulated with JELCO 22G x 1 inch catheter (Smiths Medical, Dublin, OH). The liver was perfused with EGTA solution (5.4 mmol/l KCl, 0.44 mmol/l KH_2PO_4, 140 mmol/l NaCl, 0.34 mmol/l Na_2HPO_4, and 0.5 mmol/l EGTA [pH 7.4]) and digested with DMEM containing 0.075% type I collagenase. Hepatocytes were washed twice in Hepatocyte Wash Medium (Invitrogen, Grand Island, NY). The isolated mouse hepatocytes were seeded in collagen-coated 6-well plates (BD BioCoat, San Jose, CA) at a density of 106 cells/well in DMEM containing 25 mM glucose and 10% FBS (v/v). Sixteen hours later, hepatocytes were cultured for 24 hr in serum-free DMEM containing 5.5 mM glucose. HCM was collected, centrifuged, and kept at ~80^{O}C. Islets were initially starved for 3 hr in DMEM containing 3 mM glucose and 0.1% BSA and thereafter incubated for 24 hr in DMEM/5.5mMglucose media containing 50% HCM. Islet β cell proliferation and apoptosis were analyzed by fluorescent microscopy after Ki67, TUNEL, and insulin immunostaining.

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 arrival, islets were cultured overnight in Miami Media

#1A (Cellgro). The islets were then starved in Final Wash/Culture Media (Cellgro, Manassas, VA) for 3 hr prior to stimulation with serum (diluted to 10% v/v), LECM (diluted to 10% v/v), or HCM (diluted to 50% v/v) or Miami Media #1A supplemented with Sivelestat or GW311616A for 24 hr. Islets were used for immunostaining studies as described (El Ouaamari, *et al.*, 2013).

Immunostaining Studies

Pancreases and islets were analyzed by immunostaining using anti-Ki67 (BD), anti-insulin (Abcam, Cambridge, MA), or anti-glucagon (Sigma-Aldrich, St. Louis, MO) antibodies. Quantification of replicating β- and α-cells was performed as described previously (El Ouaamari, *et al.*, 2013).

Counting Proliferating β Cells

In all experiments, cell counting was manually performed in a blinded fashion by a single observer. BrdU+ or Ki67+ β cells were assessed by immunofluorescence microscopy. Insulin+ cells showing nuclear DAPI staining were considered as β cells. Insulin+ cells showing nuclear colocalized staining for DAPI+ and Ki67+ (or BrdU+) were counted as proliferating β cells. The double-positive cells (Ins+/BrdU+ or Ins+/Ki67+) were confirmed in randomly selected cells in all experiments by confocal microscopy.

BrdU Injection Studies

Mice were injected with BrdU intraperitoneally (100 mg/kg body weight) 5 hr prior to animal sacrifice for immunostaining of the pancreas. BrdU injections in the *in vivo* serum administration experiments were performed on three occasions as denoted in Figure 2A.

Delivery of Sivelestat and GW311616A

For Sivelestat administration, mice were anesthetized, and osmotic pumps (ALZET) containing 100 µl of either vehicle or Sivelestat (Santa Cruz) were implanted subcutaneously. Sivelestat was dissolved in 50% DMSO and was administered at a dose of 150 or 300 µg/kg/day. Control mice were infused with DMSO 50% alone. GW311616A was administered into mice by daily oral gavage for two weeks. Mice received either GW311616A (2 mg/kg body weight) or vehicle.

Proteomics

Proteins extracted from hepatocyte-conditioned media were pre-enriched on nanoparticles (NanoScape AG, Germany). Samples were resolved by SDS-PAGE NuPAGE 4-12% Bis-Tris gels and stained using SilverQuest (Invitrogen). The proteins captured on nanozeolites were reduced and alkylated and then digested with LysC (1/50 w/w) for 4 hours at 37 °C and then with trypsin (1/50 w/w) for 18 hours at 37 °C. Enzymatic digests were subjected to liquid chromatography mass spectrometry (LC-MS) analysis. LC-MS experiments were performed on NanoAcquity UPLC (Waters, Milford, MA) connected to a hybrid LTQ (Linear Trap Quadropole) Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a nanoelectrospray source. Protein digests were loaded onto a nanoAcuity UPLC Trap column (Symmetry C18, 5 µm, 180 µm x 20 mm, Waters) and peptides were eluted on a C18 reverse-phase nanoAcquity column (BEH130 C18, 1.7 µm, 75 µm x 250 mm, Waters) with a linear gradient of 7-30 % solvent B (H2O/CH3CN/HCOOH, 10:90:0.2, by vol) for 120 min, 30-90% Solvent B for 20 min, and 90% solvent B for 5 min, at a flow rate of 250 nL/min. samples were injected in triplicate. LC-MS data were acquired using the Xcalibur software (version 2.07, Thermo-Fisher Scientific) and were processed using a Visual Basic program developed using XRawfile libraries (distributed by Thermo-Fisher Scientific). Data base searches were performed using MASCOT server (version 2.1, matrix Science; www.matrixscience.com/) using the Swiss-Prot database.

Western blotting

150 mg of livers were lysed in RIPA buffer and total protein concentration was determined using BCA assay (Pierce, Rockford, IL). Samples were resuspended in Laemmli buffer, boiled and resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes, blocked in blocking buffer (PBS containing 5% Bovine Serum Albumin and 0.1% tween 20) and incubated with primary antibodies SerpinB1 (Santa cruz, sc-34305; Santa Cruz Biotechnology, Santa Cruz, CA) or Actin (Santa cruz, sc-1615) for overnight or one hour, respectively. Secondary rabbit anti-goat (Santa cruz, sc-2922) were used thereafter. Quantification of protein amounts were estimated by ImageJ software (National Institutes of Health, Bethesda, Maryland).

Statistical Analysis

All data are presented as mean ± SEM and analyzed using unpaired, two-tailed Student’s t test. A p value of less than 0.05 is considered significant.

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CLAIMS

1. Use of a Serpin peptide or active fragment, or an analog thereof as medicament.

2. The use of Claim 1, wherein said Serpin peptide is SerpinB1.

3. The use of Claim 1, wherein said Serpin analog is a known functional analog.

4. The use of Claim 1, wherein said Serpin analog is a known structural analog.

5. The use of Claim 1, wherein the subject has diabetes.

6. The use of Claim 1, wherein the subject is at risk of developing diabetes.

7. A Serpin peptide or active fragment, or an analog thereof for improving the β cell function in a subject.

8. The peptide or active fragment, or analog of Claim 7, wherein said Serpin peptide is SerpinB1.

9. The peptide or active fragment, or analog of Claim 7, wherein said Serpin analog is a known functional analog.

10. The peptide or active fragment, or analog of Claim 7, wherein said Serpin analog is a known structural analog.

11. The peptide or active fragment, or analog of Claim 7, wherein the subject has diabetes.

12. The peptide or active fragment, or analog of Claim 7, wherein the subject is at risk of developing diabetes.

13. A Serpin peptide or active fragment, or an analog thereof for promoting pancreatic β cell proliferation in a subject.

14. The peptide or active fragment, or an analog of Claim 13, wherein said Serpin peptide is SerpinB1.

15. The peptide or active fragment, or an analog of Claim 13, wherein said population of pancreatic β cells are *in vivo*.

16. The peptide or active fragment, or an analog of Claim 13, wherein said population of pancreatic β cells are *in vitro*.

17. The peptide or active fragment, or an analog of Claim 13, wherein said Serpin analog is a known functional analog.

18. The peptide or active fragment, or an analog of Claim 13, wherein said Serpin analog is a known structural analog.

19. The peptide or active fragment, or an analog of Claim 13, wherein increased pancreatic β cell proliferation *in vivo* is indicated by detecting increased glycemic control in the subject.

20. An expression-construct encoding a Serpin peptide or active fragment, or analog thereof for improving the β cell function in a subject.

21. The expression-construct encoding a Serpin peptide or active fragment, or analog thereof of Claim 20, wherein said encoded Serpin peptide is SerpinB1.

22. The expression construct encoding a Serpin peptide or active fragment, or analog thereof of Claim 20, wherein said encoded Serpin peptide analog is a known functional analog.

23. The expression construct encoding a Serpin peptide or active fragment, or analog thereof of Claim 20, wherein said encoded Serpin peptide analog is a known structural analog.

24. The expression-construct encoding a Serpin peptide or active fragment, or analog thereof of Claim 20, wherein the subject has diabetes.

25. The expression-construct encoding a Serpin peptide or active fragment, or analog thereof of Claim 20, wherein the subject is at risk of developing diabetes.

26. The use of a Serpin peptide or active fragment, or an analog thereof for manufacturing of a medicament for improving the β cell function in a subject.

27. The use of Claim 26, wherein said Serpin peptide is SerpinB1.

28. The use of Claim 26, wherein said Serpin analog is a known functional analog.

29. The use of Claim 26, wherein said Serpin analog is a known structural analog.

30. The use of Claim 26, wherein the subject has diabetes.

31. The use of Claim 26, wherein the subject is at risk of developing diabetes.

32. The use of a Serpin peptide or active fragment, or an analog thereof for manufacturing of a medicament for promoting pancreatic β cell proliferation in a subject.

33. The use of Claim 32, wherein said Serpin peptide is SerpinB1.

34. The use of Claim 32, wherein said population of pancreatic β cells are *in vivo*.

35. The use of Claim 32, wherein said population of pancreatic β cells are *in vitro*.

36. The use of Claim 32, wherein said Serpin analog is a known functional analog.

37. The use of Claim 32, wherein said Serpin analog is a known structural analog.

38. The use of Claim 32, wherein increased pancreatic β cell proliferation *in vivo* is indicated by detecting increased glycemic control in the subject.

39. The use of an expression construct encoding a Serpin peptide or active fragment, or analog thereof for manufacturing of a medicament for improving the β cell function in a subject.

