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Vesiculin derived from IGF-II drives increased islet cell mass in a mouse model of pre-diabetes

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ABSTRACT

Pancreatic islet-cell function and volume are both key determinants of the maintenance of metabolic health. Insulin resistance and islet-cell dysfunction often occur in the earlier stages of type 2 diabetes (T2D) progression. The ability of the islet cells to respond to insulin resistance by increasing hormone output accompanied by increased islet-cell volume is key to maintaining blood glucose control and preventing further disease progression. Eventual β-cell loss is the main driver of full-blown T2D and insulin-dependency. Researchers are targeting T2D with approaches that include those aimed at enhancing the function of the patient's existing β-cell population, or replacing islet β-cells. Another approach is to look for agents that enhance the natural capacity of the β-cell population to expand. Here we aimed to study the effects of a new putative β-cell growth factor on a mouse model of pre-diabetes. We asked whether: 1) 4-week's treatment with vesiculin, a two-chain peptide derived by processing from IGF-II, had any measurable effect on pre-diabetic mice vs vehicle; and 2) whether the effects were the same in non-diabetic littermate controls. Although treatment with vesiculin did not alter blood glucose levels over this time period, there was a doubling of the Proliferating Cell Nuclear Antigen (PCNA) detectable in the islets of treated pre-diabetic but not control mice and this was accompanied by increased insulin- and glucagon-positive stained areas in the pancreatic islets.

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Introduction

Insulin-like growth factor II (IGF-II) has been shown to be produced and secreted by pancreatic islet β-cells. Often thought of as a neonatal growth factor, the role of IGF-II in adulthood is less well understood. Although adult humans have relatively large concentrations of circulating IGF-II, the vast majority is bound to binding proteins in complexes that suppress its bioactivity.[1] There is no clear understanding of the role of circulating IGF-II in the healthy physiology of adults, although it is clear that dysregulation in IGF-II system, for example, through loss of imprinting if the *IGF2* or the *IGF2R* locus, is related to inappropriate tissue growth.[2] Tissue-specific expression of IGF-II in adulthood is likely to be a key factor in the regeneration of adult tissues through regulating adult stem cell differentiation.[3–5]

The capacity of the adult pancreatic islet β-cell population to expand in times of need such as during pregnancy, in overweight people or following injury is of great relevance to the risk of developing type-2 diabetes (T2D). In early diabetes, insulin resistance is often the driver of an increase in insulin requirement that must be met through β-cell adaptation, if normoglycemia is to be maintained; such adaptation must comprise both increased insulin output of the existing β-cells as well as expansion of the β-cell population. The progression of disease

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eventually leads to overexertion of the β-cells with resulting apoptosis, likely via processes such as overwhelming ER stress[6] or through the action of toxic amylin oligomers on β-cell membranes produced from overexpression of the β-cell hormone amylin.[7] T2D is multifactorial and the relative importance of insulin deficiency/ β-cell failure and insulin resistance is a defining feature of disease subtypes.[8] IGF-II has been shown to be important for mediating β-cell expansion in times of need. Indeed, in the pancreas, β-cell-specific ablation of IGF-II revealed a role in β-cell expansion in responses to high fat diet and to pregnancy.[9]

Insufficient IGF-II has been shown to be responsible for the development of the spontaneous T2D phenotype in the Goto-Kakizaki rat.[10] In adult rodents, there is no detectable circulating IGF-II, although there is evidence for tissue-specific IGF-II expression suggesting that its role in tissue regeneration is conserved between rodents and humans, even though the circulating pool of IGF-II itself is not.

