Brief Communication

MOLECULAR METABOLISM

Restoration of *Lepr* in β cells of *Lepr* null mice does not prevent hyperinsulinemia and hyperglycemia

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ABSTRACT

Objective: The adipose-derived hormone leptin plays an important role in regulating body weight and glucose homeostasis. Leptin receptors are expressed in the central nervous system as well as peripheral tissues involved in regulating glucose homeostasis, including insulin-producing β cells of the pancreas. Previous studies assessing the role of leptin receptors in β cells used Cre-*loxP* to disrupt the leptin receptor gene (*Lepr*) in β cells, but variable results were obtained. Furthermore, recombination of *Lepr* was observed in the hypothalamus or exocrine pancreas, in addition to the β cells, and *Lepr* in non-β cells may have compensated for the loss of *Lepr* in β cells, thus making it difficult to assess the direct effects of *Lepr* in β cells. To determine the significance of *Lepr* exclusively in β cells, we chose to selectively restore *Lepr* in β cells of *Lepr* null mice ($Lepr^{loxTB/loxTB}$).

Materials and methods: We used a mouse model in which endogenous expression of *Lepr* was disrupted by a *loxP*-flanked transcription blocker ($Lepr^{loxTB/loxTB}$), but was restored by *Cre* recombinase knocked into the *Ins1* gene, which is specifically expressed in β cells (*Ins1*Cre). We bred $Lepr^{loxTB/loxTB}$ and *Ins1*Cre mice to generate $Lepr^{loxTB/loxTB}$ and $Lepr^{loxTB/loxTB}$ *Ins1*Cre mice, as well as $Lepr^{wt/wt}$ and $Lepr^{wt/wt}$ *Ins1*Cre littermate mice. Male and female mice were weighed weekly between 6 and 11 weeks of age and fasting blood glucose was measured during this time. Oral glucose was administered to mice aged 7–12 weeks to assess glucose tolerance and insulin secretion. Relative β and α cell area and islet size were also assessed by immunostaining and analysis of pancreas sections of 12–14 week old mice.

Results: Male and female $Lepr^{loxTB/loxTB}$ mice, lacking whole-body expression of *Lepr*, had a phenotype similar to *db/db* mice characterized by obesity, hyperinsulinemia, glucose intolerance, and impaired glucose stimulated insulin secretion. Despite restoring *Lepr* in β cells of $Lepr^{loxTB/loxTB}$ mice, fasting insulin levels, blood glucose levels and body weight were comparable between $Lepr^{loxTB/loxTB}$ *Ins1*Cre mice and $Lepr^{loxTB/loxTB}$ littermates. Furthermore, glucose tolerance and insulin secretion in male and female $Lepr^{loxTB/loxTB}$ *Ins1*Cre mice were similar to that observed in $Lepr^{loxTB/loxTB}$ mice. Analysis of pancreatic insulin positive area revealed that restoration of *Lepr* in β cells of $Lepr^{loxTB/loxTB}$ mice did not prevent hyperplasia of insulin positive cells nor did it rescue Glut-2 expression.

Conclusion: Collectively, these data suggest that direct action of leptin on β cells is insufficient to restore normal insulin secretion and glucose tolerance in mice without leptin receptor signaling elsewhere.

Keywords Leptin receptor; Hyperinsulinemia; β cell

1. INTRODUCTION

Obesity is often associated with elevated circulating insulin levels (hyperinsulinemia), glucose intolerance, and reduced insulin sensitivity [1,2]. The molecular mechanisms underlying the increased insulin secretion and islet hyperplasia that are often concomitant with obesity remain unclear, but the adipose-derived hormone leptin may be a potential link between adipose tissue and β cells. Leptin has an established role in regulating food intake, adipose metabolism, and glucose homeostasis through signaling in the central nervous system (CNS) [3,4]. In addition, leptin receptors are distributed in several peripheral tissues involved in glucose regulation including the liver, adipose, and pancreas [5,6]. Within these tissues, the long isoform of the leptin receptor (Leprb) is believed to mediate the glucoregulatory actions of leptin [7].

In vitro studies have demonstrated that *Lepr* is expressed in murine and human pancreatic β cells, as well as in β cell lines [8–11]. Previous studies have attempted to assess whether leptin has a direct action on β cell function, but the results are contradictory. While some studies have found that incubation of human or rodent islets with leptin did not alter the expression or secretion of insulin [12–14], other studies demonstrated reduced insulin expression and secretion from

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islets treated with leptin [8,12,15–18]. The inhibitory actions of leptin on β cell function are supported by *in vivo* characterization of glucose homeostasis and circulating insulin levels in mice lacking leptin or its receptor. In leptin deficient mice (*ob/ob*) and mice with mutated *Lepr* (*db/db*), hyperinsulinemia develops prior to increases in body weight, and islet hyperplasia is evident in these mice [19–21]. When *ob/ob* mice are treated with leptin, there is a reduction of insulin gene expression and secretion and a reduction of circulating insulin levels within hours [22]. Moreover, doses of leptin insufficient to reduce body weight are sufficient to reverse hyperinsulinemia, indicating that the insulin-lowering effects of leptin can occur independent of weight loss [23].