40. The use of Claim 39, wherein said encoded Serpin peptide is SerpinB1.

41. The use of Claim 39, wherein said encoded Serpin peptide analog is a known functional analog.

42. The use of Claim 39, wherein said encoded Serpin peptide analog is a known structural analog.

43. The use of Claim 39, wherein the subject has diabetes.

44. The use of Claim 39, wherein the subject is at risk of developing diabetes.

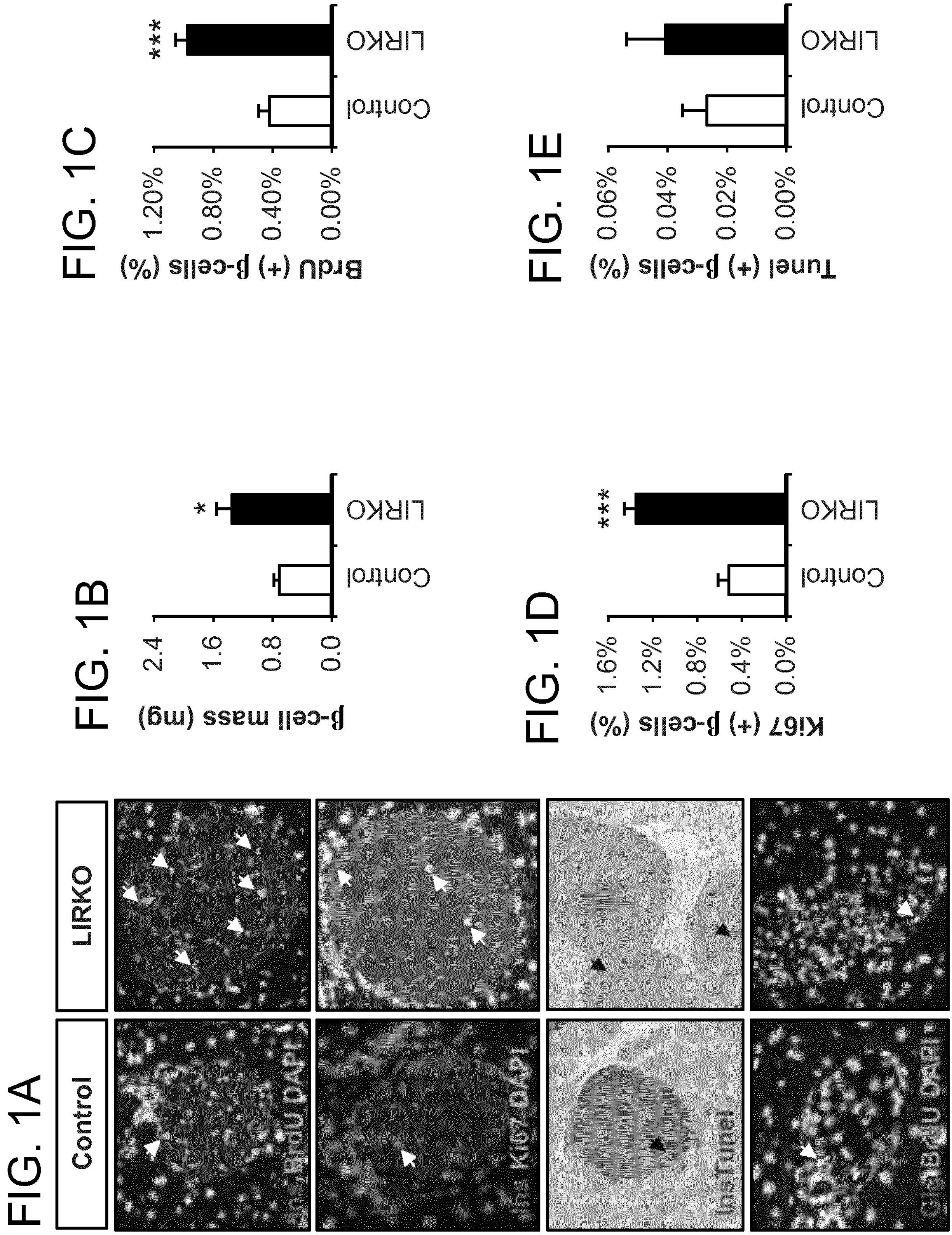


FIG. 1A FIG. 1B FIG. 1C FIG. 1D FIG. 1E

FIG. 1F

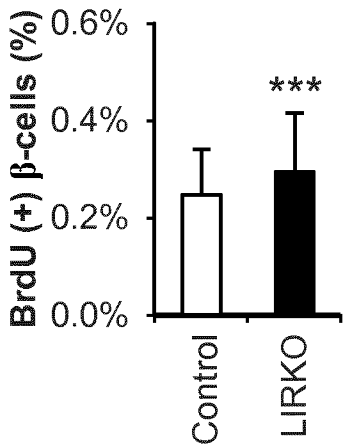


FIG. 1H

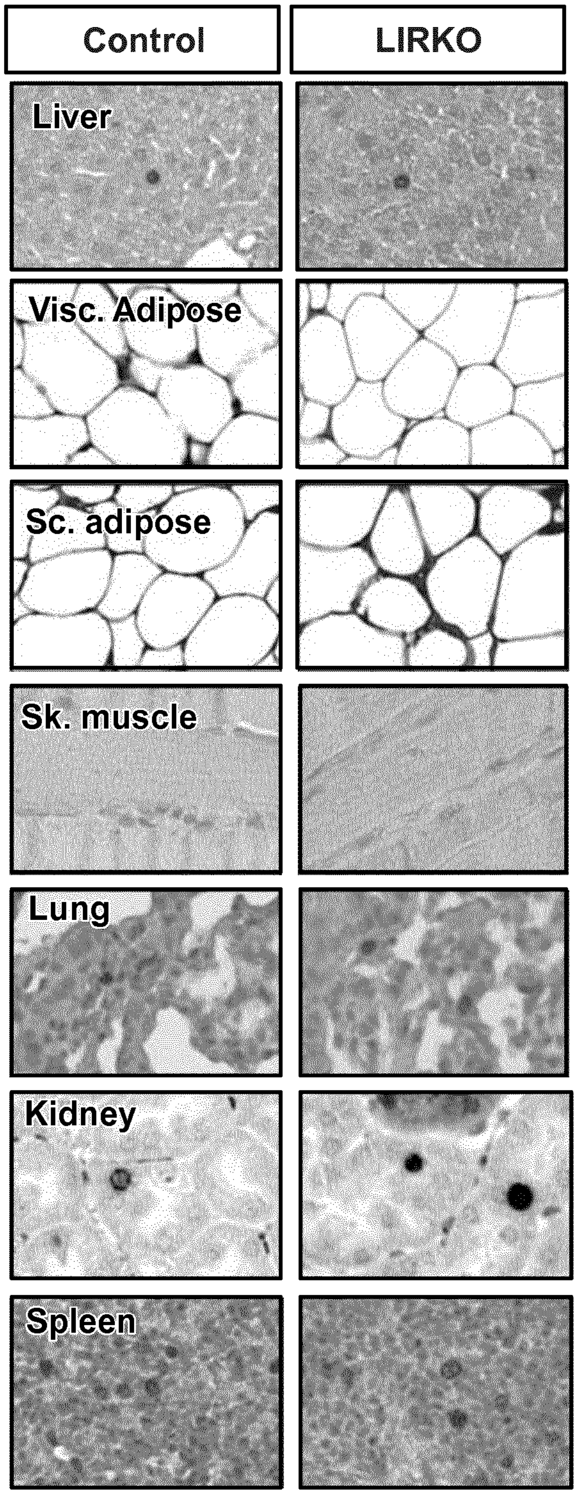


FIG. 1G

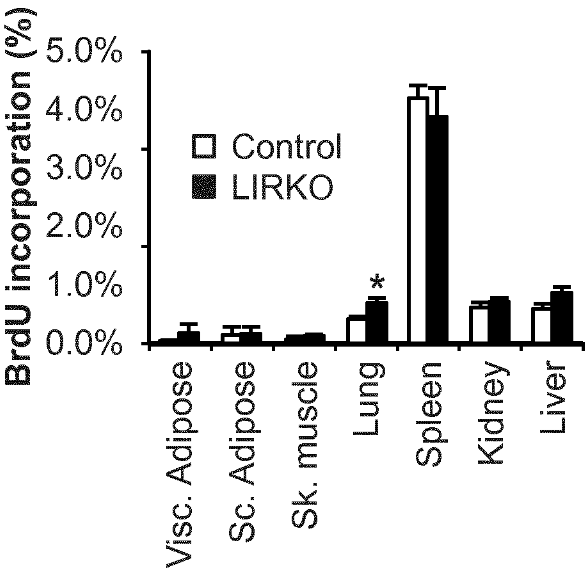


FIG. 2A

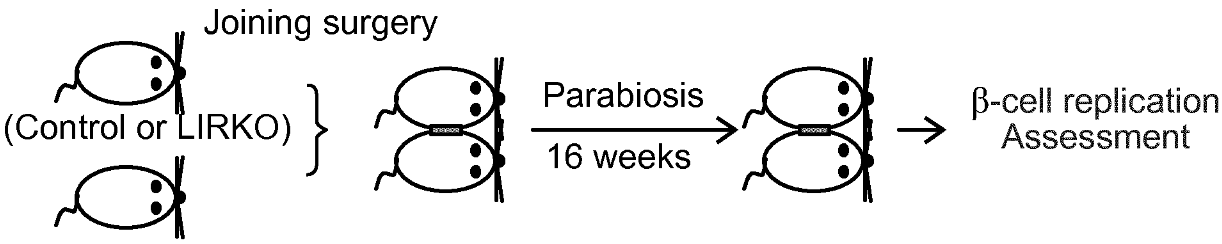


FIG. 2B

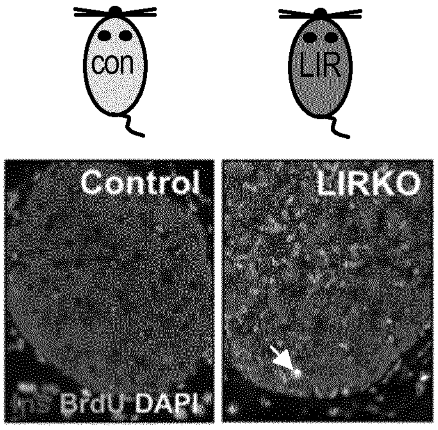


FIG. 2C

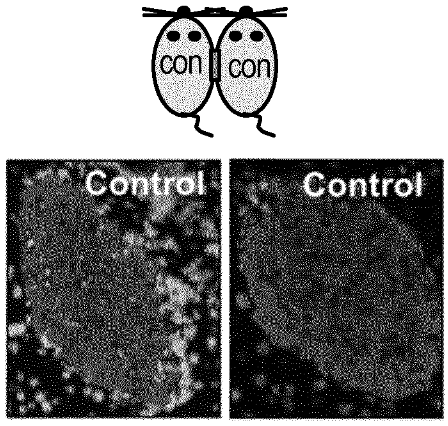


FIG. 2D

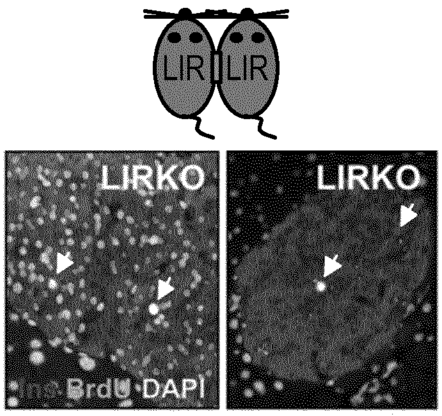


FIG. 2E

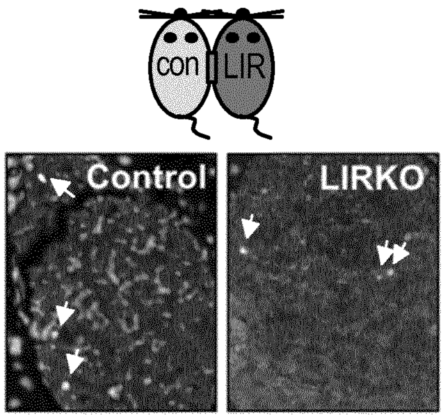


FIG. 2F

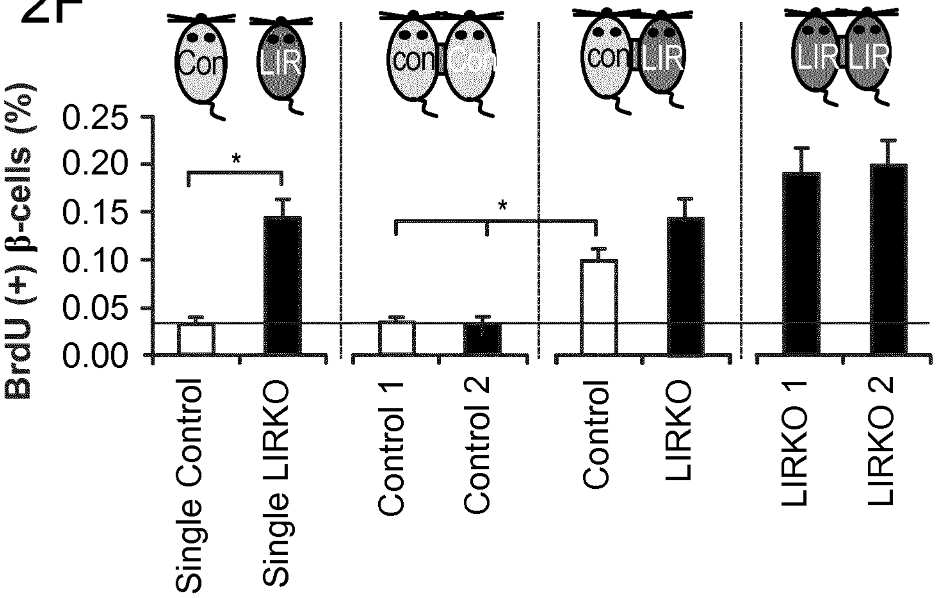


FIG. 2G

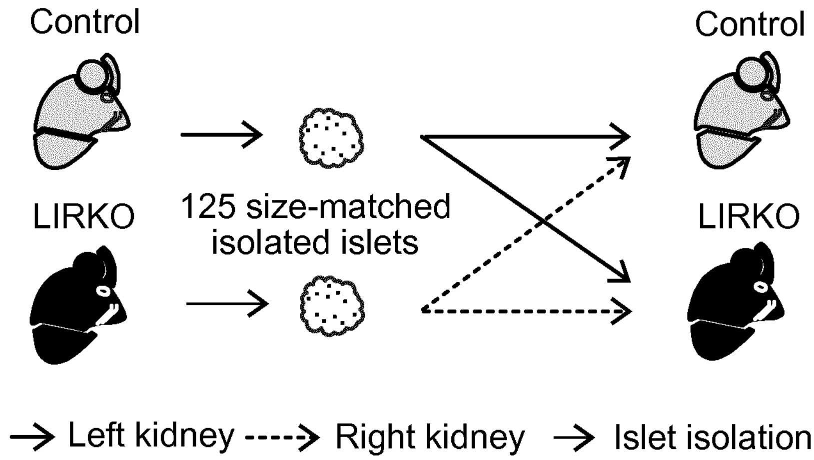


FIG. 2H

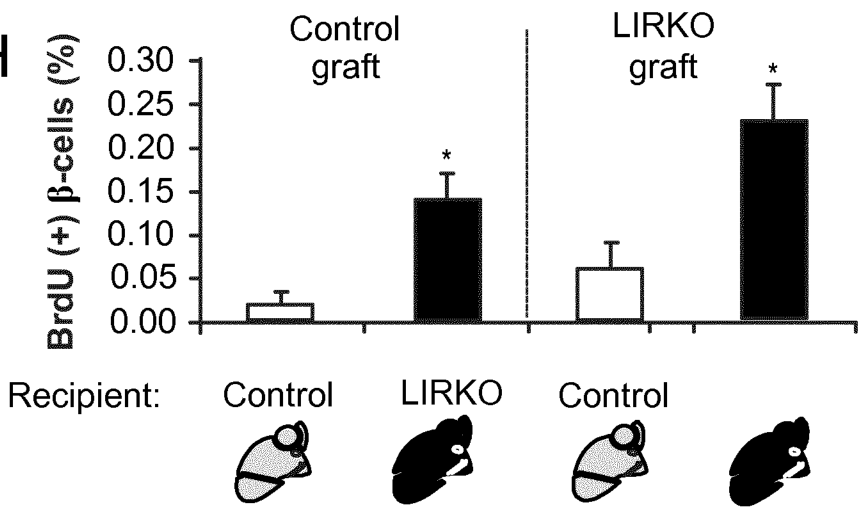


FIG. 3A

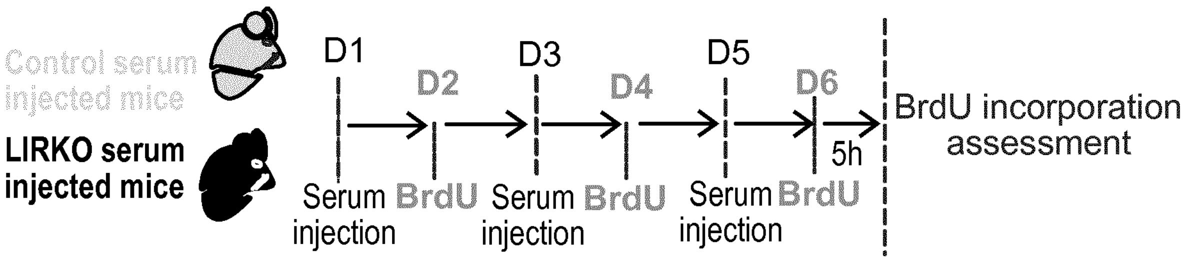


FIG. 3B

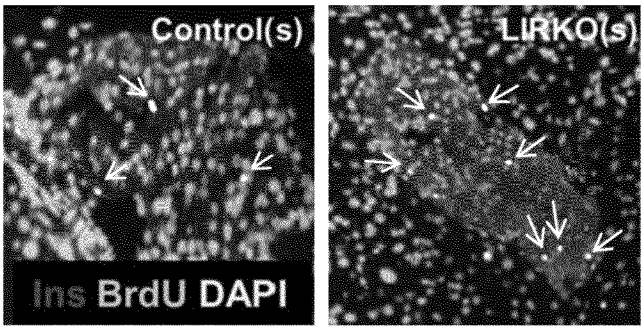


FIG. 3C

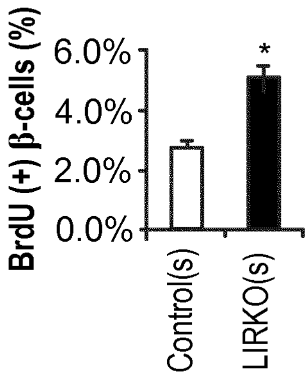


FIG. 3D

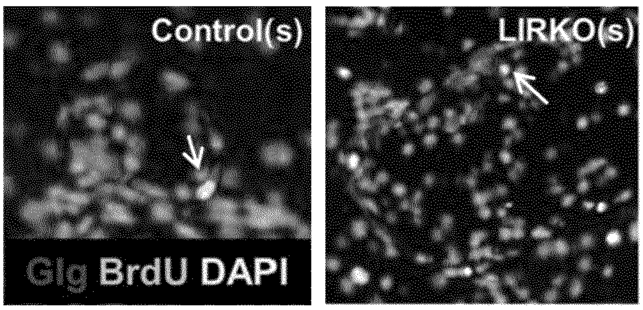


FIG. 3E

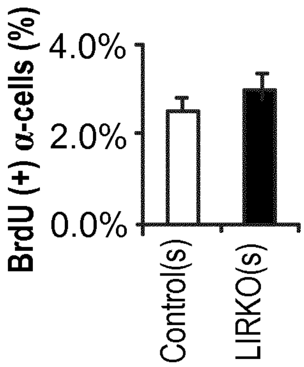


FIG. 3F

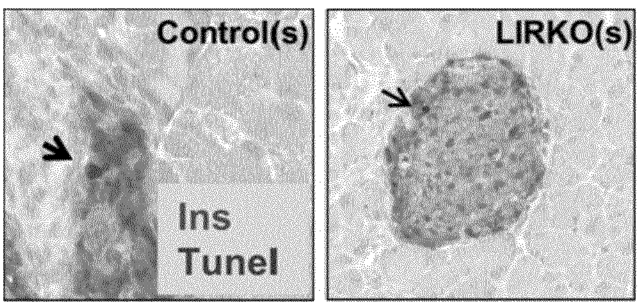


FIG. 3G

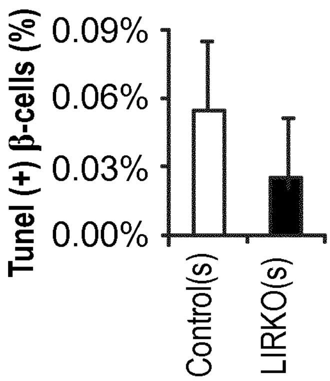


FIG. 3H

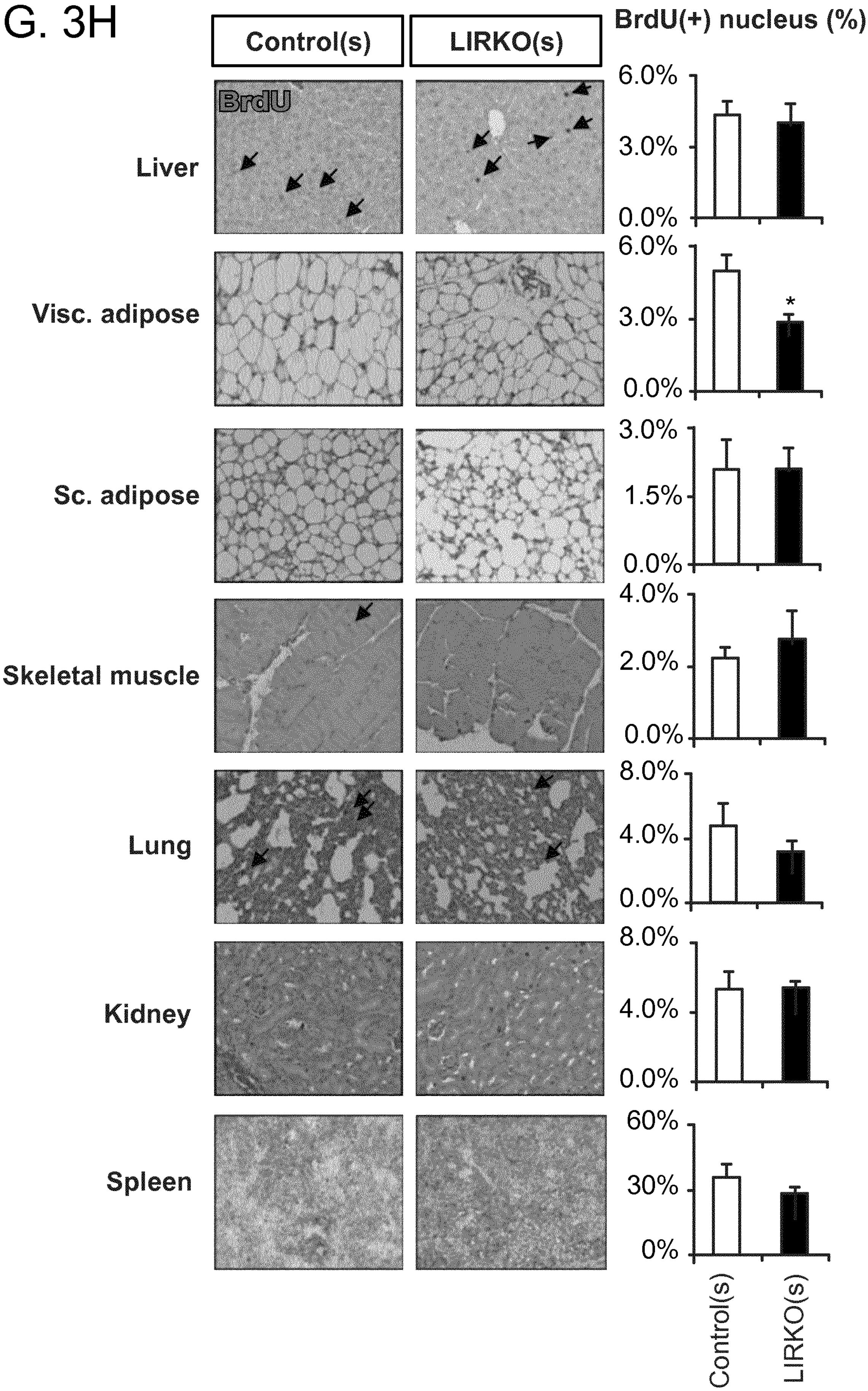

Control(s)
LIRKO(s)
BrdU(+) nucleus (%)
BrdU
Liver
Visc. adipose
Sc. adipose
Skeletal muscle
Lung
Kidney
Spleen
6.0%
3.0%
0.0%
*
1.5%
4.0%
2.0%
8.0%
60%
30%
0%
Control(s)
LIRKO(s)

FIG. 4A

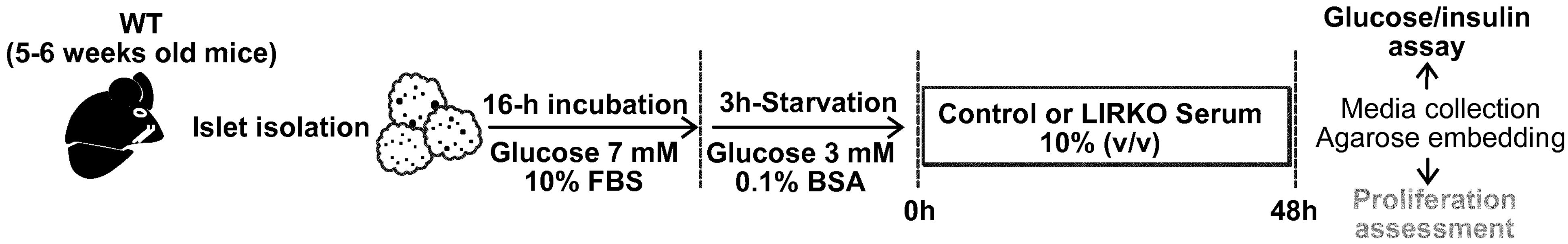

WT
(5-6 weeks old mice)
Islet isolation
16-h incubation
Glucose 7 mM
10% FBS
3h-Starvation
Glucose 3 mM
0.1% BSA
Control or LIRKO Serum
10% (v/v)
0h
48h
Glucose/insulin assay
Media collection
Agarose embedding
Proliferation assessment

FIG. 4B

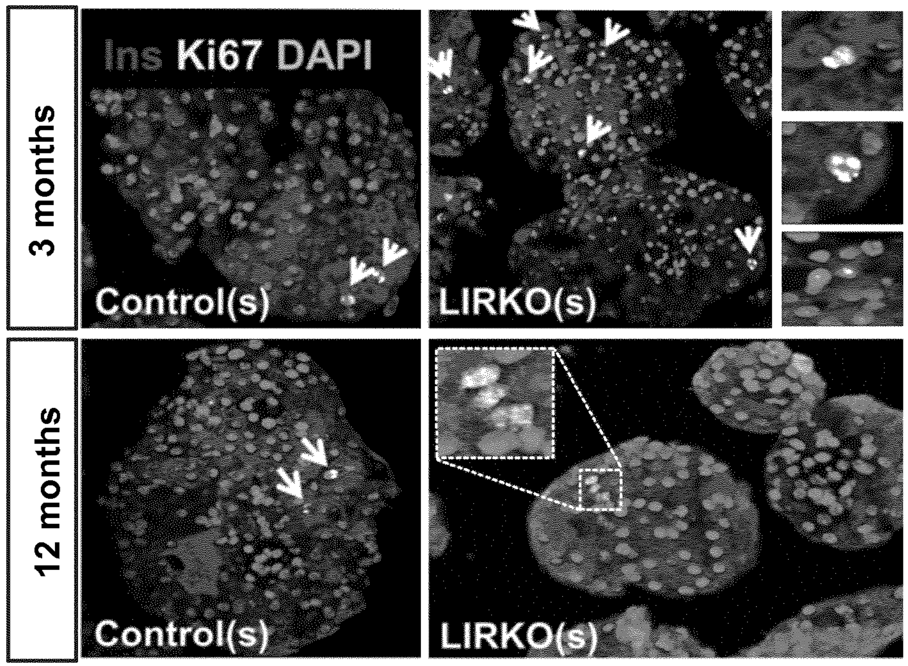

3 months
12 months
Ins Ki67 DAPI
Control(s)
LIRKO(s)
Control(s)
LIRKO(s)