IGF-II is closely related to insulin and indeed can function in the same way by signaling via the same family of receptors.[11] The glucoregulatory activity of IGF-II is approximately 50-fold lower than that of insulin yet, nevertheless, it is highly likely to have physiological relevance.[12] There are several peptide products that are derived from the *Igf2* gene. Islet β-cell granules have been shown to contain classic mature IGF-II, preptin (derived from the E-peptide of proIGF-II), and vesiculin.[13,14] The latter is a two-chain peptide, indicating that IGF-II, like other insulin-family peptides, is processed into a two-chain peptide with two inter-chain and one intra-chain disulfide bridges. The effects of vesiculin on lowering of blood glucose are equivalent to those of mature IGF-II but it is not a processing product found in the serum of adult humans or neonatal mice, it is therefore likely to be specifically produced in β-cells or at least neuroendocrine cells where expression of *Igf2* and prohormone converting enzymes converge.[15,16]

Here we aimed to study the potential for this β-cell-derived IGF-II product to limit the progression of the diabetic phenotype in human amylin transgenic (hA-tg) mice. We found that a 4-week treatment period with a sub-glucoregulatory dose of vesiculin administered via the intraperitoneal (i.p.) route at a frequency of three times per week did not result in overt changes in blood glucose levels. By contrast, however, we found substantive evidence for vesiculin-evoked expansion of islet β-cell mass and increased serum insulin levels in vesiculin-treated human amylin transgenic mice.

Materials and methods

Animals

Protocols were approved by the University of Auckland Animal Ethics Committee (AEC001984) and performed in accordance with the NZ Animal Welfare Act (1999). The model used here is the human amylin transgenic (hA-tg) mouse, which overexpress human amylin (hA) in their β-cells. When overexpressed, human amylin aggregates to form amylin oligomers that are cytotoxic to β-cells. Hemizygous hA-tg mice spontaneously develop diabetes and show symptoms similar to those seen in T2D patients, including hyperglycemia and significantly increased levels of serum insulin.[7,17–20] Control mice were non-transgenic (non-tg) littermates. Male mice were housed from weaning (day 21) in environmentally controlled conditions (temperature and humidity) with a 12-hr light/dark cycle and were assessed daily for welfare and access to food and water. They received standard rodent chow (Teklad TB 2018; Harlan, Madison, WI) and water *ad libitum*.

Weekly monitoring of male hA-tg and non-tg littermates included weights, core body temperatures and blood glucose. Weekly blood glucose concentrations were taken from age 7 weeks and the inclusion criterion was that at least 3 of the 4 readings immediately prior to inclusion were within the pre-diabetic range (8–11 mmol/L) for an hA-tg mouse and, for a non-tg, 3 of the 4 readings were <8 mmol/L. Age at study inclusion was between 11.5 and 14 weeks of age. Littermate pairs were randomized to receive vesiculin or vehicle treatment. Serum from tail blood was collected from mice matching acceptance criteria at the start of the 4-week treatment period (3 x per week i.p. delivery of vesiculin 62 pmol/g or vehicle (0.1% n-dodecyl-β-D-maltoside (DDM)),

150 µl total volume). Weekly monitoring data and tail-blood samples were collected prior to drug treatment.

Materials

Mouse vesiculin was synthesized in-house by the peptide synthesis group as per their published procedure in which the A- and B-peptide chains were first synthesized separately with protected cysteine residues before using regioselective disulfide bond formation;[21] stocks were prepared at a final concentration of 1 µg/µl in 16 mg/ml glycerol; 0.1% DDM. Insulin (Actrapid® Recombinant human-insulin 100 IU/ml; 3.47 µg/µl) was from Novo Nordisk (Bagsvaerd, Denmark). Both were diluted in physiological saline to the desired final concentrations. Blood glucose was measured in tail-vein blood (Advantage II; Roche). Core body temperature was recorded using a rectal probe (RET-3) coupled to a thermometer (Physitemp Instruments Inc).