To elucidate the mechanism by which leptin can regulate insulin secretion and expression, previous studies have employed Cre-*loxP* technology. Disruption of *Lepr* in β cells was achieved by Covey et al. using a rat insulin promoter driving Cre expression (*RIP*Cre) [24]. $Lepr^{flox/flox}$ *RIP*Cre mice were obese, hyperinsulinemic, glucose intolerant, and experienced islet hyperplasia relative to their $Lepr^{flox/flox}$ littermates. Interestingly, a similar approach by Morioka and colleagues to disrupt *Lepr* using a *Pdx-1* (pancreatic duodenal homeobox 1) promoter to drive Cre expression resulted in mice with mildly elevated fasting insulin levels but the absence of obesity [25]. Furthermore, Morioka et al. reported improved glucose tolerance and enhanced glucose stimulated insulin secretion in chow-fed $Lepr^{flox/flox}$ *Pdx-1*Cre mice compared to $Lepr^{flox/flox}$ littermates [25]. Though intended to target β cells, the Cre recombinase used in both of these studies was present in other tissues including the hypothalamus [24] and brain stem, as well as the exocrine pancreas [25–27], confounding interpretation of the direct actions of leptin in pancreatic β cells. More recently, Soedling et al. crossed mice in which Cre was knocked into the insulin I gene (*Ins1*Cre) with $Lepr^{flox/flox}$ mice to selectively delete *Lepr* in pancreatic β cells [28]. Unlike previous studies, which reported elevated plasma insulin levels following knockout of *Lepr* [24,25], knockout of *Lepr* in β cells using *Ins1*Cre did not induce elevated plasma insulin levels. Moreover, knockout of *Lepr* using *Ins1*Cre did not result in glucose intolerance or obesity. While the studies described above used very similar approaches to assess the role of *Lepr* in β cells, differences in genetic background and tissue specificity of Cre lines may have contributed to the different phenotypes observed upon deletion of *Lepr*. Furthermore, these knockout studies are limited by the potential compensation by *Lepr* expressed either in other peripheral tissues or the central nervous system (CNS) to make up for the life-long loss of direct action of leptin in β cells.

To determine the actions of leptin in β cells, we exploited a murine model in which a transcriptional blockade flanked by two *loxP* sites is placed between exon 16 and exon 17 of *Lepr* ($Lepr^{loxTB/loxTB}$), resulting in impaired leptin signaling [29]. To assess the direct actions of leptin in β cells, we crossed these mice with *Ins1*Cre mice to selectively restore *Lepr* in pancreatic β cells, while functional leptin receptors were absent in other tissues. This provided a unique model with which to assess whether the direct actions of leptin in β cells are sufficient to prevent hyperinsulinemia.

2. METHODS

2.1. Experimental animals

$Lepr^{loxTB/loxTB}$ mice (JAX no. 018989, 50% FVB, 50% C57BL/6J, <1% 129) and *Ins1*Cre mice (JAX no. 026801, C57BL/6J) [29] were obtained from Jackson Laboratory (Bar Harbor, ME, USA). $Lepr^{loxTB/wt}$ mice were then bred with *Ins1*Cre mice for two generations to yield $Lepr^{loxTB/loxTB}$ *Ins1*Cre, $Lepr^{loxTB/loxTB}$, $Lepr^{wt/wt}$ *Ins1*cre, and $Lepr^{wt/wt}$ mice on a B6.FVB hybrid background. Offspring were born at the expected Mendelian ratio. To assess the specificity of *Ins1*Cre mediated recombination, *mT/mG* reporter mice (JAX no. 007676, B6.129, Jackson Laboratory), which possess a membrane-bound Tomato fluorescent protein (mT) flanked by two unidirectional *loxP* sites, followed by the open reading frame for membrane bound enhanced green fluorescent protein (EGFP, mG) driven by the chicken β actin promoter, were crossed with *Ins1*Cre mice to generate homozygous *mT/mG* mice with or without 1 allele for *Ins1*Cre. Mice were housed on a 12 h light/12 h dark cycle and had *ad libitum* access to food (chow diet 2918, Harlan Laboratories, Madison WI, USA) and water. At 13 weeks of age, animals were fasted for 4 h prior to euthanasia. Pancreata were collected and weighed prior to fixation. All procedures with animals were approved by the University of British Columbia Animal Care Committee and carried out in accordance with the Canadian Council of Animal Care guidelines.