FIG. 4C

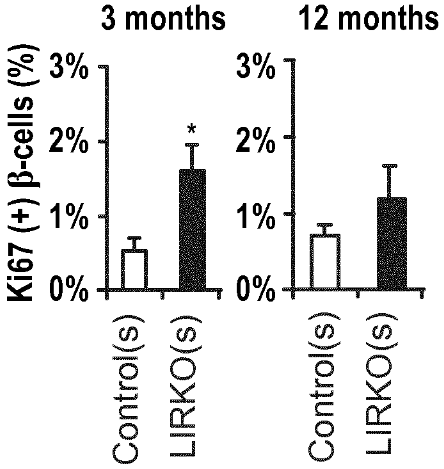


FIG. 4D

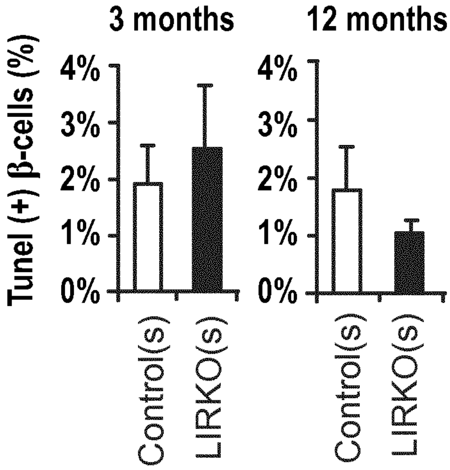


FIG. 4E

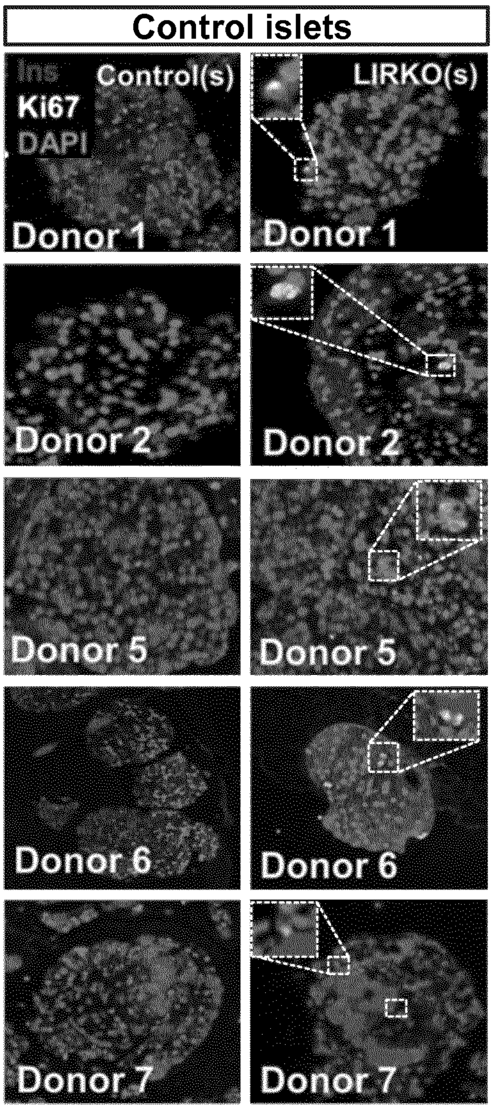


FIG. 4F

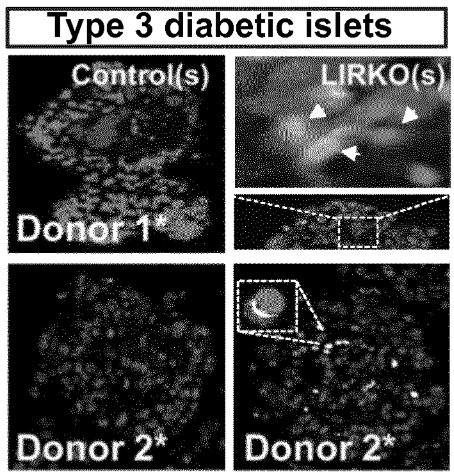


FIG. 4G

	Ki67(+) β-cells(%)	
Control islet-donors	Control(S)	LIRKO(S)
Donor 1	0.00	0.03
Donor 2	0.04	0.24
Donor 3	0.05	0.08
Donor 4	0.10	0.17
Donor 5	0.00	0.05
Donor 6	0.00	0.07
Donor 7	0.04	0.08
Donor 8	**0.00**	**0.00**
Donor 9	0.03	0.08
T2D islet-donors	Control(S)	LIRKO(S)
Donor 1*	0.30	0.60
Donor 2*	0.09	0.10

FIG. 5A

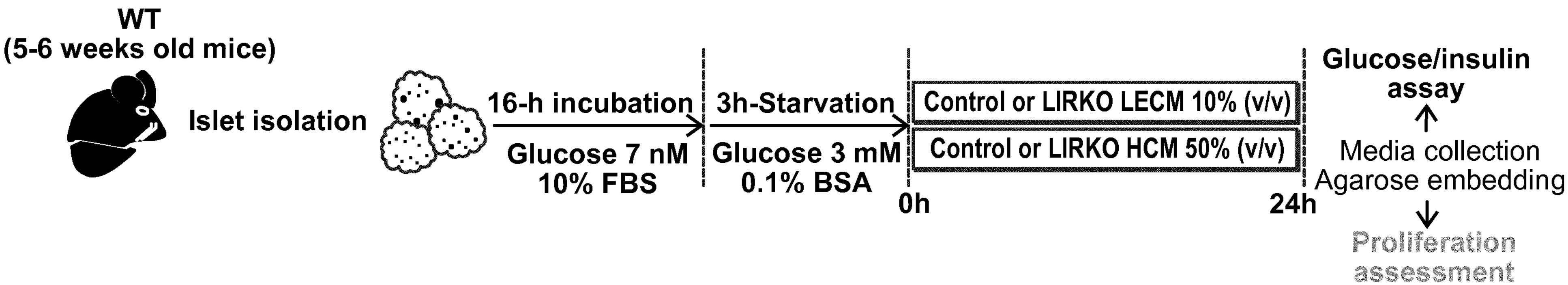


FIG. 5B

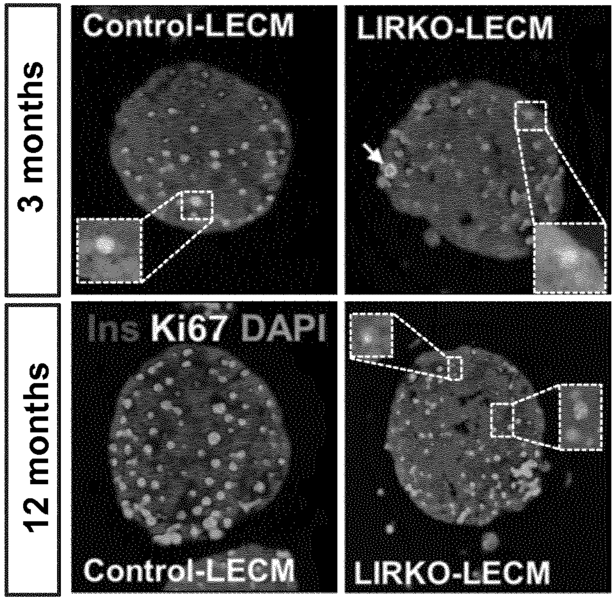


FIG. 5C

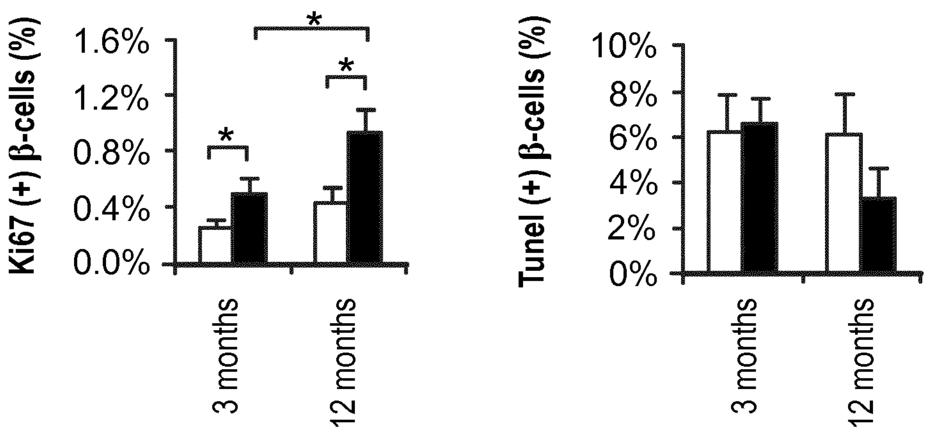


FIG. 5F

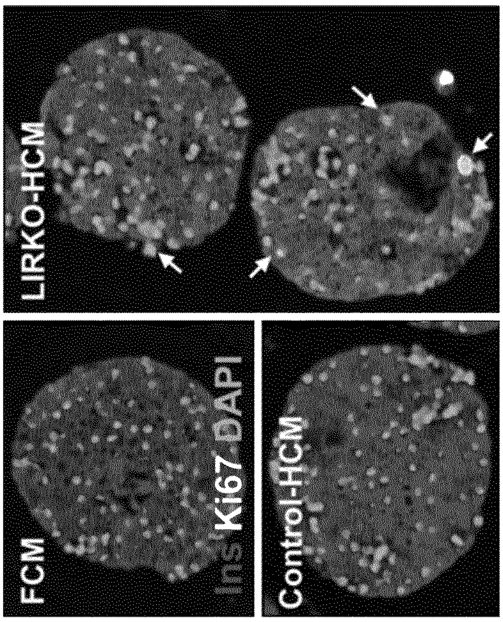


FIG. 5E

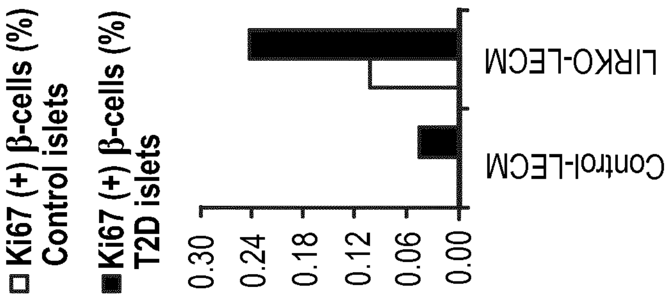


FIG. 5D

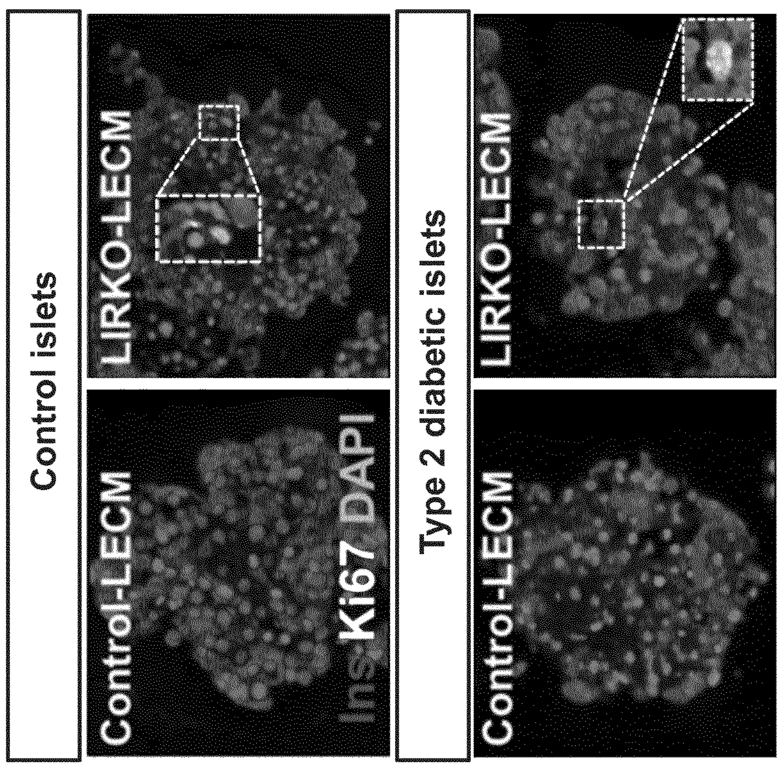


FIG. 5G

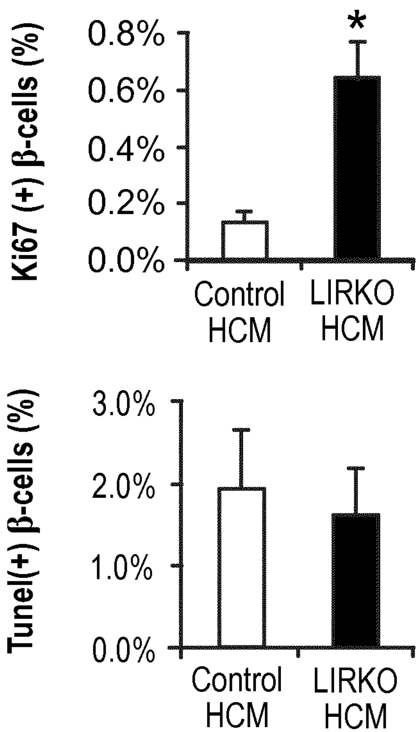


FIG. 5H

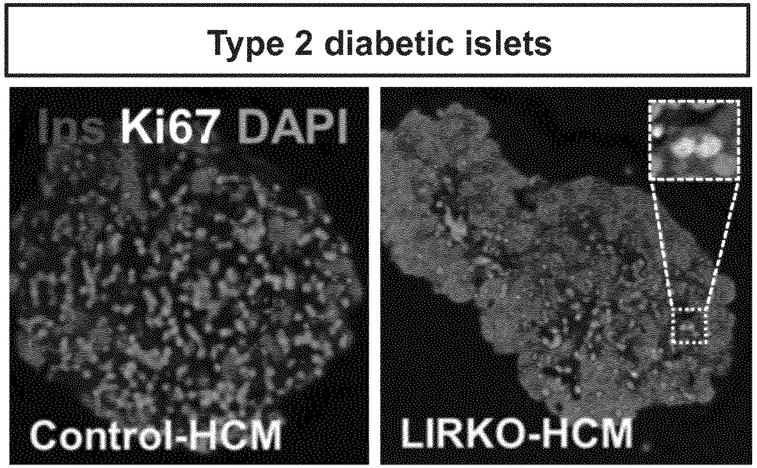
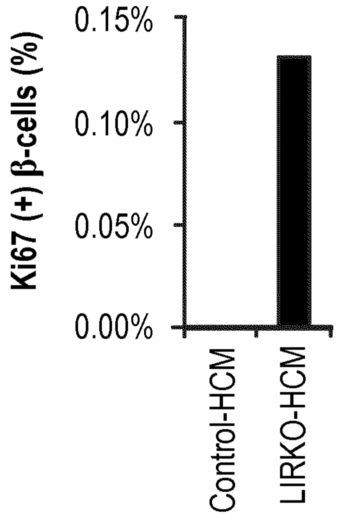
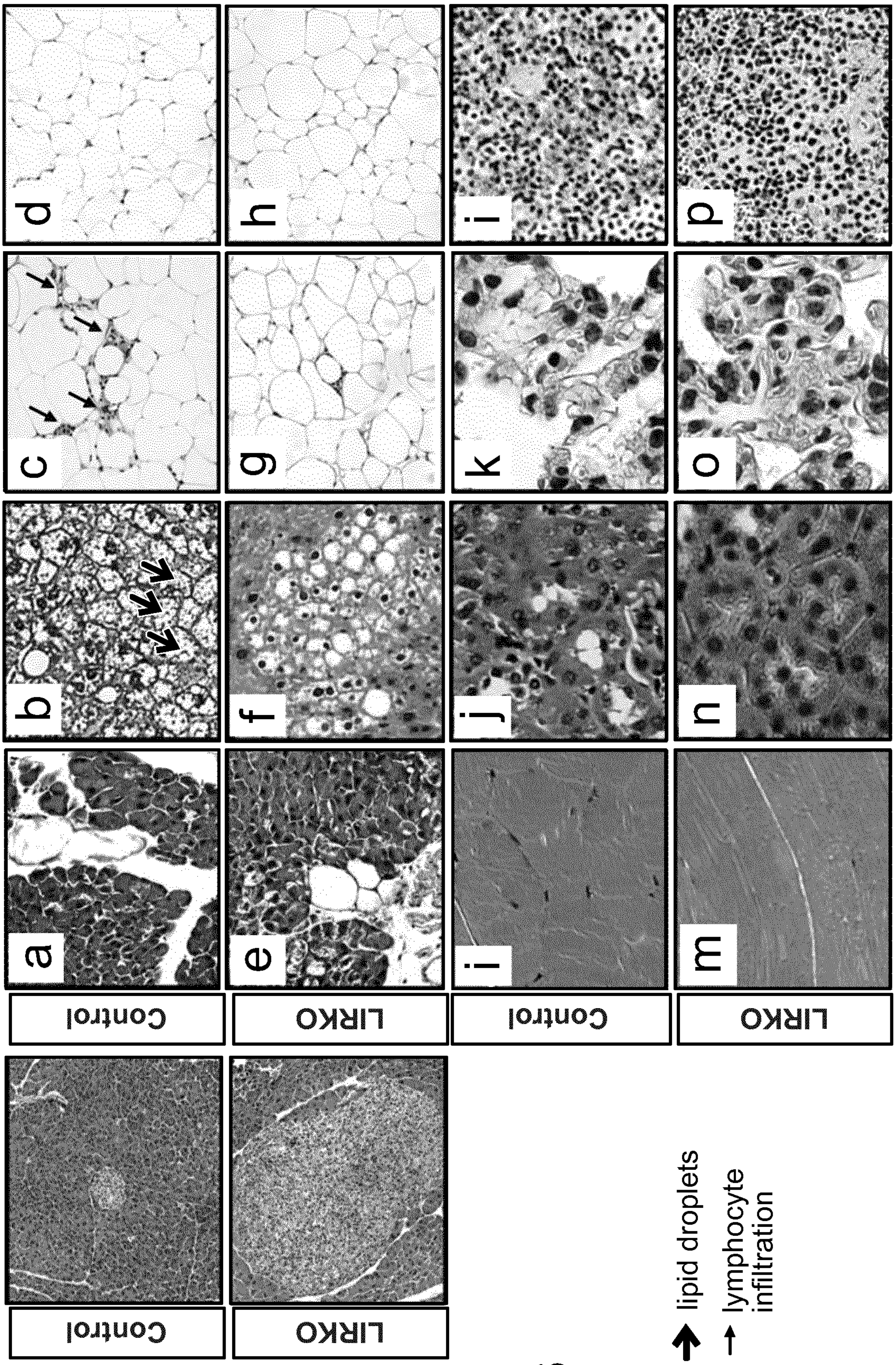


FIG. 5I





pancreas: a, e. liver: b, f. visceral adipose: c, g. subcutaneous adipose: d, h.
skeletal muscle: i, m. kidney: j, n. lung: k, o. spleen: l, p.