Vesiculin stability

Synthetic mouse vesiculin was administered i.p. in vehicle (0.1% DDM). Samples were taken from tail veins of 8-week-old male non-tg mice (n = 3 per dose) at various time-points over 24 h following peptide/treatment. Vesiculin was quantitated in serum using our published LC-MS/MS method.[15]

Hormone measurements

Tail-vein serum samples from weeks 0, 4 and 8 were used for this analysis. In the event that these samples were unavailable for some individuals, an alternative sample (week −1, 1 or 5 was used, all week 8 data were from week 8 samples). Insulin, leptin and high molecular weight (HMW) adiponectin were assayed using ELISA kits (80-INSMSU-E01, 47-ADPMS-E01 and 22-LEPMS-E01, all from Alpco, NH, USA).

Histology

Pancreas histology was performed on three representative mice from each group. Pancreas (head region) was fixed in 10% neutral-buffered formalin and paraffin embedded. Sections were cut at 7-µM thickness and groups of 15 slides with three serial sections each were taken at intervals through each block allowing approximately 6 separate sampled areas across each pancreas. 4–5 slides all from different areas were stained from each pancreas. Slides were fixed/permeabilised with acetone before blocking with 10% normal goat serum. Three sections on each slide were stained with guinea-pig anti-insulin (Abcam ab7842), 1:400; co-stained with either rabbit anti-glucagon (Cell signaling 2760) 1:400; rabbit anti-pancreatic polypeptide (Bioss bs-8543 r) 1:800; or rabbit anti-somatostatin (Dako A0566), 1:400. Secondary antibodies employed were 488 Alexfluor-labeled goat anti-guinea-pig, 1:400; and Alexfluor 594 goat anti-rabbit, 1:400 (both from Invitrogen).

Every islet on a section was imaged x40 with an Olympus FV1000 confocal microscope and images (x2.5 magnifiction) were also captured to measure total sectional area using emitted green auto-fluorescence. Images were analyzed using Fiji (Image J)[22] open-source software collecting stained areas in the red and the green channel for each islet. Data collected therefore included total section area, number of islets/section (an islet was recorded if it included more than 5 cells), insulin-positive area per islet (green) and on three serial sections either glucagon, pancreatic polypeptide, or somatostatin (red) positive area. Glucagon, somatostatin, and pancreatic polypeptide were measured to estimate areas corresponding to each hormone's specific islet cell-type.

Sections were also stained for Proliferating Cell Nuclear Antigen (PCNA) using (93–1143, Invitrogen) (Proliferating Cell Nuclear Antigen bio-tinylated antibody staining kit) with DAB as the chromogen and co-staining with hematoxylin & eosin (H&E). Analysis included 3–5 sections from different pancreas areas for each pancreas and every islet from those sections was imaged at x40 with an Olympus FV1000 confocal microscope. Images were analyzed using Fiji (Image J) open-source software and the area positive for DAB stain per islet area was measured using deconvoluted color images.