2.2. Metabolic measurements

Body weight and blood glucose were measured in mice fasted for 4 h. Blood was collected from the saphenous vein to measure blood glucose using a One Touch Ultra Glucometer (Life Scan, Burnaby, Canada). Insulin was measured in plasma (Mouse Ultrasensitive Insulin ELISA, Alpco Diagnostics, Salem, NH, USA) collected at 7 and 11 weeks of age. At 9 weeks of age, $Lepr^{loxTB/loxTB}$ *Ins1*Cre mice and littermate control mice underwent a 6h fast prior to oral gavage of dextrose (1.5 g/kg, 40% solution) followed by measurement of blood glucose and collection of plasma at time = 0, 15, 30, and 60 min to assess glucose tolerance and glucose stimulated insulin secretion.

2.3. Islet isolation

Islets were isolated from 11 to 13 week old male and female $Lepr^{loxTB/loxTB}$ *Ins1*Cre and $Lepr^{loxTB/loxTB}$ littermate mice as previously described [30]. Briefly, mice were euthanized by CO_2, and 2 ml of Hank's balanced salt solution (HBSS) containing 1000 units/ml of type XI collagenase (Sigma–Aldrich, St. Louis, MO) was immediately injected into the pancreatic duct. Pancreata were digested at 37 °C for 11 min and then washed with ice cold HBSS containing 1 mM $CaCl_2$. Islets where then hand picked into RPMI 1640 (Sigma Aldrich) containing 0.5% BSA, 100 units/ml penicillin and 100 μg/ml streptomycin. Islets were then flash frozen in liquid nitrogen and stored at −80 °C.

2.4. Gene deletion

Immediately following euthanasia, mice were decapitated and their brains were isolated. Hypothalami was then dissected on ice and flash frozen. To assess gene deletion, genomic DNA (gDNA) was extracted from flash frozen hypothalamic tissue and islets as previously described [31]. Deletion of the transcriptional blockade upstream of exon 17 in the *Lepr* gene was assessed using Taqman qPCR probes (IDT) (for primer sequences see Table 1; for location of primers in *Lepr* gene, see Figure 1A). The degree of recombination was determined using the Pfaffl method in which the deltaCT value of the floxed region of *Lepr* (target gene) was divided by the deltaCT value of a region upstream of the *loxP* sites (reference gene).

2.5. Islet morphology and immunohistochemistry

Pancreata were collected from 12 to 14 week old mice, fixed overnight in 4% PFA, rinsed in 70% ethanol, embedded in paraffin, and processed for sectioning by Wax-It Histology Services (Vancouver, Canada). Three sections separated by 200 μm per mouse were immunostained for insulin (rabbit anti-insulin antibody, Cat# C27C9, 1:1000, Cell Signaling) and glucagon (mouse anti-glucagon antibody,

Table 1 Sequences of primers used for quantitative PCR. Primer names as depicted in Figure 1A. Primer set 1 was used to amplify sequence within the transcriptional blockade region of *Lepr* while Primer set 2 was used to amplify exon 17 within *Lepr*.

Primer name	Sequence
1 (forward)	5′ GTG AGA TCA TGA GAC CCT AAA 3′
1 (reverse)	5′ GGA ACT CAA GAC CAT CTA TCA 3′
1 (HEX probe)	5′ TTC TGA ATT GGT GTC CCT GGA GCC 3′
2 (forward)	5′ CCT TTC CAG ATA ATG CCT GAT AGA 3′
2 (reverse)	5′ GCA CCA CAC TTA GCT CCA ATA 3′
2 (FAM probe)	5′ TAG GGC GGA TGA ACC AGC AAA TGT 3′

Cat# G2654, 1:1,000; Sigma–Aldrich) or Glut-2 (rabbit anti-Glut-2 antibody, Cat# 07-1402, 1:500, Millipore) overnight at 4 °C. Slides were then incubated with AlexaFluor-conjugated secondary antibodies (AF488, AF594; Life Technologies, Burlington, Ontario, Canada) for 1 h at room temperature followed by counterstaining with DAPI to identify nuclei. Sections were scanned and quantified as previously described [32]. The insulin and glucagon positive areas are expressed relative to the whole pancreas area and averaged across three pancreas sections per mouse (n = 3–5 per group). Islet sizes were approximated by measuring insulin positive area of each islet in three pancreas sections (separated by 200 μm) per mouse. Islet sizes were then binned into ranges and the histogram analysis function in Microsoft Excel (2016) was used to determine the frequency of islets per size range. Frequency was represented as a percentage of total number of islets assessed per animal.