FIG. 6

FIG. 7A

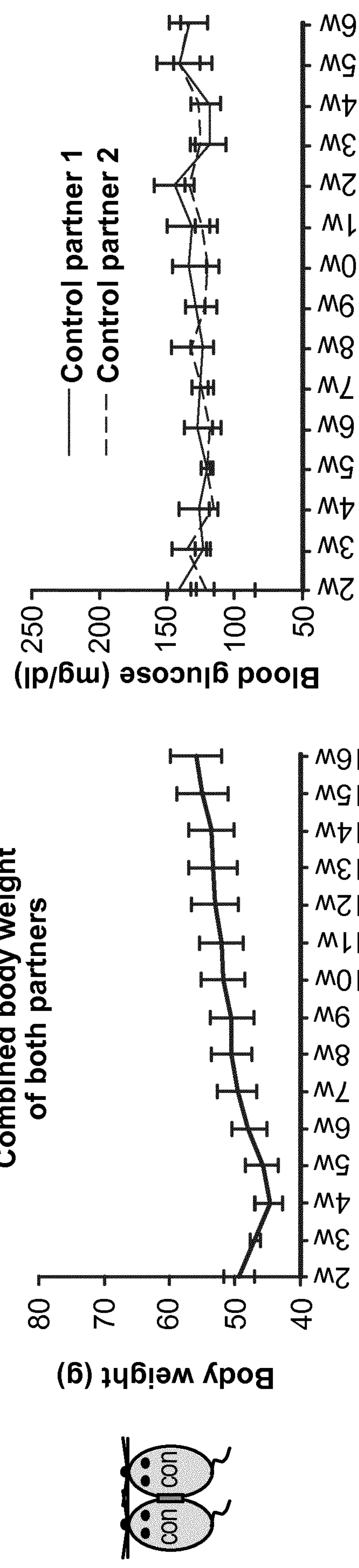
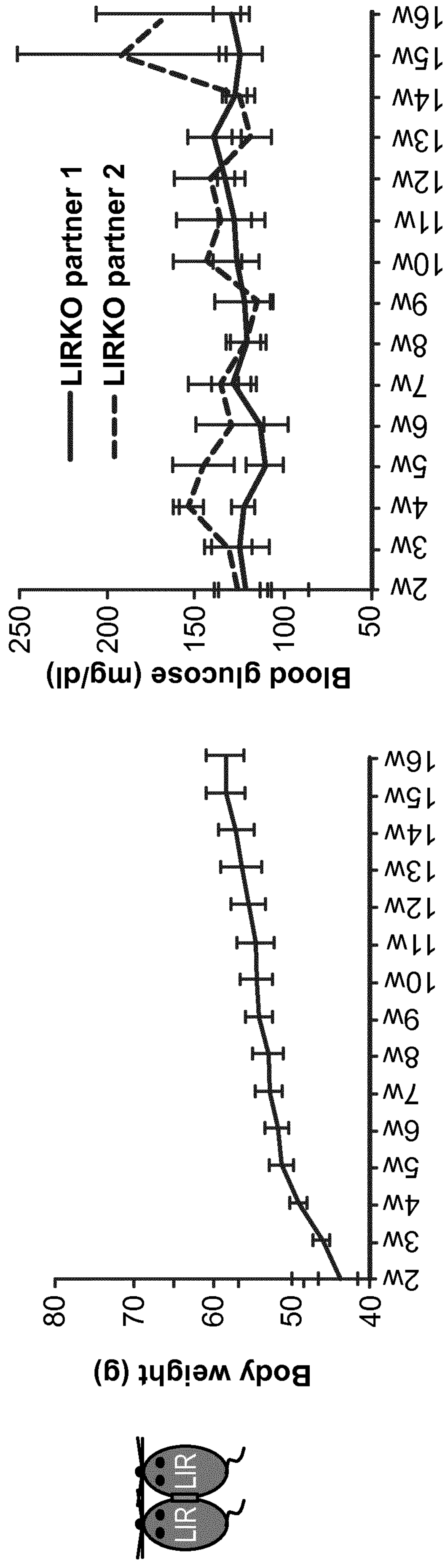

con
con
Body weight (g)
Combined body weight of both partners
Blood glucose (mg/dl)
Control partner 1
Control partner 2
2w
3w
4w
5w
6w
7w
8w
9w
10w
11w
12w
13w
14w
15w
16w

FIG. 7B


LIR
LIR
Body weight (g)
Blood glucose (mg/dl)
LIRKO partner 1
LIRKO partner 2
2w
3w
4w
5w
6w
7w
8w
9w
10w
11w
12w
13w
14w
15w
16w

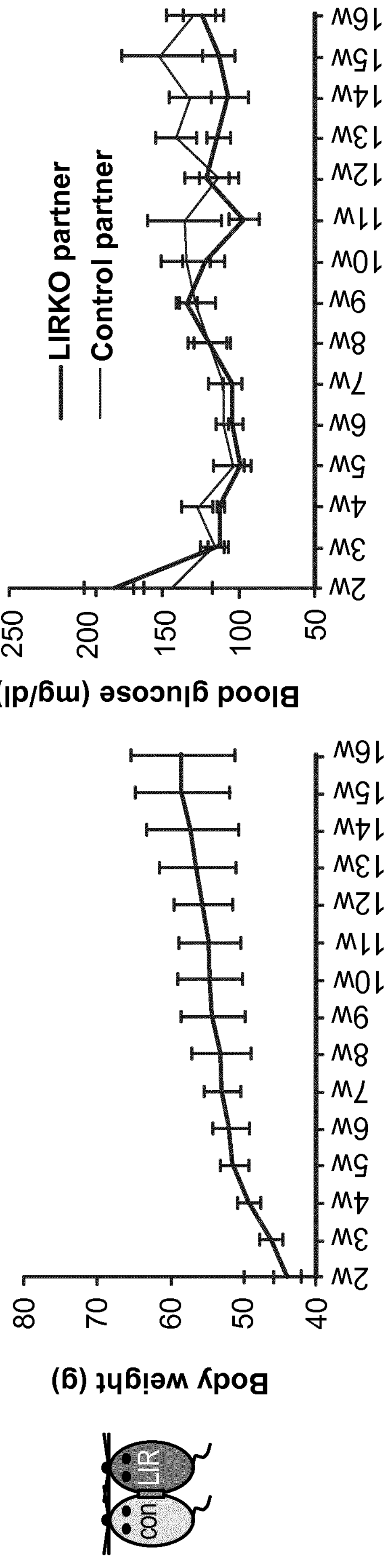

con
LIR
Body weight (g)
80
70
60
50
40
2w
3w
4w
5w
6w
7w
8w
9w
10w
11w
12w
13w
14w
15w
16w
Blood glucose (mg/dl)
250
200
150
100
50
LIRKO partner
Control partner

FIG. 7C

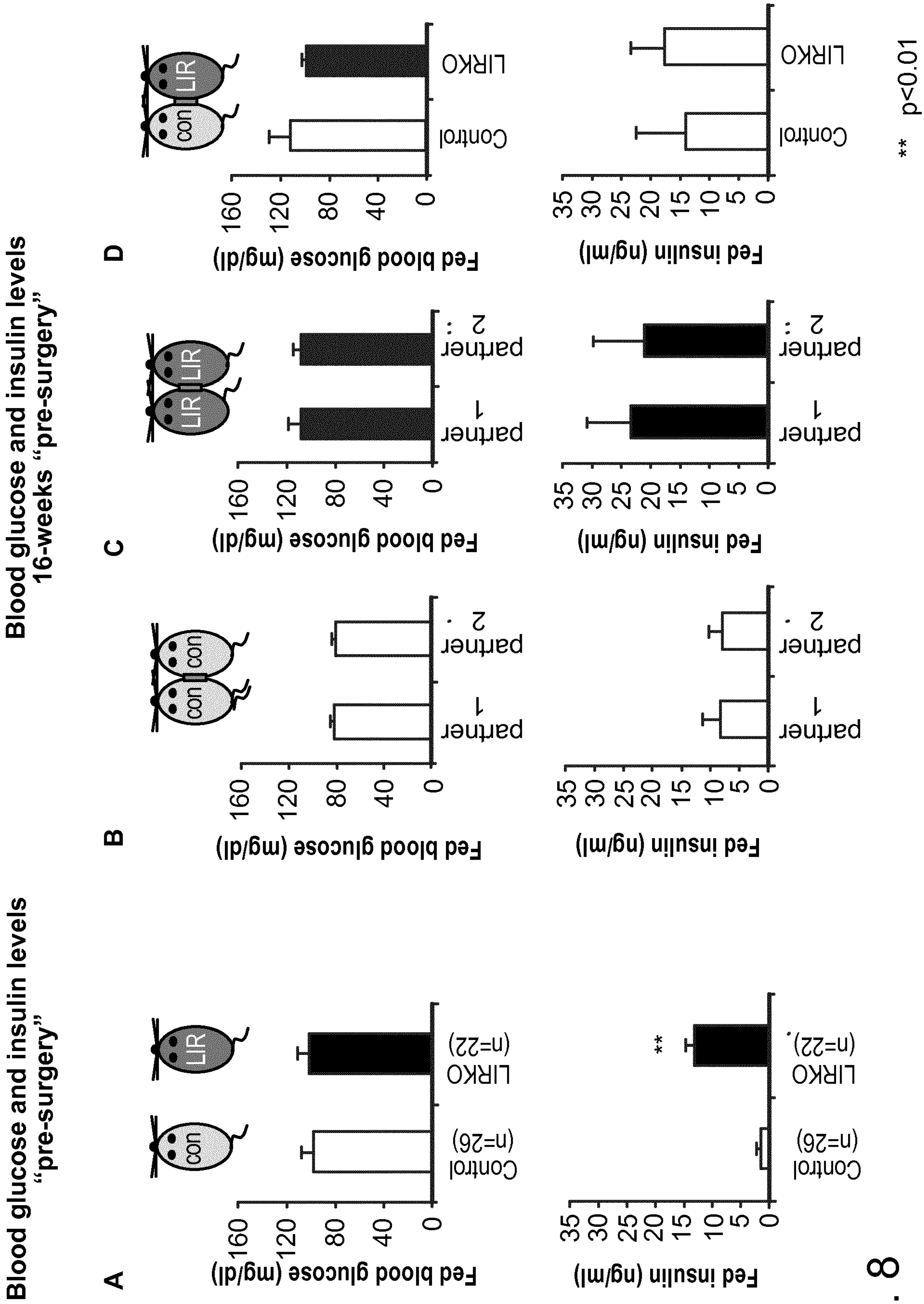

Blood glucose and insulin levels
"pre-surgery"
Blood glucose and insulin levels
16-weeks "pre-surgery"
A
B
C
D
Fed blood glucose (mg/dl)
Fed insulin (ng/ml)
con
LIR
Control
(n=26)
LIRKO
(n=22)
partner 1
partner 2
**
** p<0.01

FIG. 8

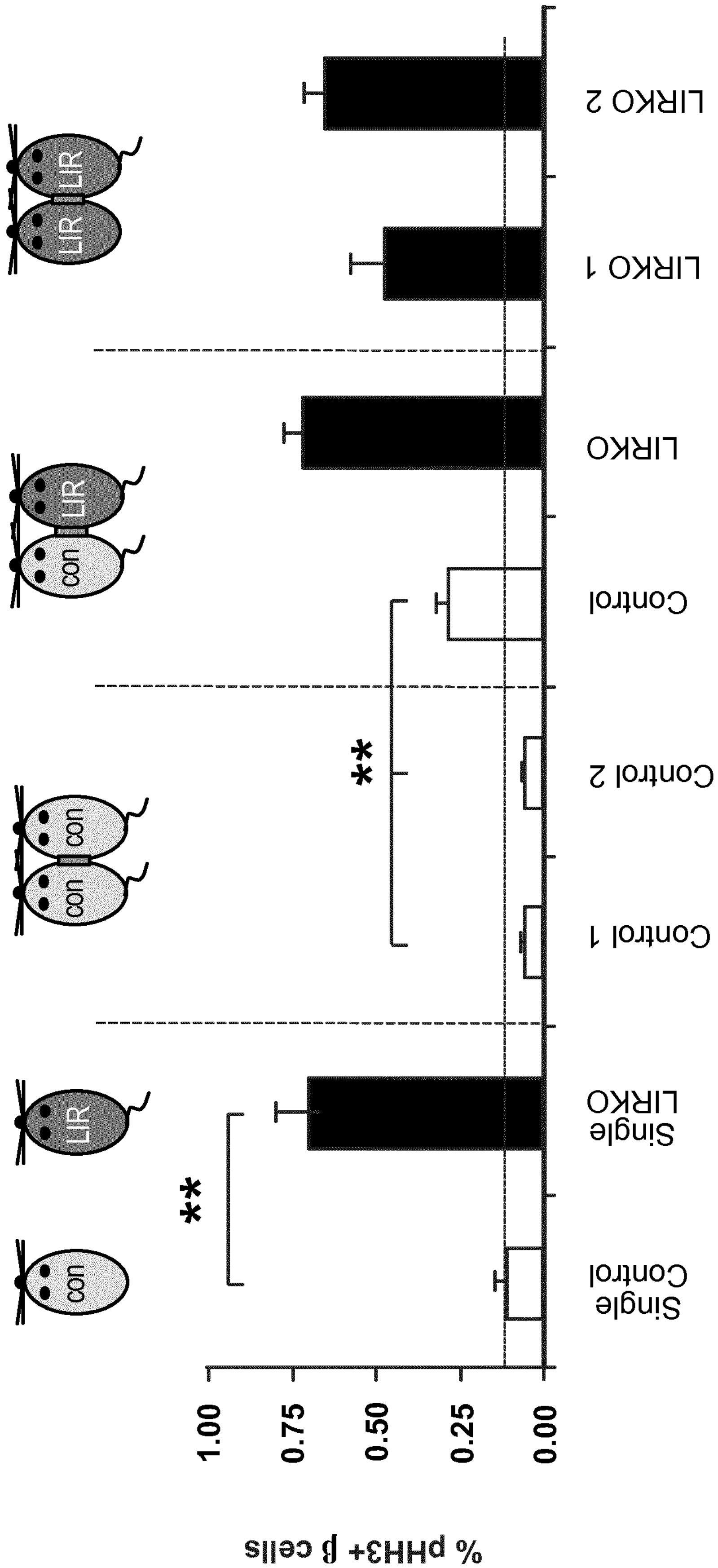

con
LIR
con
con
con
LIR
LIR
LIR
**
**
% pHH3+ β cells
1.00
0.75
0.50
0.25
0.00
Single Control
Single LIRKO
Control 1
Control 2
Control
LIRKO
LIRKO 1
LIRKO 2