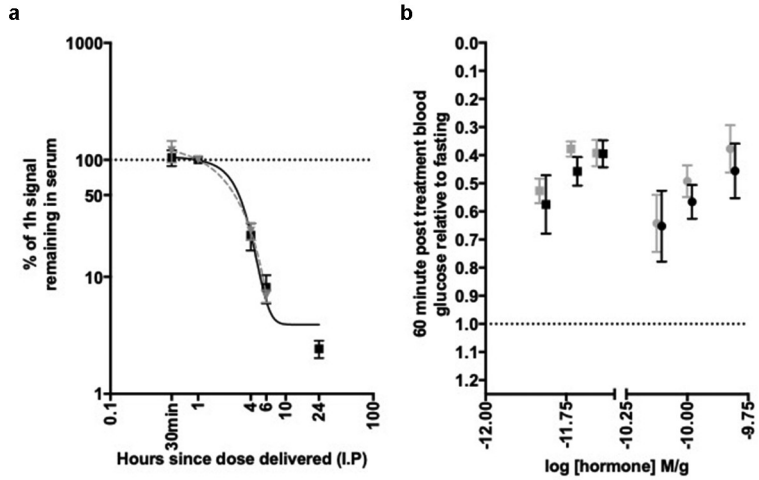

Figure 1. a) In vivo serum stability of vesiculin. Peptide quantification calculated as peak area from mass spectral analysis and presented relative to 1 h signal. 150 pmol/g black squares, 68 pmol/g gray inverted triangles. Half-life of 150 pmol/g was 2.64 h. **b) Blood glucose lowering activity of peptides in hA-tg (gray) and non-tg (black) mice**. X-axis shows log of the hormone molar concentration per mouse weight (g). Recombinant human insulin (Actrapid®), 1.47, 1.84 and 2.21 pmol/g (0.4, 0.5 and 0.6 mU/g) in square symbols. Synthetic mouse vesiculin, 75, 100 and 150 pmol/g in circle symbols.

Results

In vivo stability of vesiculin in mice at two doses was assessed using our previously described LC-MS/MS method[15] (Figure 1A). The half-life was calculated as 2.64 h (SEM 1.12 h). As previously shown,[16] vesiculin can lower blood glucose at a potency of approximately 50-fold lower than insulin but equivalent to IGF-II. The blood-glucose lowering capacity of vesiculin did not differ between young hemizygous hA-tg mice and non-tg littermates (Figure 1B).

We recruited 9 mice per group and the ages and weights did not differ significantly between groups (Table 1A). As the hA-tg mouse has been shown to have elevated core body temperature as well as elevated blood glucose we assessed both parameters over the course of the study. Core body temperature trended toward elevation in hemizygous hA-tg mice compared to non-tg littermates but this difference was not statistically significant; however, blood glucose levels were significantly higher in hemizygous hA-tg

Table 1. A) Initial age, weight, body temperature (CBT) and blood glucose (BG) of mice in each study group. Age and weight are week 0 (starting values), CBT and BG are average values from weeks −3 to 0. Statistical analysis is by 2-way ANOVA with Sidak's multiple testing between genotype within treatment. (****p < .0001). No other statistical differences were found either between genotypes or treatment groups.
B) Tissue wet weight (g) of mice in each study group. 2-way ANOVA found no differences.

A

Treatment	Genotype	Age (d)	Weight (g)	Ave CBT (°C)	Ave BG (mmol/L)
vehicle	hA-Tg	85.8 (5.8)	29.0 (2.5)	37.33 (0.5)	9.5 (0.5)
vesiculin	hA-Tg	87.4 (6.6)	28.7 (2.8)	37.26 (0.46)	9.4 (0.7)
vehicle	non-tg	85.8 (5.8)	29.4 (1.75)	37.15 (0.57)	7.7 (0.2)****
vesiculin	non-tg	86.8 (5.4)	29.9 (2.8)	37.17 (0.43)	7.6 (0.3)****

B

Treatment	Genotype	pancreas (g)	heart (g)	kidney (g)	liver (g)
vehicle	hA-Tg	1.28 (0.16)	0.60 (0.25)	1.05 (0.25)	4.66 (0.26)
vesiculin	hA-Tg	1.3 (0.26)	0.50 (0.12)	0.87 (0.23)	5.01 (0.41)
vehicle	non-tg	1.22 (0.18)	0.60 (0.14)	1.01 (0.27)	4.80 (0.34)
vesiculin	non-tg	1.17 (0.21)	0.60 (0.18)	0.98 (0.19)	4.84 (0.31)

mice (Table 1A). All mice gained weight over the 4 weeks prior to treatment but weight gain stopped over the 4 weeks of i.p. injections. Weight gain resumed in the 4 weeks following treatment (Figure 2 A-D). Body weights of both vehicle- (p = .006) and vesiculin-treated (p = .0001) hA-tg mice were higher at week 8 than at week 4. Both non-transgenic treatment groups had significantly higher body weights at week 8 compared to week 0, but only the vesiculin-treated group had a statistically significant increase from weeks 4 to 8 (p = .0003). Post-hoc testing also revealed that weight gain was significantly higher in vesiculin-treated hemizygous hA-tg mice between 1–4 and 5–8 weeks (p = .015), but not in the vehicle treated group, nor the non-tg groups.