2.6. Statistical analysis

Statistical analysis was performed using Graphpad Prism 7.0 software (Graphpad Software, La Jolla, California) with a p value <0.05

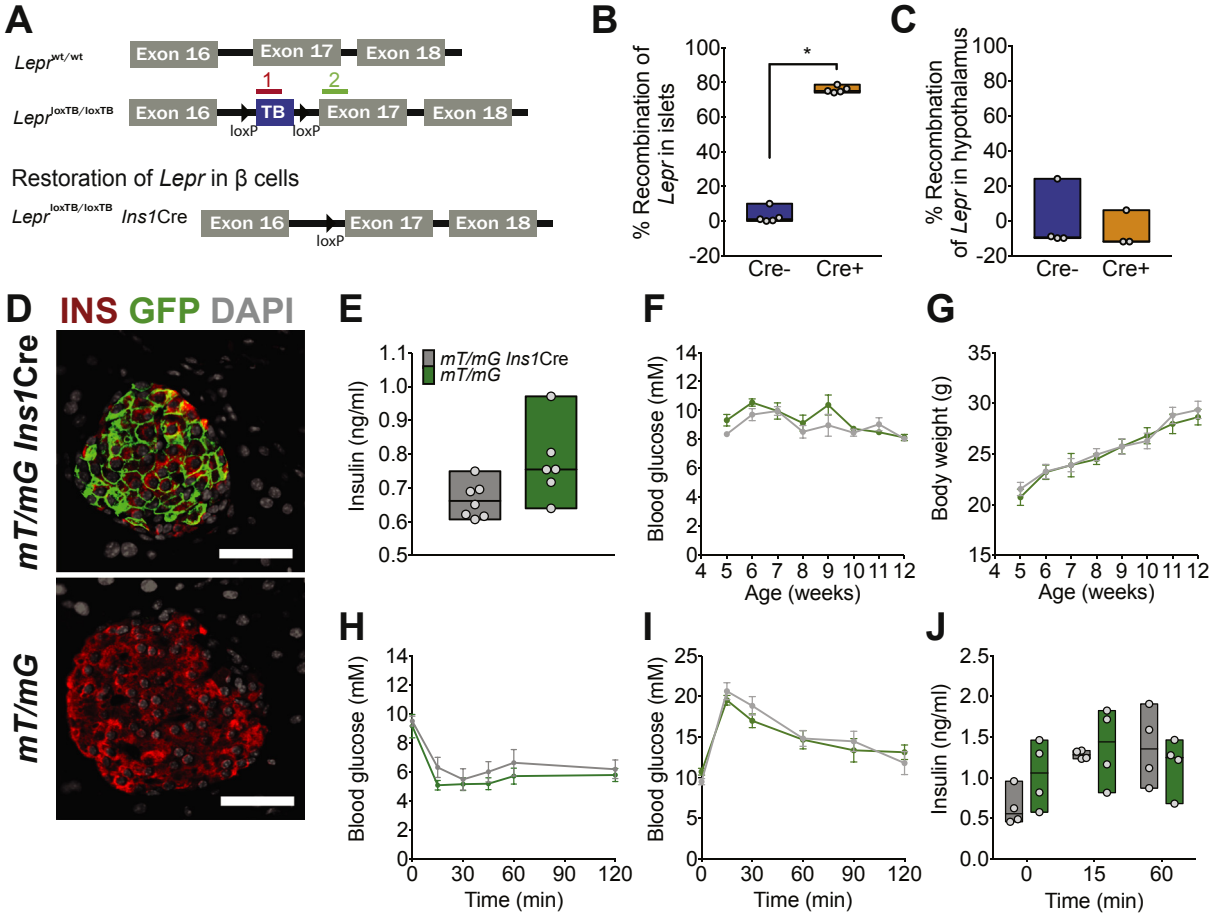

Figure 1: *Lepr* was restored in mice with a transcriptional blockade (TB) upstream of exon 17 in *Lepr* by recombination of *loxP* sites flanking the TB using *Ins1*Cre (A). Percent recombination was assessed by measuring the ratio of qPCR product obtained using primers within and outside of the TB region (primers 1 and 2, respectively in A) of gDNA extracted from islets (B) and hypothalamus (C). Mice expressing *mT/mG* were used to determine the extent of recombination in islets with *Ins1*Cre. Representative images were collected from pancreata of *mT/mG Ins1*Cre and *mT/mG* mice co-stained for insulin (red) and EGFP (green) and DAPI (gray) (D; n = 3–4). Expression of GFP indicates Cre-mediated recombination. Scale bar represents 100 μm. Fasted insulin levels were measured at 5 weeks of age (E), along with fasting glucose levels (F) and body weight (G) between 5 and 12 weeks of age in male *mT/mG Ins1*Cre and *mT/mG* mice. Blood glucose was measured following an i.p. injection of insulin (0.75 U/kg body weight) to assess insulin sensitivity in 6-week old mice (H). Plasma insulin levels were measured in 8-week old mice following a gavage of 40% glucose (1.5 g/kg body weight) to assess glucose tolerance and glucose stimulated insulin secretion (I, J). Values are presented as individual data points in B, C, E, and J with a line indicating the median and the shaded area spanning the range of values. A student's t-test with $P < 0.05$ was used to determine statistical significance. Values in F-I are presented as mean ± SEM and a repeated one-way ANOVA with Tukey post-hoc test was used to assess statistical significance (n = 4–6/group).

considered statistically significant. All values are expressed either as individual data points with median or as mean ± SEM where indicated. Unless otherwise specified, a one-way ANOVA (or non-parametric Kruskal–Wallis test), two-way ANOVA, or Student's t-test were performed, with a Tukey post-hoc test.