FIG. 9

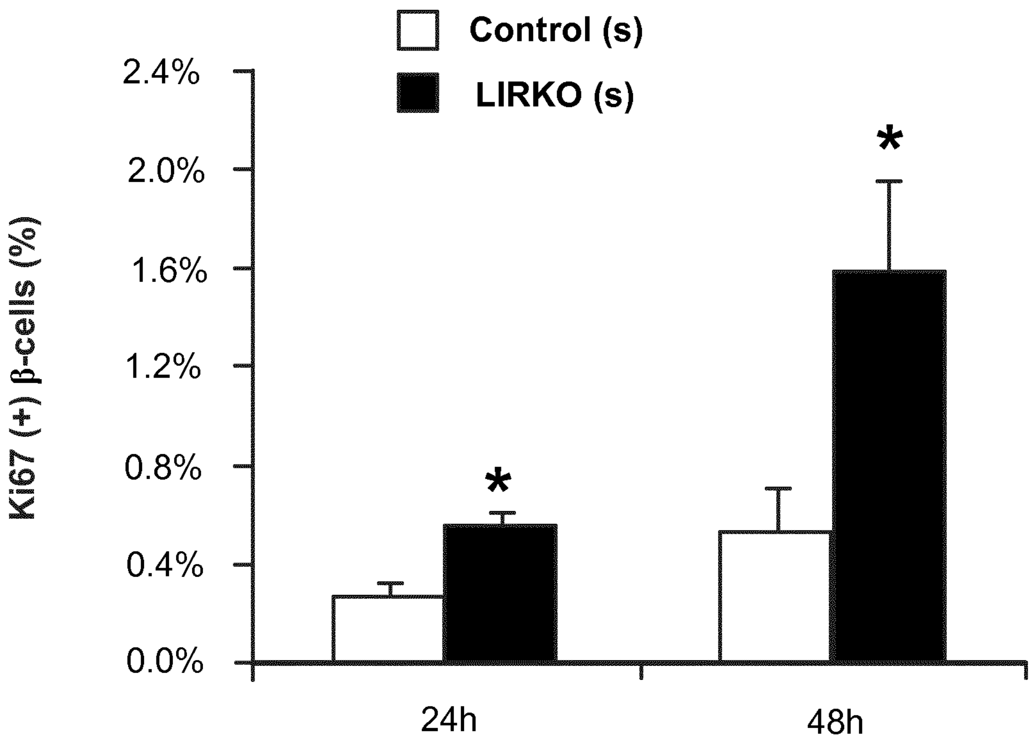


FIG. 10

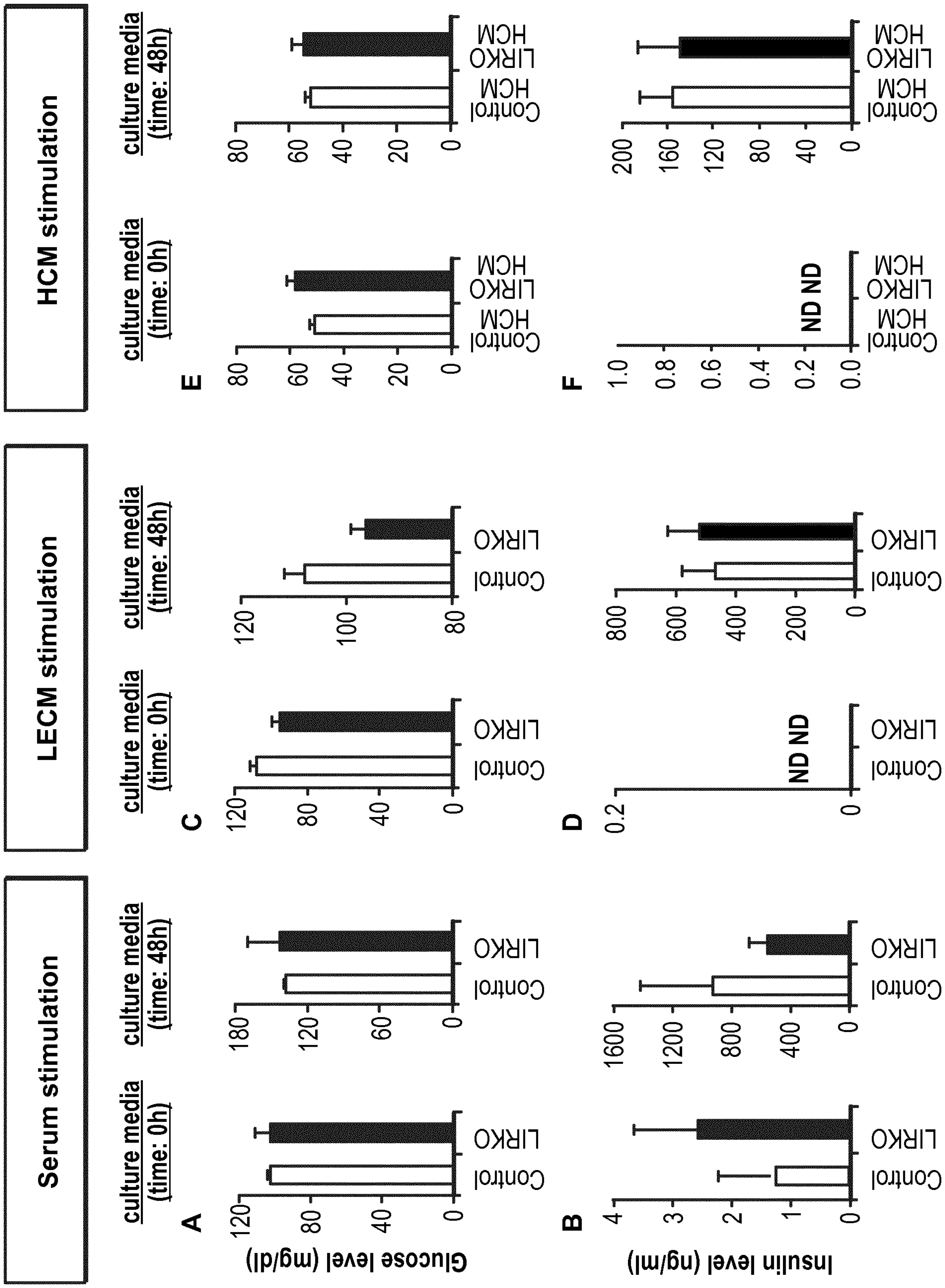
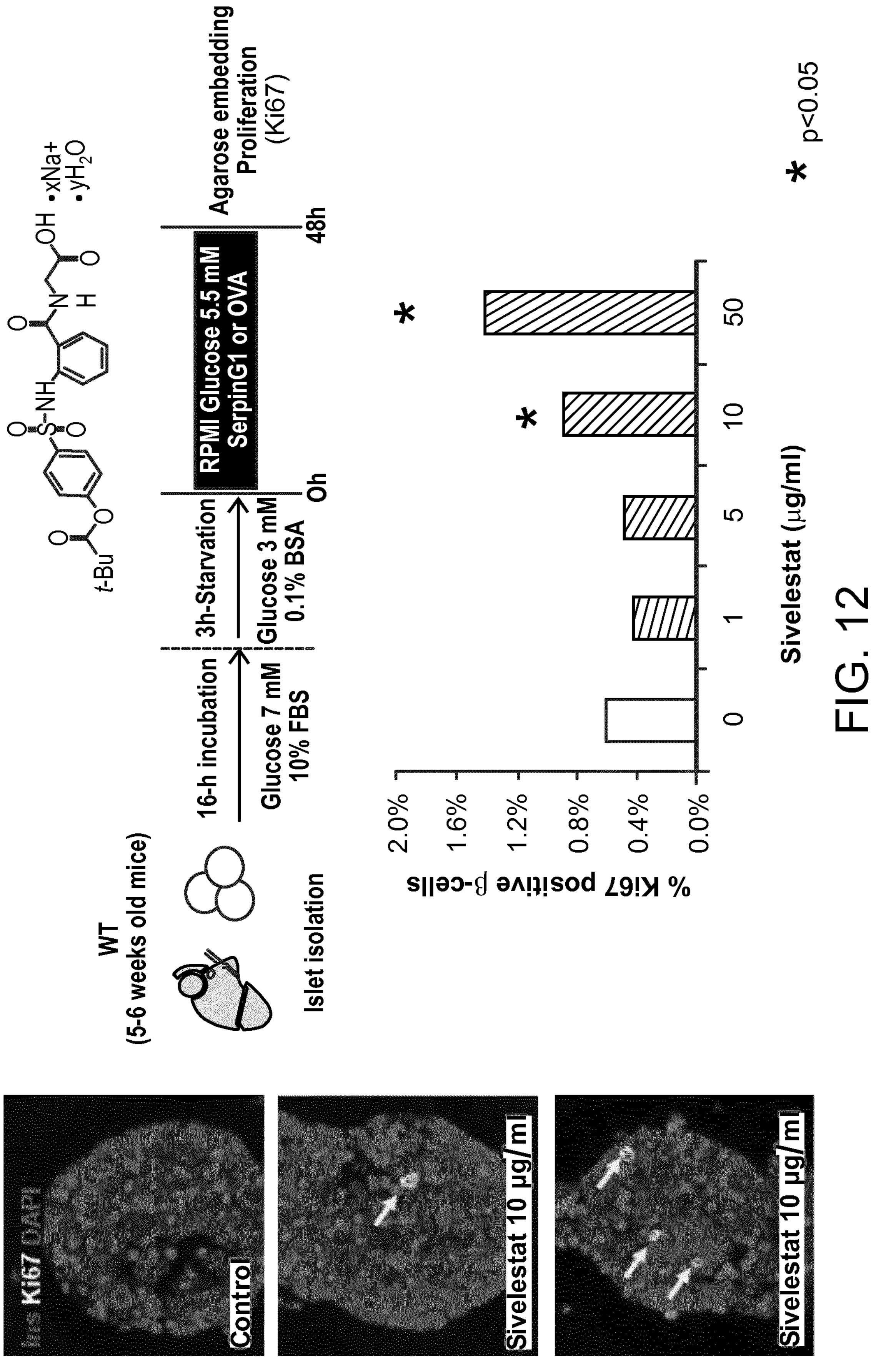

Serum stimulation
LECM stimulation
HCM stimulation
culture media (time: 0h)
culture media (time: 48h)
A
B
C
D
E
F
Glucose level (mg/dl)
Insulin level (ng/ml)
Control
LIRKO
Control HCM
LIRKO HCM
ND ND
120
80
40
0
180
60
1600
1200
800
400
4
3
2
1
100
0.2
600
200
20
1.0
0.8
0.6
0.4
0.0
160

FIG. 11



Ins Ki67 DAPI
Control
Sivelestat 10 µg/ml
Sivelestat 10 µg/ml
WT
(5-6 weeks old mice)
Islet isolation
16-h incubation
Glucose 7 mM
10% FBS
3h-Starvation
Glucose 3 mM
0.1% BSA
RPMI Glucose 5.5 mM
SerpinG1 or OVA
0h
48h
Agarose embedding
Proliferation
(Ki67)
t-Bu
•xNa+
•yH2O
% Ki67 positive β-cells
2.0%
1.6%
1.2%
0.8%
0.4%
0.0%
0
1
5
10
50
Sivelestat (µg/ml)
* p<0.05

FIG. 12

Mouse:

>gi|114158674|ref|NM_025429.2| Mus musculus serine (or cysteine) peptidase inhibitor, clade B, member 1a (Serpinb1a), mRNA (SEQ ID NO: 1).

```
ACTTCATCCTAGCTGTAAGTGGAGCCAGACCTGCTAAGCAAGAGACTTCACCATGGAGCAGCTGAGTTCA
GCCAACACCCTCTTCGCCTTGGAGCTGTTCCAAACCCTGAATGAAAGCAGCCCCACAGGAAACATCTTCT
TCTCTCCCTTCAGCATTTCTTCTGCCTTGGCCATGGTCATTCTGGGGGCCAAAGGCAGCACTGCAGCTCA
GCTTTCTAAGACTTTTCATTTTGACTCTGTTGAGGACATCCATTCACGCTTCCAAAGCCTGAATGCTGAA
GTGAGCAAACGTGGAGCATCTCACACTCTGAAACTTGCTAACAGACTGTATGGAGAGAAAACCTACAACT
TCCTTCCTGAATACTTGGCTTCAACCCAGAAAATGTATGGTGCTGACTTGGCCCCTGTGGATTTTCTGCA
TGCCTCTGAGGATGCAAGGAAGGAGATAAACCAGTGGGTCAAAGGTCAAACAGAAGGGAAAATCCCAGAA
CTGTTGTCTGTGGGTGTGGTGGACAGTATGACCAAACTTGTGCTGGTGAATGCCATCTACTTTAAGGGAA
TGTGGGAGGAGAAATTCATGACAGAGGACACAACGGATGCTCCATTCCGACTGAGTAAGAAAGACACAAA
AACAGTGAAGATGATGTATCAAAAGAAAAAATTTCCATTTGGTTACATTTCGGACCTGAAGTGCAAGGTG
CTGGAGATGCCTTACCAGGGTGGAGAACTTAGCATGGTCATTCTGCTGCCTAAAGACATTGAGGACGAGT
CCACGGGTCTTAAGAAGATTGAAAAGCAAATAACTTTGGAAAAACTGCTTGAATGGACCAAACGTGAGAA
CTTGGAATTCATTGATGTCCACGTCAAACTGCCCCGGTTCAAGATAGAAGAGAGCTATACCCTCAACTCT
AACCTGGGCCGCCTGGGAGTGCAGGATCTCTTTAGCAGTAGCAAGGCTGATCTCTCTGGCATGTCAGGAT
CCAGAGATCTTTTCATATCAAAAATTGTCCACAAGTCCTTTGTGGAAGTGAATGAGGAAGGAACAGAGGC
AGCCGCTGCTACAGGAGGCATTGCTACATTCTGTATGTTGTTGCCTGAGGAAGAATTCACAGTGGACCAT
CCGTTCATTTTCTTCATTCGGCACAATCCCACATCTAATGTGCTCTTCCTTGGCAGGGTTTGTTCCCCAT
AGAAGAAGGAGACTTTACAGATACAAGGCAGAGCTTAGAGTTTCATTCCCTGAGATTTTAATAGTGATTA
TTTTCATTTGTACTTGACAATAAAAACTCTAACCAGAAACCAATCTTTCTTTTGTATGTTCAACCCTGTT
AGCTCTTTATATCCATGACTTTTGGCATGGGTATGTCTATTTTGATTGTACAATGAAAGCAGGACTCCTG
TTTTCCTCCTCGGCTTTTGCATGACCTCCAGAGTACATCAAAGGTTCATAGCTAGGCTGAAAATTCTGGA
CGACTCCATCCTCAAACTTTATGGACTGTAGGTGGGTGCCTGCAGATGCTAACTGAAGTCATATCCATCT
GGGGTAGCGTGGATACCCTTAAGCCTCAAATCATTATTACAATCTGCTTTTCAAGTACAACATCCAGAGT
ATAATCAAAGATAACTGTTTGGGTGGGCAGCCATGGCAACAGAGATACAAAGCAGCACAAACAAAAGAGA
AGGACAACAGTGGAAGGCTCTAAATGCTGCTGCCGCCCATACAAACCAGAGAACAACAGTCTGTGAAGAT
AATATTGACGAAATCCAAGGTCAGATACTTTAGCAGGCTATTGCAAACTTACAAACAACATTTCATGTCT
GGATGAAAAGGAACTAGAAACCCCAGAGCTTAAACATACAATAAATTATTTCCATTGAAAACTTAAATAA
TAAAGAATTTGTGGATTTTTAAGTCTGAAAAAAAAAAAAAA
```

FIG. 13

FIG. 14A
FIG. 14B

FIG. 14

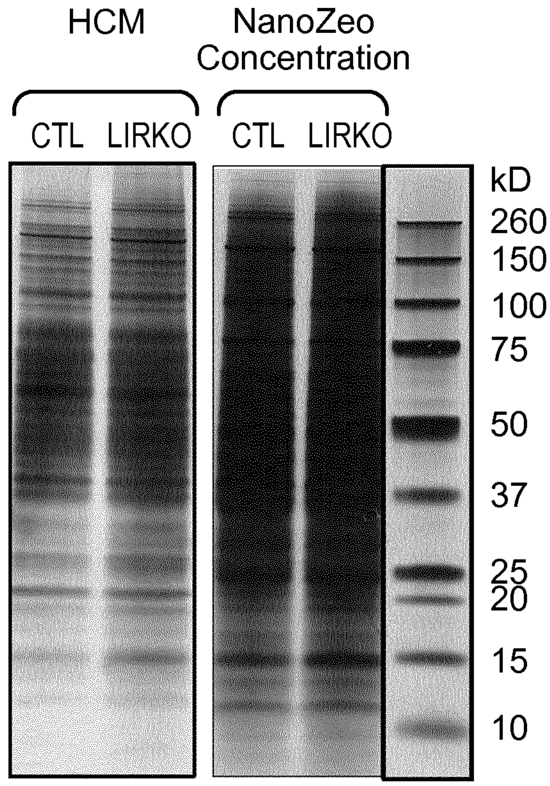
FIG. 14A

Human:

```
>gi|401709928|ref|NM_030666.3| Homo sapiens serpin peptidase
inhibitor, clade B (ovalbumin), member 1 (SERPINB1), transcript
variant 1, mRNA (SEQ ID NO: 2).
AGAAAGAAGCCGCGCCCCTGAGGAGGGCGCTGCCCGGAAGCCACGCTCACTTCTGCTTGCACTTAGGCGA
CCTCGGGAGCTCGGACTCCTACGCAGTCACCGGGAAGGGCCGCCGCCCCGCCCGCGGCTGCTGGCCCGGG
TGACGCTTCCGCCTGCTATAAGAGCAGCGGCCCTCGGTGCCTCCTTCCTGACCTCGCACCCAGCTCGGAG
CCCGGAGCGTGCCTCGGCGGCCTGTCGGTTTTCACCATGGAGCAGCTGAGCTCAGCAAACACCCGCTTCG
CCTTGGACCTGTTCCTGGCGTTGAGTGAGAACAATCCGGCTGGAAACATCTTCATCTCTCCCTTCAGCAT
TTCATCTGCTATGGCCATGGTTTTTCTGGGGACCAGAGGTAACACGGCAGCACAGCTGTCCAAGACTTTC
CATTTCAACACGGTTGAAGAGGTTCATTCAAGATTCCAGAGTCTGAATGCTGATATCAACAAACGTGGAG
CGTCTTATATTCTGAAACTTGCTAATAGATTATATGGAGAGAAAACTTACAATTTCCTTCCTGAGTTCTT
GGTTTCGACTCAGAAAACATATGGTGCTGACCTGGCCAGTGTGGATTTTCAGCATGCCTCTGAAGATGCA
AGGAAGACCATAAACCAGTGGGTCAAAGGACAGACAGAAGGAAAAATTCCGGAACTGTTGGCTTCGGGCA
TGGTTGATAACATGACCAAACTTGTGCTAGTAAATGCCATCTATTTCAAGGGAAACTGGAAGGATAAATT
CATGAAAGAAGCCACGACGAATGCACCATTCAGATTGAATAAGAAAGACAGAAAAACTGTGAAAATGATG
TATCAGAAGAAAAAATTTGCATATGGCTACATCGAGGACCTTAAGTGCCGTGTGCTGGAACTGCCTTACC
AAGGCGAGGAGCTCAGCATGGTCATCCTGCTGCCGGATGACATTGAGGACGAGTCCACGGGCCTGAAGAA
GATTGAGGAACAGTTGACTTTGGAAAAGTTGCATGAGTGGACTAAACCTGAGAATCTCGATTTCATTGAA
GTTAATGTCAGCTTGCCCAGGTTCAAACTGGAAGAGAGTTACACTCTCAACTCCGACCTCGCCCGCCTAG
```

FIG. 14B

GTGTGCAGGATCTCTTTAACAGTAGCAAGGCTGATCTGTCTGGCATGTCAGGAGCCAGAGATATTTTTAT
ATCAAAAATTGTCCACAAGTCATTTGTGGAAGTGAATGAAGAGGGAACAGAGGCGGCAGCTGCCACAGCA
GGCATCGCAACTTTCTGCATGTTGATGCCCGAAGAAAATTTCACTGCCGACCATCCATTCCTTTTCTTTA
TTCGGCATAATTCCTCAGGTAGCATCCTATTCTTGGGGAGATTTTCTTCCCCTTAGAAGAAAGAGACTGT
AGCAATACAAAAATCAAGCTTAGTGCTTTATTACCTGAGTTTTTAATAGAGCCAATATGTCTTATATCTT
TACCAATAAAACCACTGTTCAGAAACAAGTCTTTCATTTTCTTTGTAAGTTTGGCTCTGTTGGCTGTTTA
CACCCATGAATTTTGGCATGGGTATCTATTTTTCTTTTTTACATTGAAAAAAATCCAGTGGTTGCTTTTG
AATGCATCAAGTAAAGAAGAAGAAAAGAATACATCCGATGCGTAGATTCTTGACCATGTAGTAATCTATA
AAATTGCTATATCCTCCTGATAGCCATGGGAAAACATGATAAGATGGTCATTTATTTTGCAGTTAGAATT
TTGGAAGCCACAAAATAGACAGACACCCTGACTGTTGAAGGGAGGTTTAAAAACAGATATTCAATTGAAA
TGTAAGAGAGCACCCCAATTGAGAGCCCAGGTTACGAAGACAAGCTTGCCTCGCCTGACTTTTCTGTCCC
TTGTTCTGCAGGATTAGTATTCTGTTACAGACCTCTAGTTTTTAGACTCTTCAATTAAAGGGCCAATGGT
TATAACCTGCATTCCCTTTTTTGTTCTTCTTTATGTATAATATATAGTTCATGTGGCGCTGCATGAAATC
AAGAAGTGGGTGTCTTAGGATAAAAGATACCAAGAGTCTACAAAAATAACCATGTAGTAAGATAAACTGC
TGAACAAAGGTTTTACTGTTAGCCACCTTCTCATGTGTTTTCTTTTCTCTTTTTCTTTTTCTTTCTTTCT
TTCTTTTTTTTTTTTTTGAGACAGAGTCTTGCTCTGTTACCCAGGCTGGAGTGCAGTGGCACGATCTCAG
CTCACCGCAACCTCTGCCTCCTGGGTTCAAGTGATTCTCTTGCTTCAGCCTCCTGAGTAGCTGGGATTAT
AGGCATGCACCACTAGGCCTGGCTAATTTTTGTATTTTTAGTAGAGATGGGGTTTTTCCATGTTGGCCAG
GCTGGTCCCGAACTCCTGACCTCAGGTGATCCGCGCACCTCAGCCTCCCAAAGTGCTGGGATTACAGGCA
TGAGCTACCATGCCTGGCCTTCTCATGTGTTTTCTGATTAAGGCTCTTGACTTCCAAGGCTGTGTGGGGA
GATGGGGTGGGGGCTCTTGGACTGATATAAAACTTTGTCAAATGTAGTTCTTTGAATGGAGCTTGAAACG
CCGCATATTCTTGCTCCCACAAGGATAGTGGGCATCATGAATTAATAAAACGTCCTAGGATTCTGCAAGC
TAAAAAAAAAAAAAA



SDS/PAGE analysis of secreted proteins from LIRKO and control mouse hepatocytes

FIG. 15

A

MEQLSSANTL FALELFQTLN ESSPTGNIFF SPFSISSALA MVILGAKGST
AAQLSK**TFHF** **DSVEDIHSRF** **QSLNAEVSK**R GASHTLKLAN RLYGEK**TYNF**
LPEYLASTQK **MYGADLAPVD** **FLHASEDAR**K EINQWVKGQT EGK**IPELLSV**
GVVDSMTKLV LVNAIYFKGM WEEK**FMTEDT** **TDAPFR**LSKK DTKTVKMMYQ
KKK**FPFGYIS** **DLK**CKVLEMP YQGGELSMVI LLPKDIEDES TGLKKIEKQI
TLEKLLEWTK **RENLEFIDVH** **VK**LPRFK**IEE** **SYTLNSNLGR** **LGVQDLFSSS**
KADLSGMSGS RDLFISKIVH KSFVEVNEEG TEAAAATGGI ATFCMLLPEE
EFTVDHPFIF FIR**HNPTSNV** **LFLGR**VCSP

Murine Serpinβ peptide: SEQ ID NO: 3

B

HUGO-ID	Description	Ratio LIRKO/Control Experiment 1	p value exp. 1	Ratio LIRKO/Control Experiment 2	p value exp. 2	Ratio LIRKO/Control Experiment 3	p value exp. 3
SERPINB1	serpin peptidase inhibitor, clade B, member 1	17,5	1,50E-03	11,6	3,80E-15	18,0	3,70E-14