Core body temperature and blood glucose measurements can vary from reading to reading for various reasons including handling stress and therefore data is shown as a mean of 4 readings (core body temp Figure 2 E&F, blood glucose G&H). Core body temperature trended toward elevation in all groups over the 4 weeks of treatment although the difference was not statistically significant in any individual group. In general, core body temperature was lowered again in weeks 5–8 and this reduction was statistically significant in both vehicle-treated groups (hA-tg, p = .02; non-tg, p = .013). Core body temperature was not statistically different between hemizygous hA-tg and non-tg mice at any of the time points tested.

Blood glucose levels of hemizygous hA-tg mice were higher than non-tg at the start of the study as per the inclusion criteria. Through the 4 weeks of peptide hormone or vehicle treatment, the blood glucose levels of all groups remained steady. Average blood glucose in all groups was lower in week 5–8 than in week 1–4; post-hoc testing revealed the difference was only statistically significant in the vehicle-treated hemizygous hA-tg mice (p = .04) (Figure 2G). Overall, for body weight, core body temperature and blood glucose, there were no significant difference between treatment and vehicle groups over the course of the study in either hemizygous hA-tg or non-tg mice.

Serum insulin levels remained consistent through the 4-week treatment period for all groups (Figure 3A&B). However, insulin levels were elevated in week 8 compared to week 4 for vesiculin and vehicle-treated non-tg mice with no significant difference due to treatment. For the hemizygous hA-tg mice the increase in week 8 was of a larger magnitude and occurred only in the vesiculin-treated group. Insulin in the vesiculin-treated hemizygous mice was

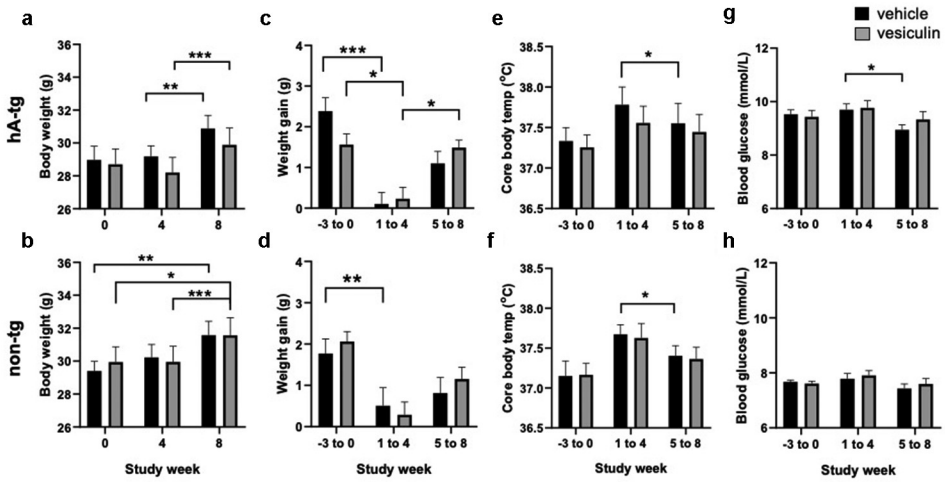

Figure 2. Weight, body temperature and blood glucose from 8-week study period. Upper panels (a, c, e, g) hemizygous hA-tg mice, lower panels (b, d, f, h) non-tg mice. Vehicle treated (black bars) vesiculin treated (gray bars). Statistics are 2-way ANOVA with Sidak's multiple comparisons tests comparing time-points within treatment (*p < .05, **p < .01, ***p < .001).