3. RESULTS

3.1. Validation of *Lepr* restoration in β cells

To assess recombination of the *Lepr*$^{loxTB/loxTB}$ site in the presence of *Ins1*Cre, islets were isolated from 11 to 13 week old *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, and gDNA was extracted for analysis by qPCR. A ~75% rate of recombination of *Lepr* was observed in islets of *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, while the recombination of *Lepr* in islets of *Lepr*$^{loxTB/loxTB}$ mice was less than 10% (Figure 1B). Recombination of *Lepr* in islets was compared to recombination of *Lepr* in the hypothalamus to assess the tissue specificity of *Ins1*Cre. The percent of recombination in the hypothalamus was minimal and was comparable between *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre and *Lepr*$^{loxTB/loxTB}$ mice (Figure 1C). Next, we crossed *Ins1*Cre mice with *mT/mG* reporter mice to determine whether *Ins1*Cre produced recombination in β cells. These double fluorescent reporter mice express membrane-targeted dimer Tomato prior to Cre-mediated excision and membrane-targeted enhanced green fluorescent protein (EGFP) after excision [33]. Immunostaining of EGFP in fixed pancreas from *mT/mG Ins1*Cre and *mT/mG* control mice revealed EGFP expression co-localized in β cells of *mT/mG Ins1*Cre mice (Figure 1D), indicating that Cre-induced recombination occurred in β cells. To determine whether replacing 1 insulin allele with Cre influenced glucose homeostasis and metabolism, we also assessed body weight, fasting glucose, glucose tolerance, and insulin sensitivity in male *mT/mG Ins1*Cre mice between the ages of 4–12 weeks. Fasting insulin, blood glucose, and body weight levels were similar between *mT/mG Ins1*Cre mice and *mT/mG* mice (Figure 1E–G). Similarly, no differences in insulin sensitivity, glucose tolerance, or glucose stimulated insulin secretion were observed (Figure 1H–J). Therefore, our characterization of the *Ins1*Cre line confirms and extends previously published data [34] and demonstrates that knock-in of Cre into one allele of *Ins1* does not appear to significantly affect glucose homeostasis.

3.2. *Lepr* in β cells is not sufficient to regulate islet morphology or β cell area

It has been previously reported that whole body mutation of *Lepr* [21] or knockout of *Lepr* in the pancreas of chow-fed mice [25] results in increased β cell area. To determine if restoration of *Lepr* in β cells is sufficient to prevent the increase in β cell area, we immunostained pancreas sections collected from 12 to 14 week old *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice with insulin, glucagon, and DAPI (Figure 2A). Quantification revealed that *Lepr*$^{loxTB/loxTB}$ insulin positive area was ~10 fold higher compared to *Lepr*$^{wt/wt}$ *Ins1*Cre mice ($p < 0.01$; Figure 2C). Restoration of *Lepr* in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice resulted in a trend for reduced insulin positive area compared to *Lepr*$^{loxTB/loxTB}$ mice ($p = 0.06$). In contrast, no differences in α cell area, as determined by glucagon positive immunostaining, were observed between experimental groups (Figure 2D). Analysis of islet size revealed a significantly lower proportion of small islets (<500 μm^2) in *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice compared to *Lepr*$^{wt/wt}$ mice, while *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice had a greater number of large (>10,000 μm^2) islets than *Lepr*$^{wt/wt}$ littermates. To determine if restoration of *Lepr* in β cells is able to prevent the loss of Glut-2 expression in *Lepr*$^{loxTB/loxTB}$ mice, pancreas sections were co-immunostained for Glut-2, insulin, and DAPI (Figure 2B). Glut-2 immunoreactivity was clearly present in *Lepr*$^{wt/wt}$ *Ins1*Cre mice, but generally lacking in *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Together, these findings suggest that direct action of leptin in β cells may not be sufficient to modify islet size or rescue Glut-2 expression in mice lacking leptin receptors elsewhere.

3.3. Restoration of *Lepr* in β cells has no significant impact on body weight, insulin levels, or fasting glucose levels