FIG. 16

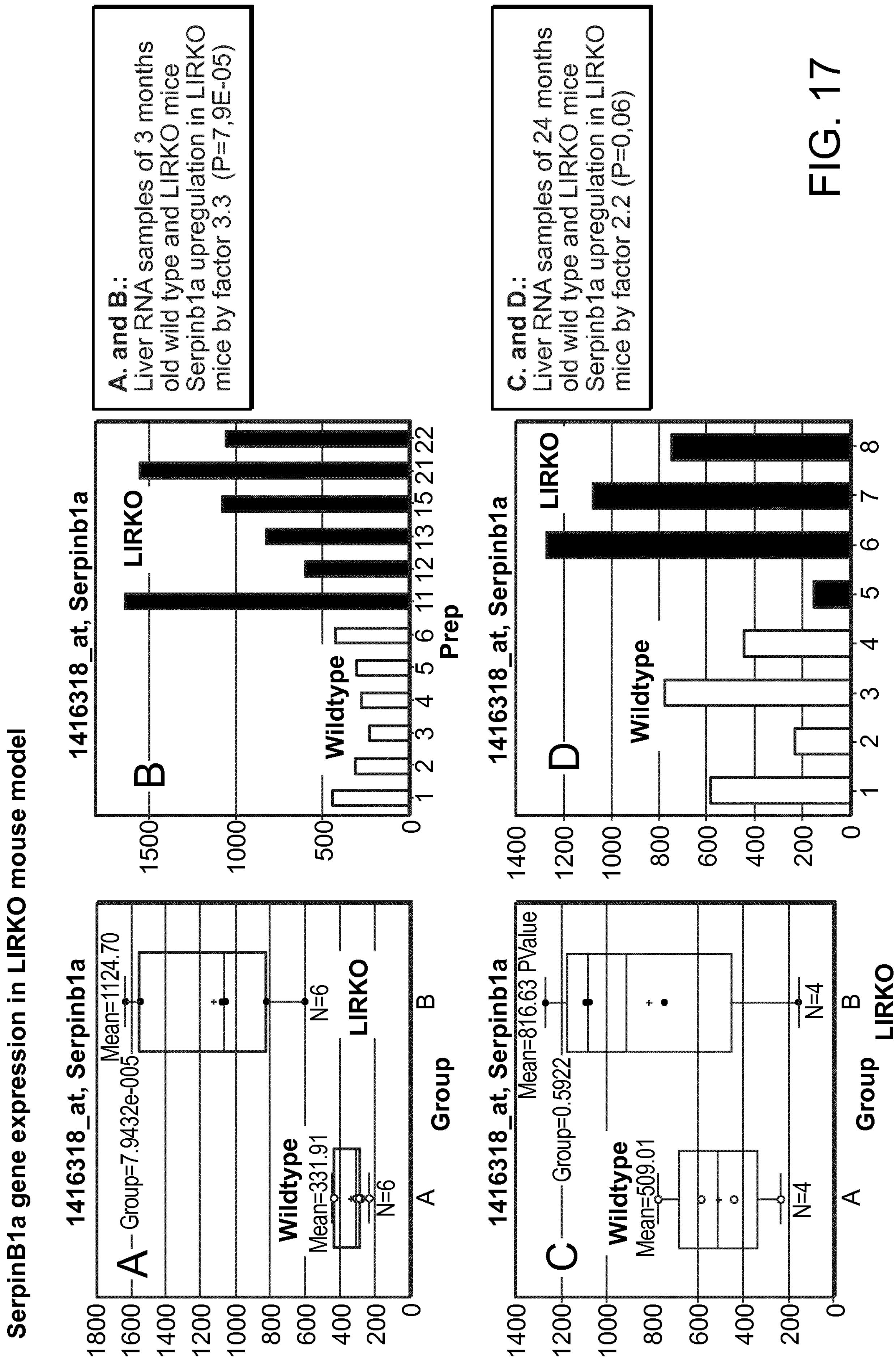
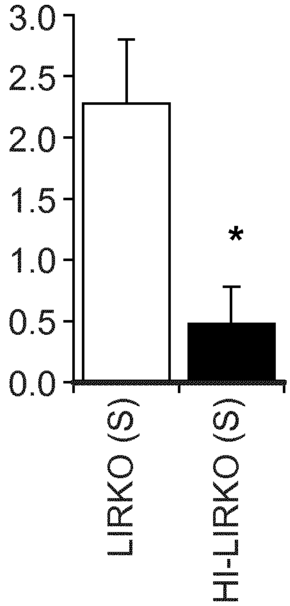

SerpinB1a gene expression in LIRKO mouse model
A
1416318_at, Serpinb1a
Group=7.9432e-005
Mean=1124.70
Wildtype
Mean=331.91
N=6
N=6
LIRKO
Group
B
1416318_at, Serpinb1a
Wildtype
LIRKO
Prep
A. and B.:
Liver RNA samples of 3 months old wild type and LIRKO mice
Serpinb1a upregulation in LIRKO mice by factor 3.3 (P=7,9E-05)
C
1416318_at, Serpinb1a
Group=0.5922
Mean=816.63 PValue
Wildtype
Mean=509.01
N=4
N=4
LIRKO
Group
D
1416318_at, Serpinb1a
Wildtype
LIRKO
C. and D.:
Liver RNA samples of 24 months old wild type and LIRKO mice
Serpinb1a upregulation in LIRKO mice by factor 2.2 (P=0,06)

FIG. 17



Fold change–Ki67 (+) β-cells

FIG. 18

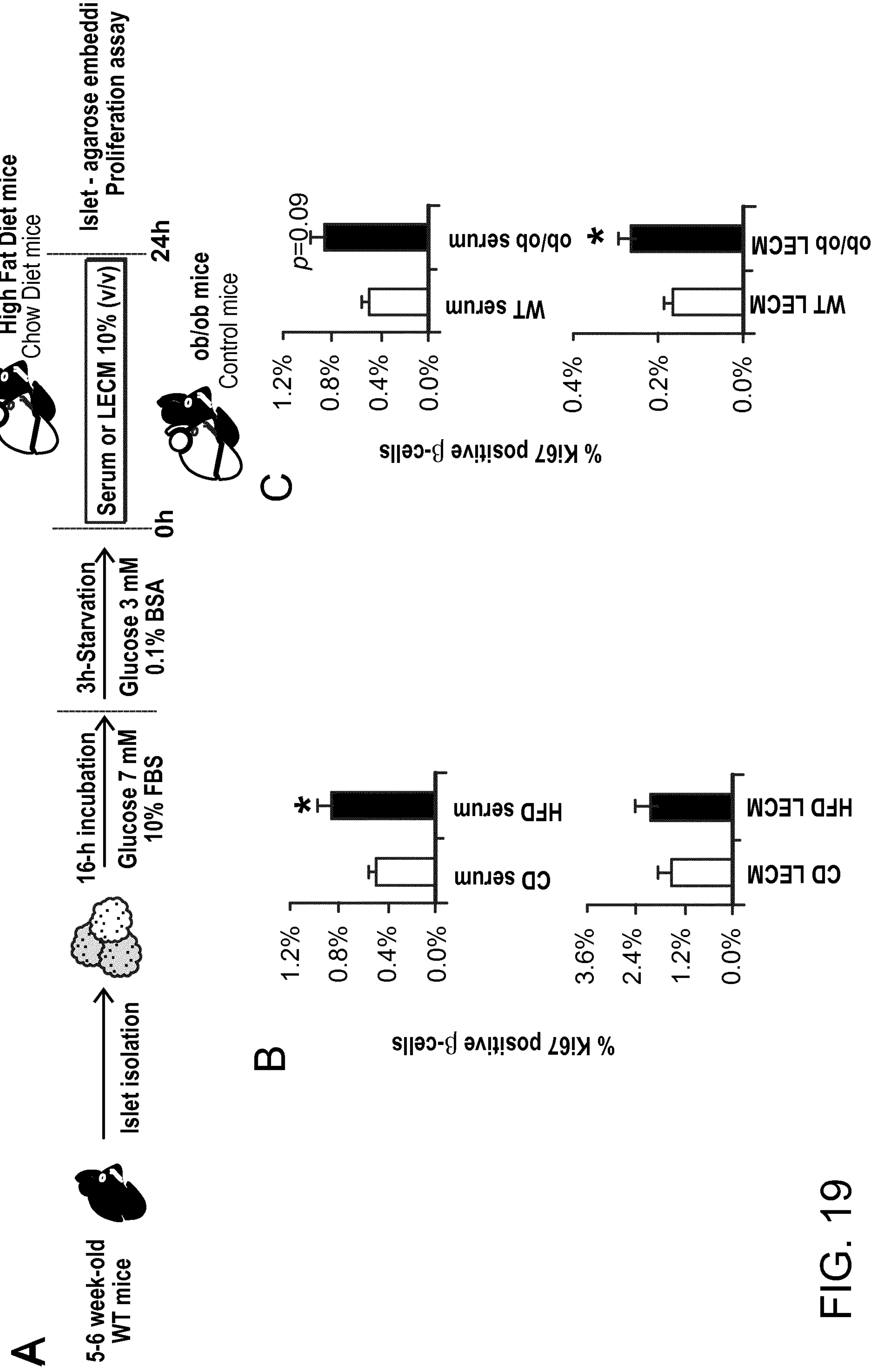

A
5-6 week-old WT mice
Islet isolation
16-h incubation
Glucose 7 mM
10% FBS
3h-Starvation
Glucose 3 mM
0.1% BSA
0h
Serum or LECM 10% (v/v)
24h
High Fat Diet mice
Chow Diet mice
ob/ob mice
Control mice
Islet - agarose embedding
Proliferation assay
B
% Ki67 positive β-cells
1.2%
0.8%
0.4%
0.0%
CD serum
HFD serum
*
3.6%
2.4%
1.2%
0.0%
CD LECM
HFD LECM
C
% Ki67 positive β-cells
1.2%
0.8%
0.4%
0.0%
WT serum
ob/ob serum
p=0.09
0.4%
0.2%
0.0%
WT LECM
ob/ob LECM
*

FIG. 19

FIG. 20A

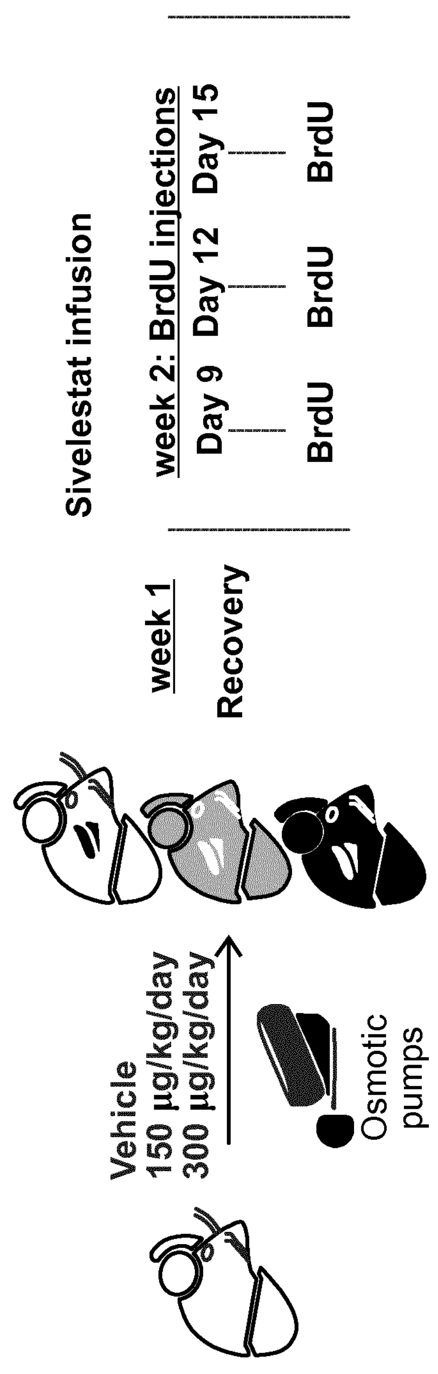


FIG. 20B

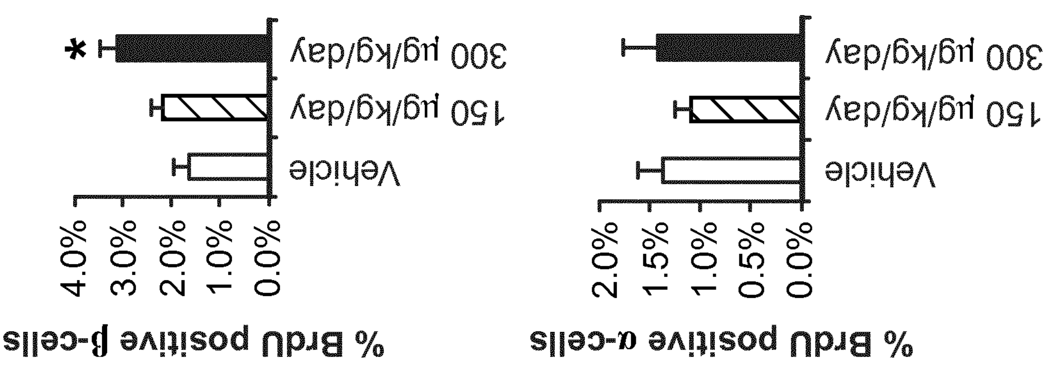


FIG. 20C

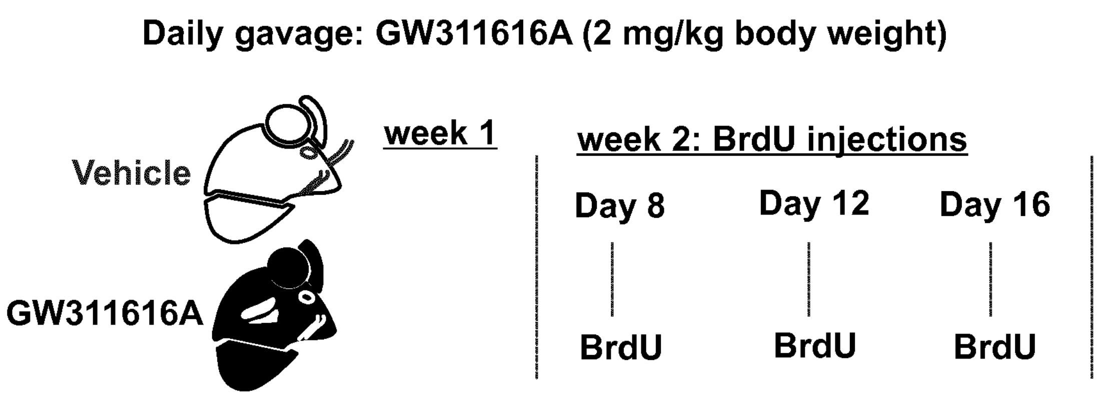
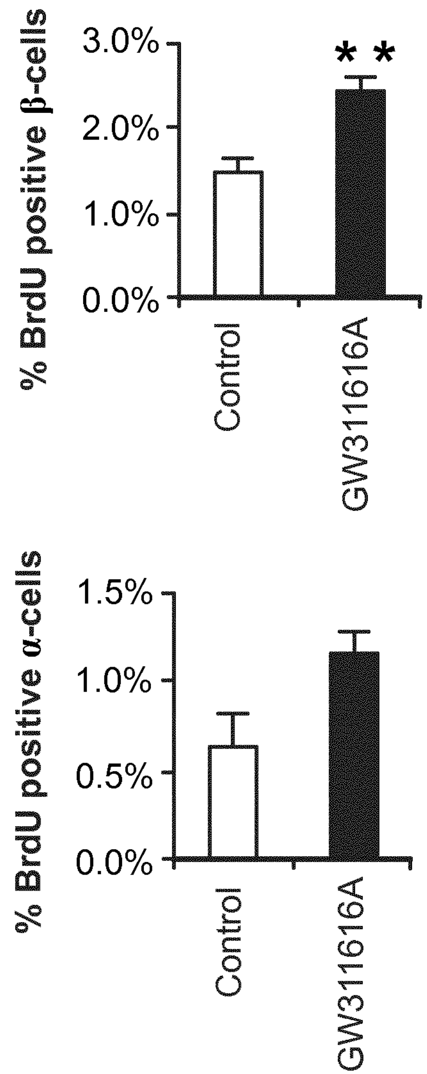


FIG. 20D



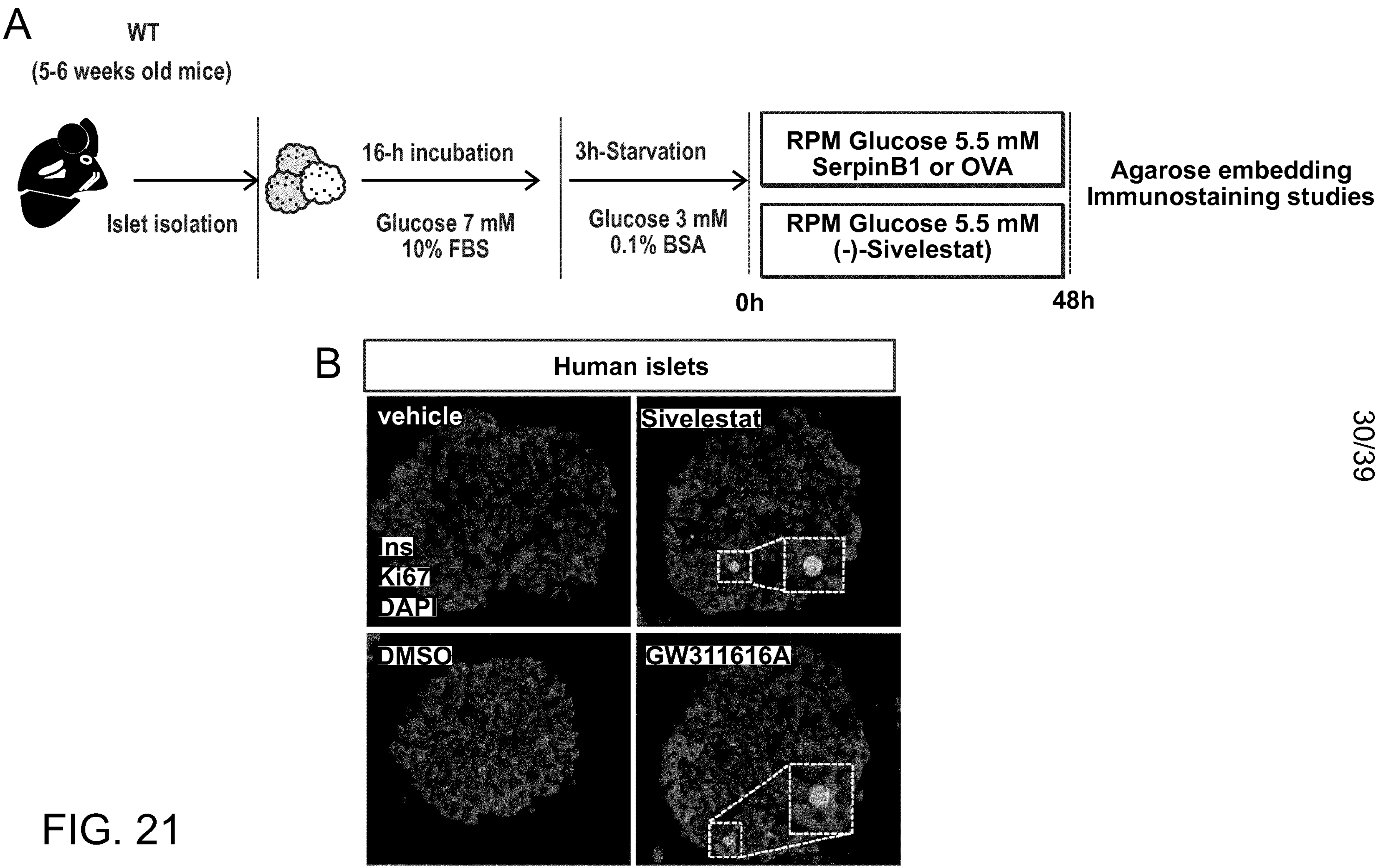

A
WT
(5-6 weeks old mice)
Islet isolation
16-h incubation
Glucose 7 mM
10% FBS
3h-Starvation
Glucose 3 mM
0.1% BSA
RPM Glucose 5.5 mM
SerpinB1 or OVA
RPM Glucose 5.5 mM
(-)-Sivelestat)
Agarose embedding
Immunostaining studies
0h
48h
B
Human islets
vehicle
Ins
Ki67
DAPI
Sivelestat
DMSO
GW311616A