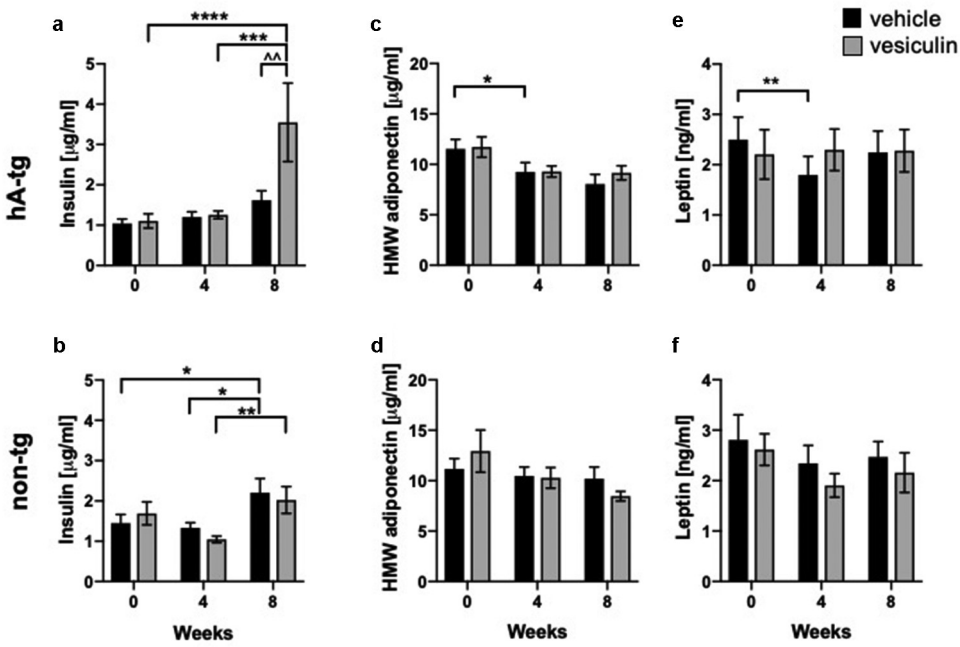

Figure 3. Serum hormone levels. Serum insulin (a, b), HMW adiponectin (c, d) and leptin (e, f). Upper panels (a, c, e) hemizygous hA-tg mice, lower panels (b, d, f) non-tg mice. Vehicle treated (black bars) vesiculin treated (gray bars). Statistics are 2-way ANOVA with Sidak's multiple comparisons test. Post-hoc *p* value between time-points within treatment **, *p* value between treatments within time-points ^^.

significantly higher than in vehicle-treated controls (p = .003). The higher serum insulin levels at week 8 in all groups was associated with a drop in blood glucose in week 5–8 (post treatment) for all groups (Supplemental Figure S1 C). The largest elevation in serum insulin was in the hA-tg mice and this did not equate to a significantly larger decrease in blood glucose in this group (Suppl Figure 1D). Both leptin (p = .005) and high molecular weight (HMW) adiponectin (p = .023) decreased in the vehicle-treated hA-tg mice following 4 weeks of treatment whereas vesiculin-treated mice did not show a corresponding decrease (Figure 3 C-F).

At the end of the 8 weeks, organ weights did not indicate any difference for pancreas, heart, kidney and liver (Table 1B). Histological analysis of the pancreas was performed on three representative animals per group. A total of 147 sections were analyzed. In total, 424 individual islets were analyzed on 49 sections co-stained for insulin and glucagon, 457 individual islets were analyzed on 49 sections co-stained insulin and PP, and 477 individual islets were analyzed on 49 sections co-stained for insulin and somatostatin. Immunofluorescent labeling of islet hormones revealed larger proportions of both insulin and glucagon staining per section area in the vesiculin-treated hemizygous hA-tg mice (Figure 4A and B). By contrast, there were no significant differences in pancreatic polypeptide or somatostatin (Figure 4C and D). Section area per islet indicated that the vesiculin-treated non-tg group had the lowest number of islets but this finding may have been driven by some sections from one individual which contained no discernible islet tissue. There was no difference between the other groups (Figure 4E).