Along with increased insulin levels, obesity has been reported in *Lepr*$^{flox/flox}$ *RIP*Cre mice [24]. To examine the effects of restoration of *Lepr* in β cells, we measured body weight weekly between 6 and 11 weeks of age and collected plasma at 13 weeks of age to measure leptin levels. Body weight of male and female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice was comparable to *Lepr*$^{loxTB/loxTB}$ mice. Male *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice weighed 50% more than *Lepr*$^{wt/wt}$ littermate mice, while female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice weighed 80% more than their respective control littermates (Figure 3A, B). Similar to prior studies with *Lepr*$^{loxTB/loxTB}$ mice [29], we observed hyperleptinemia in *Lepr*$^{loxTB/loxTB}$ male and female mice, with leptin levels that were ~150 times higher compared to *Lepr*$^{wt/wt}$ *Ins1*Cre littermates (*Lepr*$^{loxTB/loxTB}$ = 252.9 ± 26.7 ng/ml, *Lepr*$^{wt/wt}$ *Ins1*Cre = 1.65 ± 0.20 ng/ml). Despite restoring *Lepr* in β cells, fasting leptin levels of *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice (279.8 ± 11.67 ng/ml) were comparable to those of *Lepr*$^{loxTB/loxTB}$ mice. To assess the effects of β cell-specific signaling of leptin on insulin secretion and glucose homeostasis, we monitored blood glucose weekly between 6 and 11 weeks of age and collected plasma from male and female mice at 7 and 11 weeks of age to measure insulin levels. Hyperinsulinemia was evident in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice and plasma insulin levels were comparable to that of *Lepr*$^{loxTB/loxTB}$ mice (Figure 3C, D). In contrast, plasma insulin levels of *Lepr*$^{wt/wt}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice were ~20 fold lower than *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Similar to fasting insulin levels, fasting glucose levels were elevated in *Lepr*$^{loxTB/loxTB}$ mice and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice compared to *Lepr*$^{wt/wt}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice (Figure 3E, F). However, by 10–11 weeks of age, female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice exhibited significantly higher glucose levels than their *Lepr*$^{loxTB/loxTB}$ littermates. Together these findings suggest that selective restoration of *Lepr* in β cells does not prevent obesity and hyperinsulinemia in mice lacking leptin receptors elsewhere. Moreover, restoration of *Lepr* in β cells does not improve fasting glucose levels of male or female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice relative to their respective littermate *Lepr*$^{loxTB/loxTB}$ mice.

3.4. *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice are glucose intolerant and have an exaggerated insulin response to glucose

We next assessed the effects of restoring *Lepr* in β cells on insulin secretion in both male and female *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Following a 6 h fast, *Lepr*$^{wt/wt}$ mice were euglycemic, while *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice were hyperglycemic (blood glucose >11 mM; Figure 4A, D). Fifteen minutes after glucose gavage, blood glucose levels of male and female *Lepr*$^{wt/wt}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice peaked between 15 and 18 mM. In contrast, glucose levels of *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice peaked above the limit of detection (33.3 mM, represented as a dashed line on the graph), indicating they were severely glucose intolerant. Analysis of area under the curve (AUC) revealed that *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice were significantly glucose intolerant compared to control littermate mice (Figure 4B, E). In response to glucose gavage, male and female *Lepr*$^{wt/wt}$ and *Lepr*$^{wt/wt}$ *Ins1*Cre mice had fasting plasma insulin levels between 0.1 and 0.7 ng/ml, and insulin levels peaked at 0.3–