FIG. 21

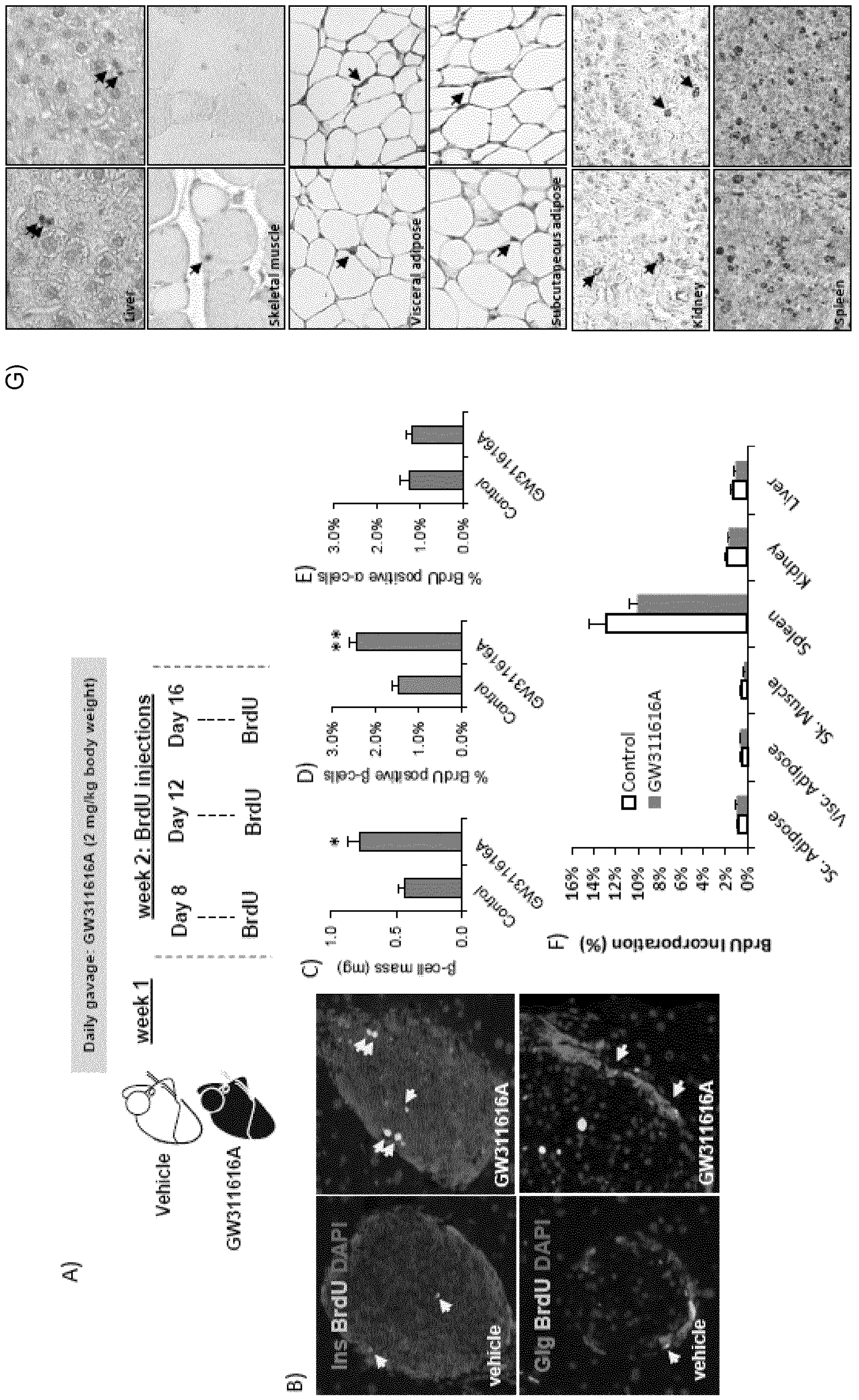

A)
Vehicle
GW311616A
Daily gavage: GW311616A (2 mg/kg body weight)
week 1
week 2: BrdU injections
Day 8
Day 12
Day 16
BrdU
BrdU
BrdU
B)
Ins BrdU DAPI
vehicle
GW311616A
Glg BrdU DAPI
vehicle
GW311616A
C)
β-cell mass (mg)
1.0
0.5
0.0
Control
GW311616A
*
D)
% BrdU positive β-cells
3.0%
2.0%
1.0%
0.0%
Control
GW311616A
**
E)
% BrdU positive α-cells
3.0%
2.0%
1.0%
0.0%
Control
GW311616A
F)
BrdU Incorporation (%)
16%
14%
12%
10%
8%
6%
4%
2%
0%
Control
GW311616A
Sc. Adipose
Visc. Adipose
Sk. Muscle
Spleen
Kidney
Liver
G)
Liver
Skeletal muscle
Visceral adipose
Subcutaneous adipose
Kidney
Spleen

FIG. 22

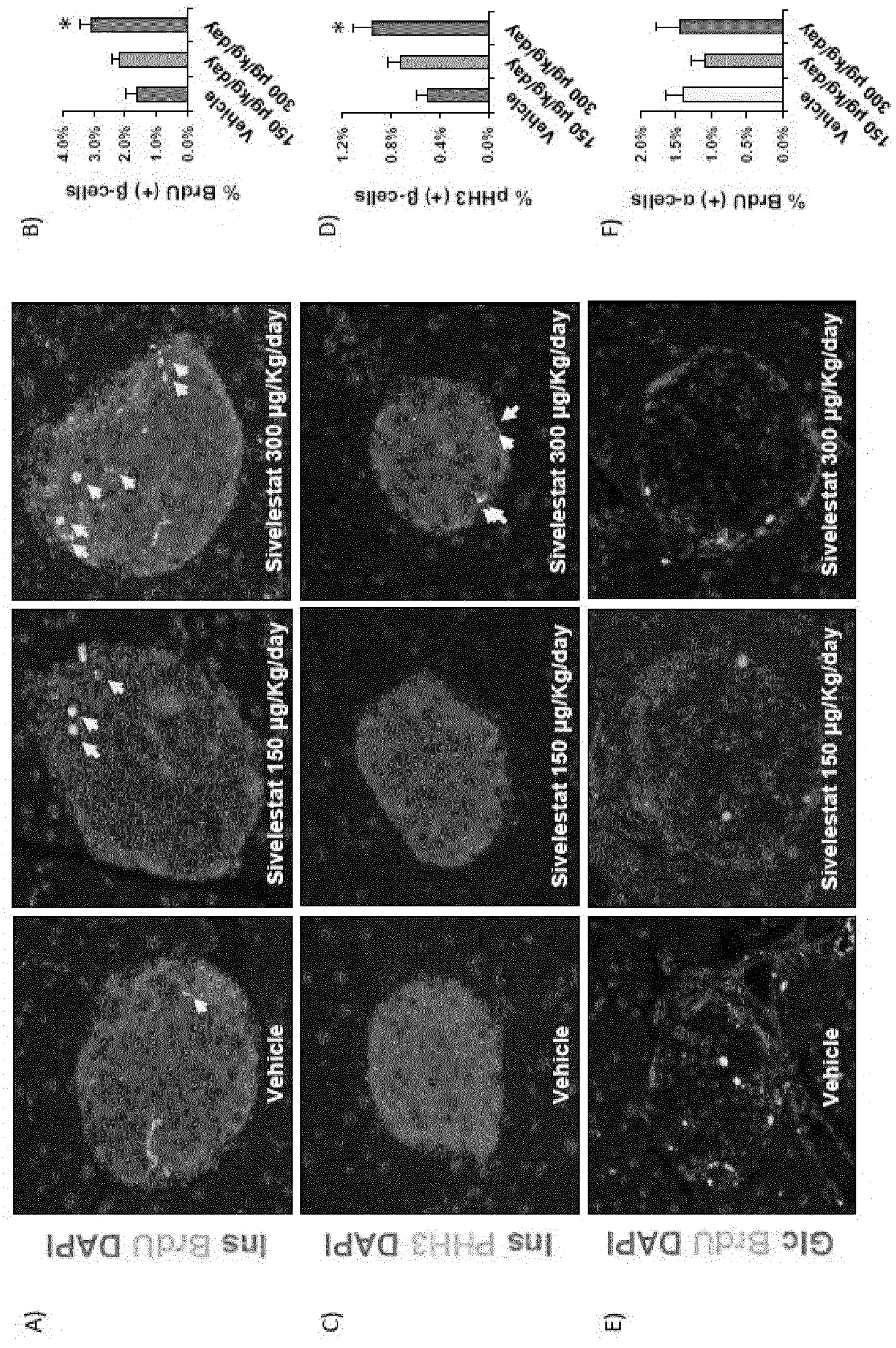

A)
Ins BrdU DAPI
Vehicle
Sivelestat 150 µg/Kg/day
Sivelestat 300 µg/Kg/day
B)
% BrdU (+) β-cells
4.0%
3.0%
2.0%
1.0%
0.0%
Vehicle
150 µg/kg/day
300 µg/kg/day
*
C)
Ins PHH3 DAPI
Vehicle
Sivelestat 150 µg/Kg/day
Sivelestat 300 µg/Kg/day
D)
% pHH3 (+) β-cells
1.2%
0.8%
0.4%
0.0%
Vehicle
150 µg/kg/day
300 µg/kg/day
*
E)
Glc BrdU DAPI
Vehicle
Sivelestat 150 µg/Kg/day
Sivelestat 300 µg/Kg/day
F)
% BrdU (+) α-cells
2.0%
1.5%
1.0%
0.5%
0.0%
Vehicle
150 µg/kg/day
300 µg/kg/day

FIG. 23

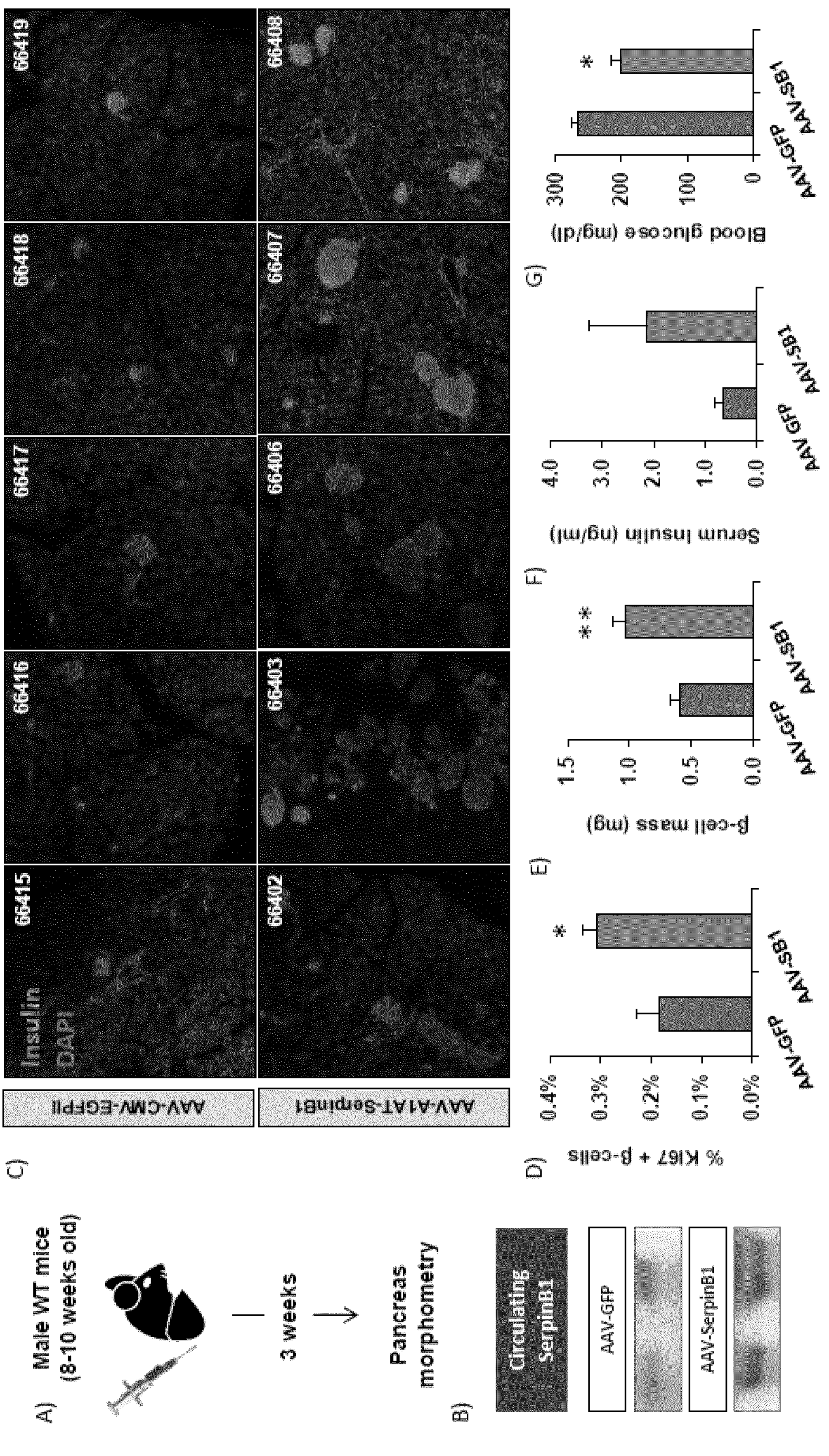

A)
Male WT mice
(8-10 weeks old)
3 weeks
Pancreas
morphometry
B)
Circulating
SerpinB1
AAV-GFP
AAV-SerpinB1
C)
AAV-CMV-EGFPII
AAV-A1AT-SerpinB1
Insulin
DAPI
66415
66416
66417
66418
66419
66402
66403
66406
66407
66408
D)
% Ki67 + β-cells
0.4%
0.3%
0.2%
0.1%
0.0%
AAV-GFP
AAV-SB1
*
E)
β-cell mass (mg)
1.5
1.0
0.5
0.0
AAV-GFP
AAV-SB1
**
F)
Serum Insulin (ng/ml)
4.0
3.0
2.0
1.0
0.0
AAV GFP
AAV-SB1
G)
Blood glucose (mg/dl)
300
200
100
0
AAV-GFP
AAV-SB1
*

FIG. 24

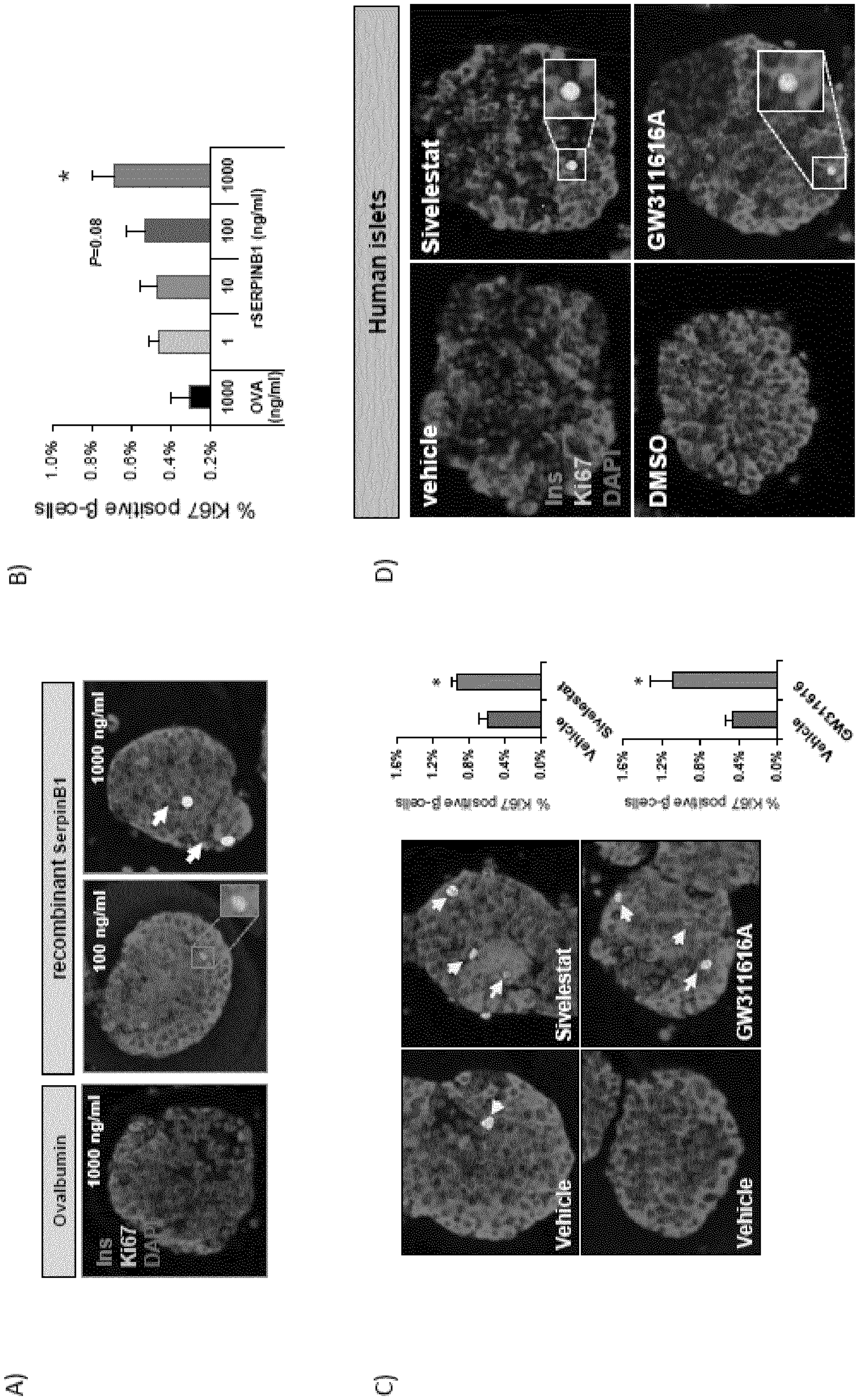

A)
Ovalbumin
recombinant SerpinB1
Ins
Ki67
DAPI
1000 ng/ml
100 ng/ml
1000 ng/ml
B)
% Ki67 positive β-cells
1.0%
0.8%
0.6%
0.4%
0.2%
P=0.08
*
1000
1
10
100
1000
OVA (ng/ml)
rSERPINB1 (ng/ml)
C)
Vehicle
Sivelestat
Vehicle
GW311616A
% Ki67 positive β-cells
1.6%
1.2%
0.8%
0.4%
0.0%
Vehicle
Sivelestat
GW311616
D)
Human islets
vehicle
Ins
Ki67
DAPI
Sivelestat
DMSO
GW311616A