3,3′-Diaminobenzidine (DAB)-labeled PCNA antibody showed the highest levels in both vesiculin- and vehicle-treated non-tg mice and the lowest levels in the vehicle-treated hemizygous hA-tg mice. The proportion of PCNA- labeled nuclei in the islets from the vesiculin-treated hemizygous hA-tg group was significantly higher than in vehicle-treated hemizygous hA-tg (p = .02) and was not different to that in the non-tg mice (Figure 4F).

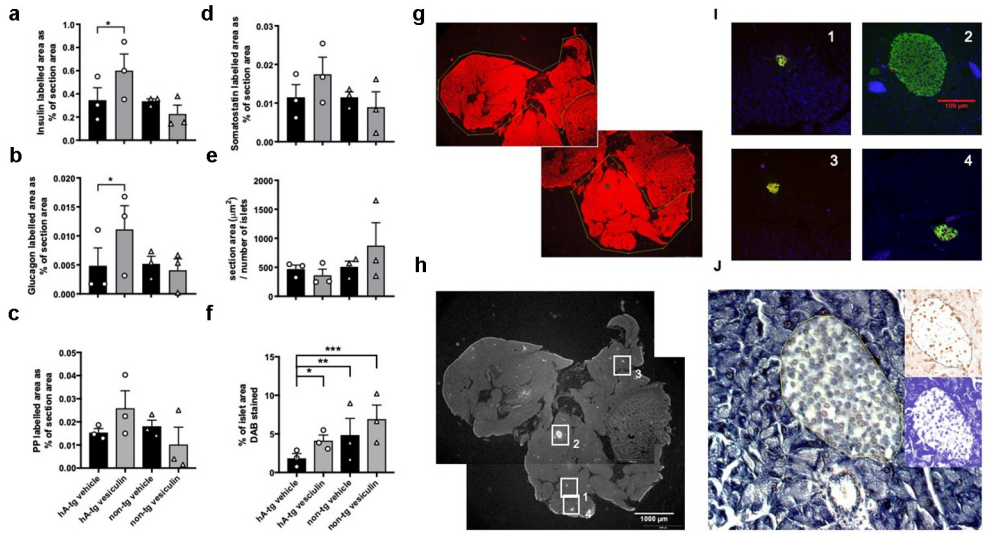

Figure 4. Histological analysis of the pancreas. Percentage of total section area stained positive for insulin (a), glucagon (b), pancreatic polypeptide (c), and somatostatin (d). Pancreas section area (µm) /number of islets on section (e). % of islet area stained positive for PCNA (f). A-F are average values for all sections imaged for an individual pancreas. Image showing total section imaging at 2.5x magnification illustrating autofluorescence used to measure total section area of each section using signal thresholding and ROI. In this case, the section was too large to see in a single image and area was calculated from two images. The area of GI tract visible on each section was excluded from the calculation (g). Image of total section shown in G with position of 4 islets indicated, 2.5x magnification. Scale bar indicates 1000 µm (h). Images of four islets at 40x magnification indicated in h, insulin (green), glucagon (red) and DAPI (blue). Scale bar indicates 100 µm (I). Image of H&E stained islet x40 co-labeled for PCNA indicated by DAB brown-stained nuclei. Insets shows color deconvolution of hematoxylin (purple) and DAB (brown) staining used to measure 'stained area' within 'islet area'(J). Statistical analysis for A-F is by 2-way ANOVA with Dunnett's multiple comparisons test.