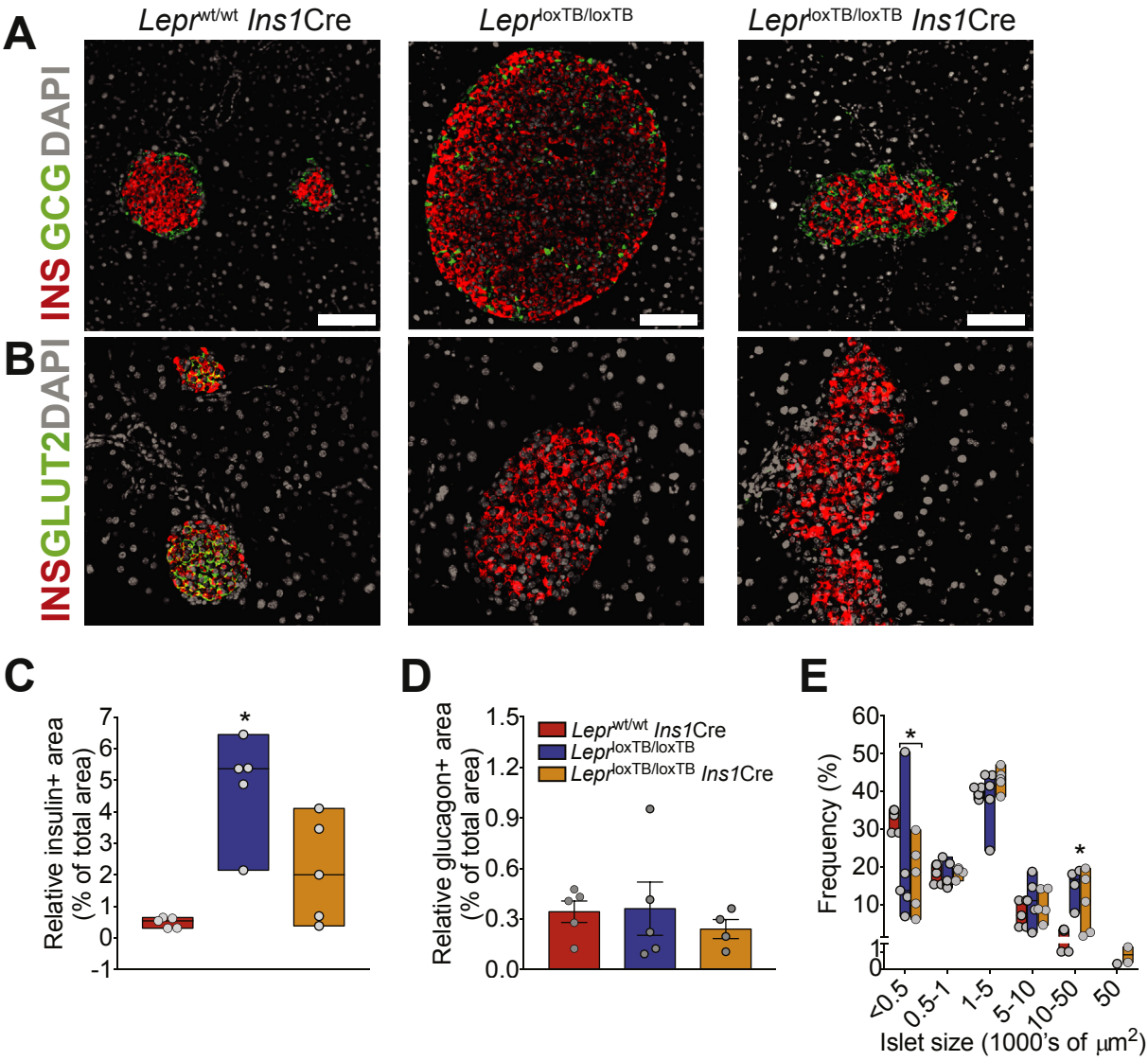

Figure 2: Representative images were taken from pancreas sections of *Lepr*$^{wt/wt}$, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice immunostained for insulin (INS; red), glucagon (GCG; green), and DAPI (gray) (A). Presence of Glut-2 in β cells was determined by co-staining pancreas sections for insulin (red) Glut-2 (green) and DAPI (white) in *Lepr*$^{wt/wt}$, *Lepr*$^{loxTB/loxTB}$, and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice (B). Scale bar represents 100 μm. Relative β and α cell area were calculated by measuring total insulin and glucagon positive areas, respectively, relative to total pancreas area (C, D). Islet size was approximated by measuring insulin positive area per islet. Islet size was divided into 6 categories and frequency of islets in each category was determined relative to total number of islets measured per animal (E). Values are presented as individual data points with a line representing the median and the shaded area indicating the range of values. *P $<$ 0.05 compared to *Lepr*$^{wt/wt}$ *Ins1*Cre. A non-parametric one-way ANOVA with Kruskal–Wallis post hoc analysis was used to determine statistical significance (n = 4–5 per group).

1.1 ng/ml at the 15-minute time point (Figure 4C, F). In contrast, *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice had fasting insulin levels that were ~10-fold higher than *Lepr*$^{wt/wt}$ mice and peak glucose stimulated insulin secretion levels that were between 15 and 30-fold higher than *Lepr*$^{wt/wt}$ mice. Fasting as well as glucose stimulated plasma insulin levels were comparable between *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Despite similar glucose levels in response to oral glucose gavage, basal plasma insulin levels and glucose stimulated insulin secretion was different between male and female mice. Female *Lepr*$^{loxTB/loxTB}$ and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice had lower basal insulin levels compared to males (Figures 3C, D and 4C, F), and insulin secretion in response to glucose was much higher in female *Lepr*$^{loxTB/loxTB}$ as compared to male mice of the same genotype (Figure 4C, F).

4. DISCUSSION

To examine the effects of restoring *Lepr* in β cells on insulin secretion and morphology, we crossed mice expressing Cre knocked into the *Ins1* gene with *Lepr*$^{loxTB/loxTB}$ mice, which have a transcriptional blockade inserted between exon 16 and exon 17 of *Lepr*, resulting in impaired leptin signaling. Disruption of the leptin receptor signaling domain in *Lepr*$^{loxTB/loxTB}$ mice results in a phenotype similar to *db/db* mice, characterized by obesity, hyperinsulinemia, hyperglycemia, insulin resistance, and islet hyperplasia [35,36]. Cre-mediated removal of the transcriptional blockade and restoration of *Lepr* can be achieved in *Lepr*$^{loxTB/loxTB}$ mice in a cell-type selective manner, enabling the assessment of the physiological impact of *Lepr* in specific tissues.