FIG. 25

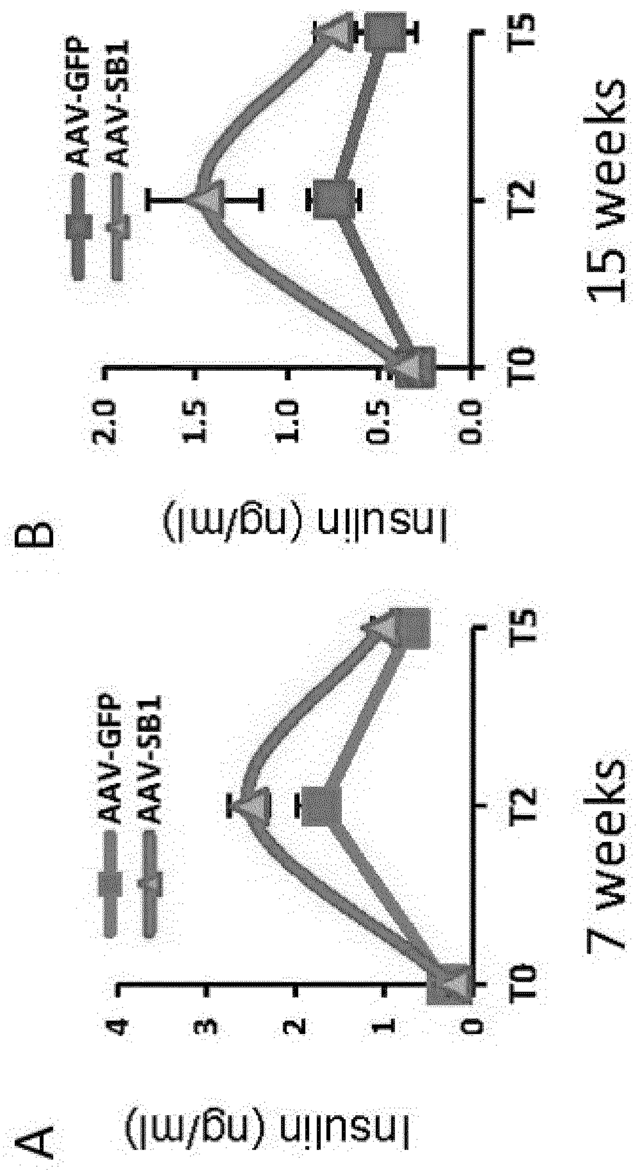

A
Insulin (ng/ml)
4
3
2
1
0
AAV-GFP
AAV-SB1
T0
T2
T5
7 weeks
B
Insulin (ng/ml)
2.0
1.5
1.0
0.5
0.0
AAV-GFP
AAV-SB1
T0
T2
T5
15 weeks

FIG. 26

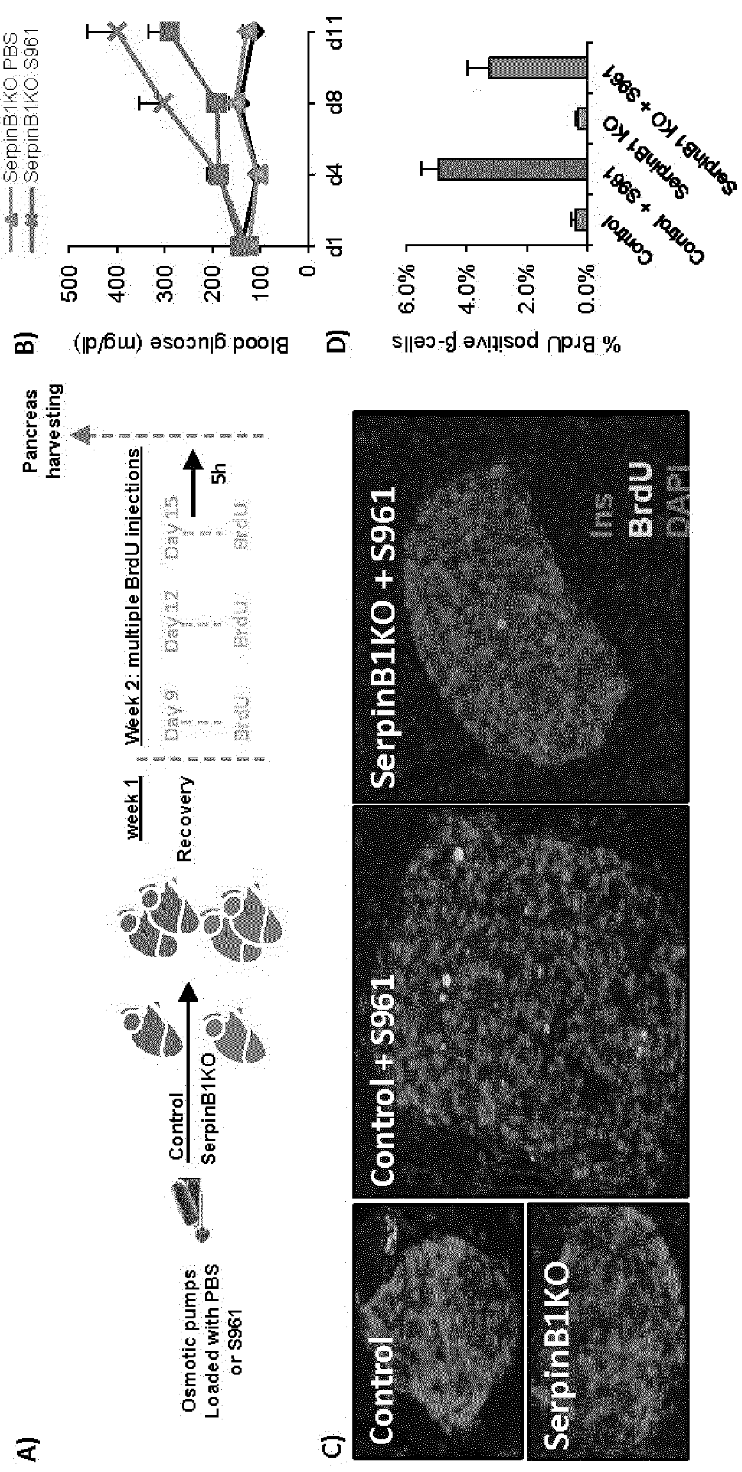

A)
Osmotic pumps Loaded with PBS or S961
Control
SerpinB1KO
week 1
Recovery
Week 2: multiple BrdU injections
Day 9
Day 12
Day 15
BrdU
BrdU
BrdU
5h
Pancreas harvesting
B)
Control PBS
Control S961
SerpinB1KO PBS
SerpinB1KO S961
Blood glucose (mg/dl)
500
400
300
200
100
0
d1
d4
d8
d11
C)
Control
SerpinB1KO
Control + S961
SerpinB1KO + S961
Ins
BrdU
DAPI
D)
% BrdU positive β-cells
6.0%
4.0%
2.0%
0.0%
Control
Control + S961
SerpinB1 KO
SerpinB1 KO + S961

FIG. 27

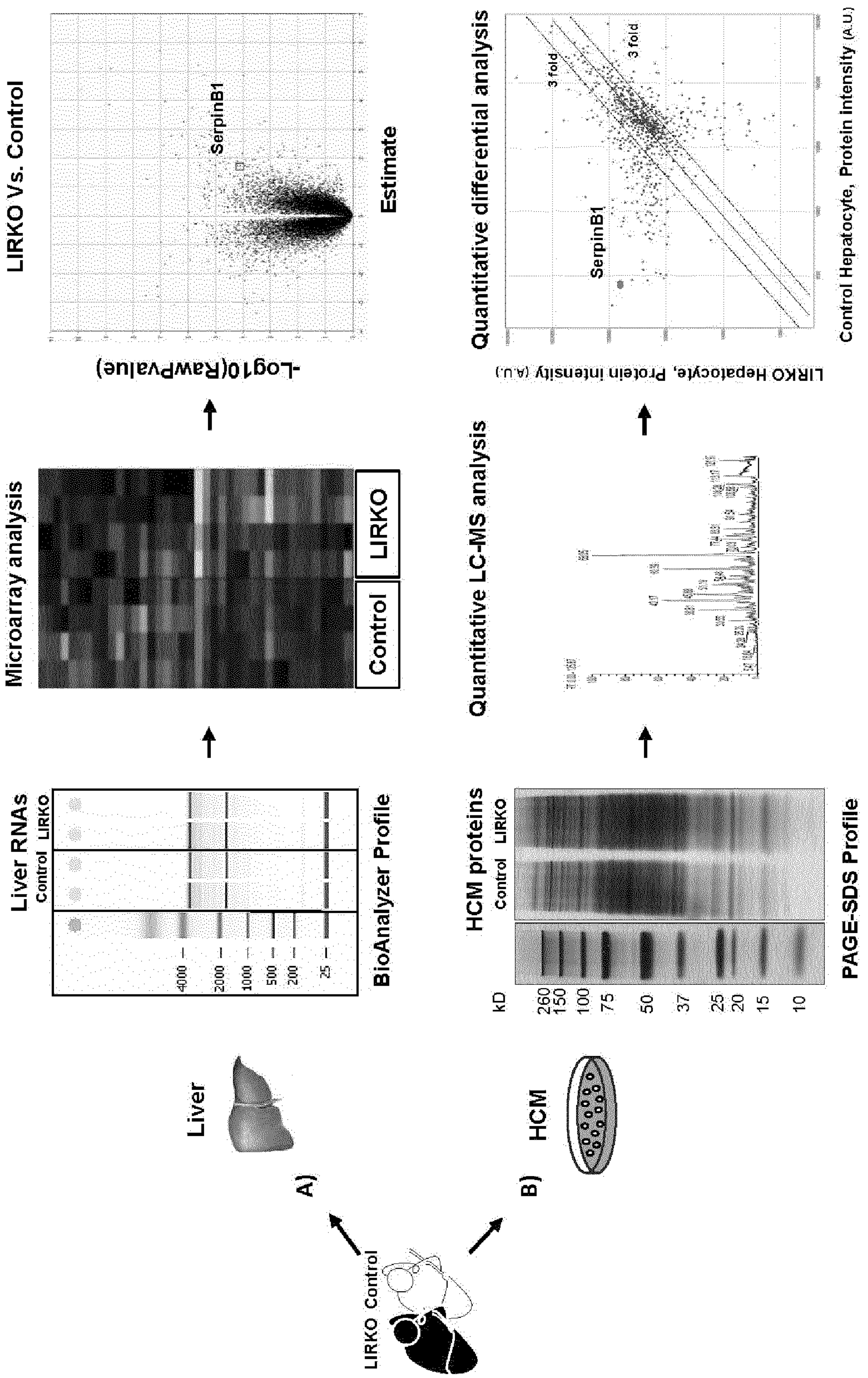

LIRKO Control
A)
Liver
B)
HCM
Liver RNAs
Control
LIRKO
4000
2000
1000
500
200
25
BioAnalyzer Profile
HCM proteins
Control
LIRKO
kD
260
150
100
75
50
37
25
20
15
10
PAGE-SDS Profile
Microarray analysis
Control
LIRKO
Quantitative LC-MS analysis
LIRKO Vs. Control
SerpinB1
-Log10(RawPvalue)
Estimate
Quantitative differential analysis
3 fold
3 fold
SerpinB1
LIRKO Hepatocyte, Protein intensity (A.U.)
Control Hepatocyte, Protein intensity (A.U.)

FIG. 28

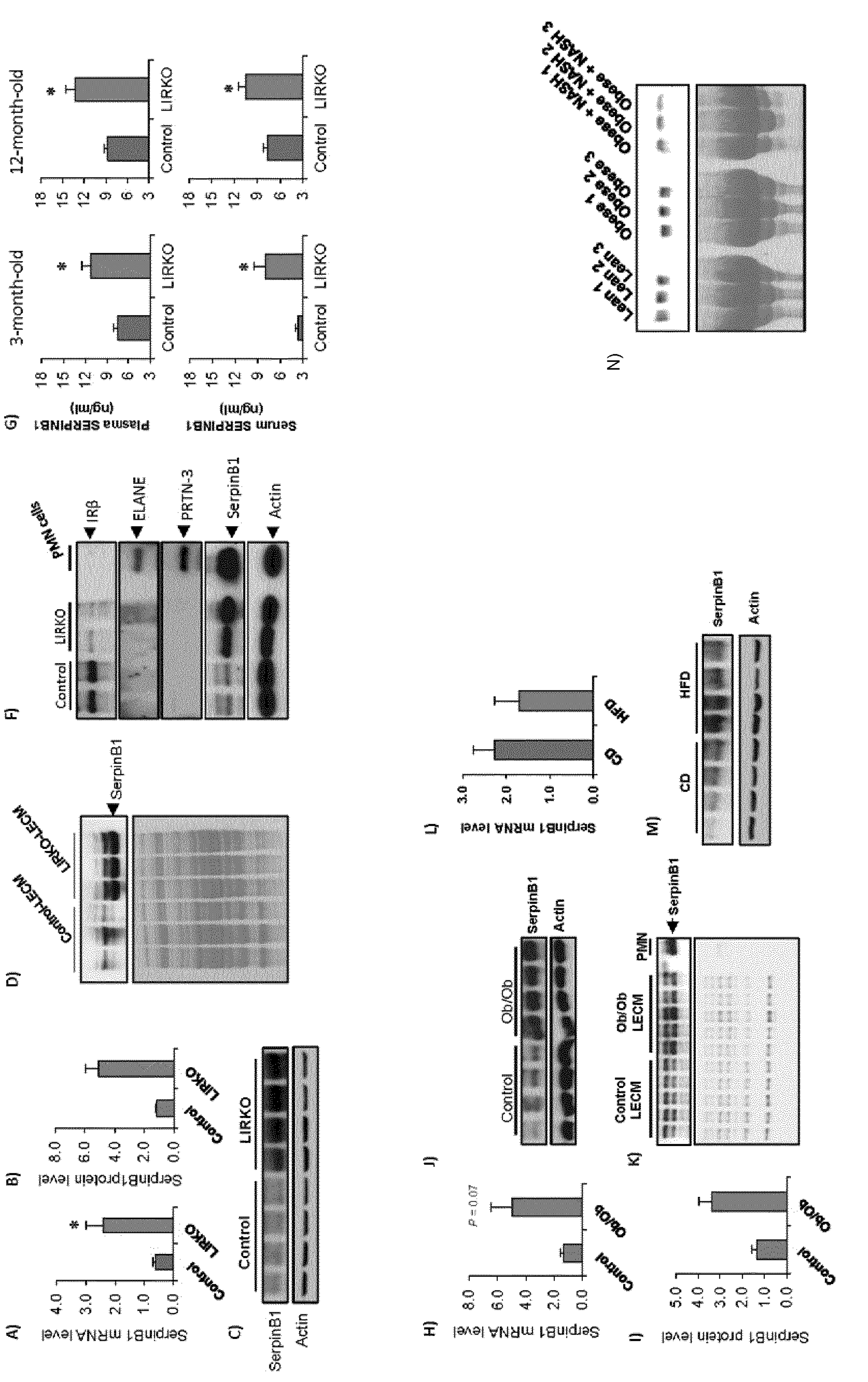

A)
Serpin B1 mRNA level
Control
LIRKO
B)
SerpinB1protein level
C)
SerpinB1
Actin
D)
Control-LECM
LIRKO-LECM
SerpinB1
F)
PMN cells
IRβ
ELANE
PRTN-3
SerpinB1
Actin
G)
3-month-old
12-month-old
Plasma SERPINB1 (ng/ml)
Serum SERPINB1 (ng/ml)
H)
SerpinB1 mRNA level
P = 0.07
Ob/Ob
I)
SerpinB1 protein level
J)
K)
Control LECM
Ob/Ob LECM
PMN
L)
CD
HFD
M)
N)
Lean 1
Lean 2
Lean 3
Obese 1
Obese 2
Obese 3
Obese + NASH 1
Obese + NASH 2
Obese + NASH 3

FIG. 29

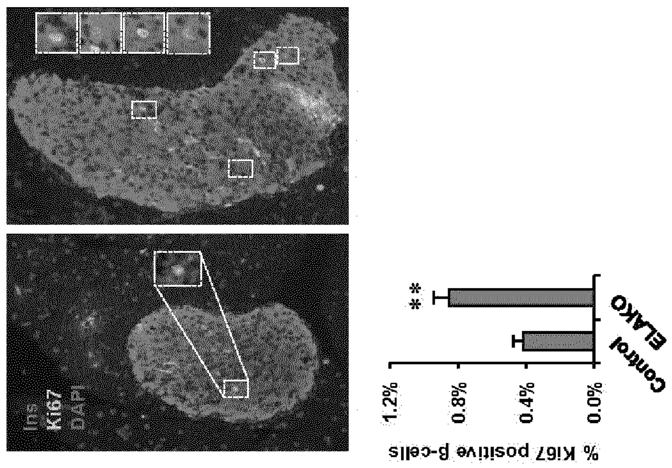


FIG. 30

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/053419

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/57 A61P3/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EL OUAAMARI A ET AL: "Liver-derived systemic factors drive [beta] cell hyperplasia in insulin-resistant states", CELL REPORTS, ELSEVIER INC, US, vol. 3, no. 2, 21 February 2013 (2013-02-21), pages 401-410, XP002707329, ISSN: 2211-1247, DOI: 10.1016/J.CELREP.2013.01.007 [retrieved on 2013-01-31] cited in the application abstract ----- -/--	1-44

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 April 2014	11/04/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vandenbogaerde, Ann

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GONG D ET AL: "Critical role of serpinB1 in regulating inflammatory responses in pulmonary influenza infection", THE JOURNAL OF INFECTIOUS DISEASES UNITED STATES 15 DEC 2010, INFECTIOUS DISEASES SOCIETY OF AMERICA, US, vol. 204, no. 4, 15 August 2011 (2011-08-15), pages 592-600, XP002707330, ISSN: 1537-6613, DOI: 10.1093/INFDIS/JIR352 abstract -----	1-44
X	WO 2007/006858 A2 (JURILAB LTD OY [FI]; SALONEN JUKKA T [FI]; TODOROVA BORYANA [FI]; AALT) 18 January 2007 (2007-01-18) claims 5, 13 paragraph [0216] -----	1-44
X	WO 2006/037606 A2 (SWITCH BIOTECH AG [DE]; UNIV MUENCHEN L MAXIMILIANS [DE]; SCHMIDT ROLA) 13 April 2006 (2006-04-13) claim 43 -----	1-44

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/053419

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007006858 A2	18-01-2007	US 2007072798 A1	29-03-2007
		WO 2007006858 A2	18-01-2007
WO 2006037606 A2	13-04-2006	AU 2005291398 A1	13-04-2006
		CA 2582474 A1	13-04-2006
		EP 1666075 A1	07-06-2006
		EP 1888134 A2	20-02-2008
		NZ 553752 A	27-08-2010
		WO 2006037606 A2	13-04-2006