Discussion

Human amylin transgenic mice become spontaneously diabetic with increases in blood glucose, insulin and amylin levels and thus mimic the signs present in T2D patients.[17] Vesiculin is an IGF-II-derived peptide hormone that has been shown to be present in insulin-secreting mouse β-cells.[14,15] Processing of IGF-II to vesiculin is likely performed by the same prohormone convertase enzymes responsible for conversion of pro-insulin to mature two-chain insulin. We have previously shown that IGF-II and vesiculin are both co-secreted with insulin in response to glucose.[15] We have also shown that both are equally active in terms of signaling via the IGF1R and in glucose lowering *in vivo* or glucose uptake *in vitro*. By contrast, we have shown that pre-diabetic mice have an improved glucose lowering response to vesiculin compared to IGF-II.[16]

Hemizygous hA-tg mice spontaneously develop diabetes in the absence of external drivers (such as high fat or high glucose diets or streptozotocin) and show symptoms similar to those seen in T2D patients, including hyperglycemia, significantly increased levels of serum insulin and amylin and eventual loss of β-cells. Thus, hA-transgenic mice are an informative model in understanding the interactions of β-cell-specific hormones in the underlying mechanisms leading to β-cell dysfunction and onset of diabetes and the possible delay of β-cell loss. Over a 4-week treatment period, vesiculin elicited no measurable treatment effect on weight gain, body temperature or blood glucose (non-fasted) compared to vehicle. Leptin and adiponectin are fat-derived hormones that play substantive roles in the regulation of systemic glucose homeostasis.[23] Leptin levels in hA-tg mice decreased in the vehicle treated mice in week 4 whereas vesiculin treated mice did not have a corresponding decrease in leptin, similar to HMW adiponectin. These hormones normally correlate inversely with weight gain in this mouse model. Previously we have seen leptin and adiponectin are decreased in the in the hA-tg mice compared to non-tg, but the genotype-driven differences were minimal in this study. The fact that leptin did not

decrease in the vesiculin treated hA-tg mice is a potential difference related to treatment. Serum insulin levels did not differ between vehicle- and vesiculin-treated groups immediately following the 4-week treatment period; however, following a further 4 weeks' monitoring, serum insulin was elevated in the vesiculin-treated hA-tg mice. This finding was accompanied by histological evidence of increased islet-cell area as demonstrated by larger insulin- and glucagon-stained areas, and increased PCNA staining in islet cell nuclei, although there was no increase in islet number. The significantly elevated serum insulin levels in the hA-tg group at week 8 were not accompanied by significantly lower blood glucose levels. As alpha cell area was shown to be increased in this group, there is potential for elevated glucagon secretion that could counteract elevated insulin. Due to limited serum sample size collected from the tail vein glucagon was not measured. This effect did not occur in the vesiculin-treated non-tg mice. The inclusion criteria used here specified a pre-diabetic stage immediately following the development of elevated blood glucose whereupon eligible mice had blood glucose levels <10 mmol/L). This is before increased buildup of amyloid deposits in this model results in a loss of islet β-cell volume.[18]

Our current results indicate that although vesiculin is capable of driving expansion of islet cell mass, the pre-diabetic state is also important to enable it to trigger this response. This effect may be mediated through a difference in receptor levels or stoichiometry of receptor types (IGF1R, IR-A, IR-B), but we have previously shown IGF-II and vesiculin to have the same signaling capacity through the IGF1R and the IR; these prior findings suggested that the activity of IGF-II in prediabetic states is blunted whereas that of vesiculin is not. Therefore, it is likely that another feature of pre-diabetes allows vesiculin to drive islet cell proliferation.

Conclusion

We present evidence that vesiculin, a proIGF-II peptide made and secreted by pancreatic islet β-cells, may form part of the mechanism by which β-cell expansion is driven in times of need such as diabetes.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Abbreviations

Type-2 diabetes (T2D), Proliferating Cell Nuclear Antigen (PCNA), Insulin-like growth factor II (IGF-II), n-dodecyl-β-D-maltoside (DDM), tandem mass spectrometry coupled to liquid chromatography (LC-MS/MS), high molecular weight (HMW) enzyme-linked immunosorbent assay (ELISA).

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