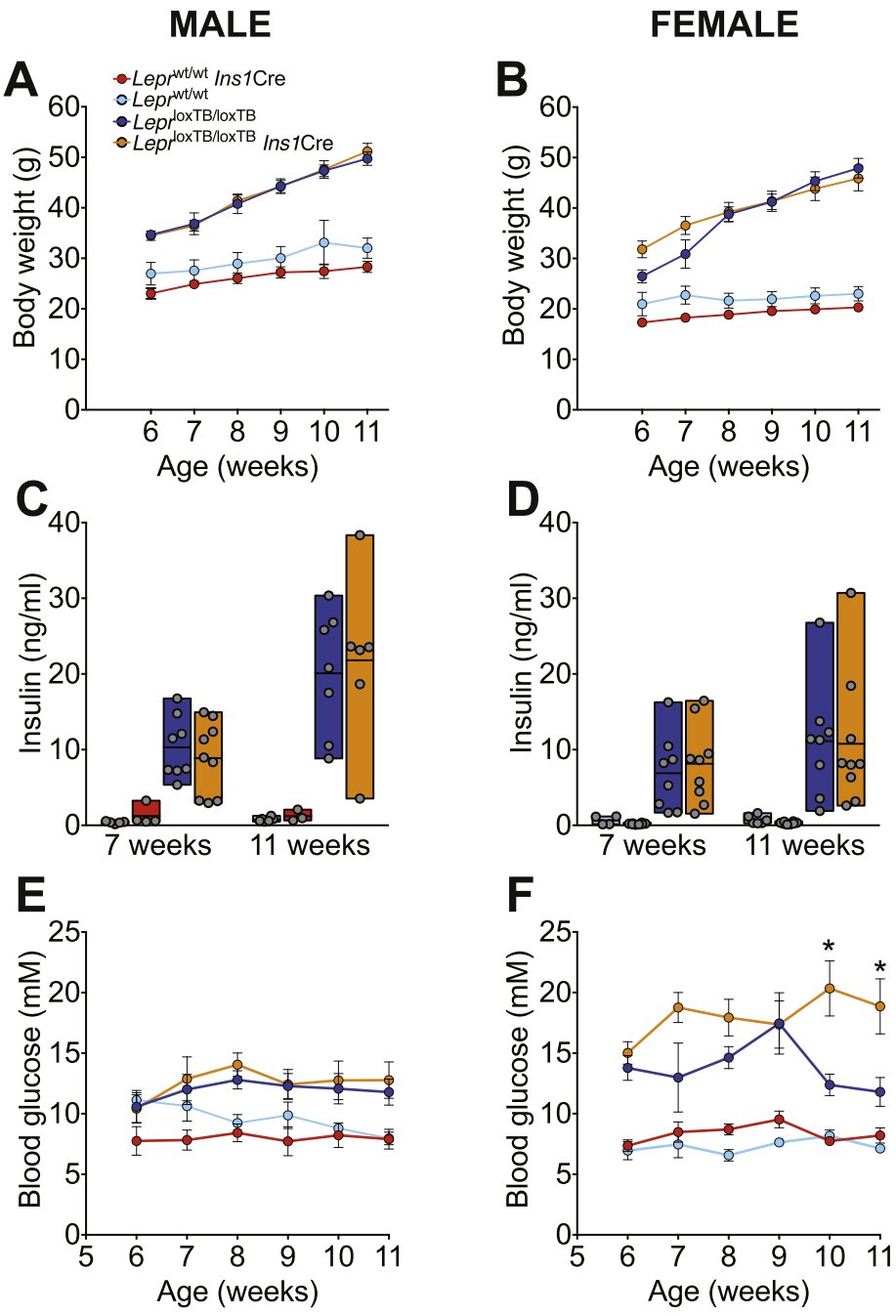

Figure 3: Body weight was measured weekly between 6 and 11 weeks of age in male (A) and female (B) *Lepr*$^{wt/wt}$, *Lepr*$^{wt/wt}$ *Ins1*Cre, *Lepr*$^{loxTB/loxTB}$, and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre, mice. Fasting plasma insulin levels at 7 and 11 weeks of age in male (C) and female mice (D). Values are presented as individual data points with a line indicating the median and the shaded region spanning the range of values. Fasting blood glucose levels were monitored in male (E) and female (F) mice. Values in A, B, E, & F are presented as mean ± SEM. *P $<$ 0.05 comparing *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre and *Lepr*$^{loxTB/loxTB}$ using a two-way repeated measures ANOVA with Tukey post hoc analysis (n = 4–13 females, n = 3–5 males).

Restoration of *Lepr* in β cells of *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice did not alter the development of hyperinsulinemia, hyperglycemia, glucose intolerance, and obesity, suggesting that leptin signaling in β cells does not suppress insulin secretion. Moreover, islet hyperplasia was still present in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, similarly to what is reported in *db/db* mice [35]. However, as we did not follow the mice for an extended period of time, our studies cannot rule out the possibility that the restoration of *Lepr* in β cells may have prevented age related β cell failure that eventually results in overt severe diabetes, like in *db/db* mice [37,38].

A distinct difference between the present study and previous studies in which *Lepr* was selectively deleted [24,25,28] is the inability of compensation to occur from non-β cells in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. In prior studies that examined the role of leptin signaling in β cells by deletion of *Lepr*, it is unclear whether *Lepr* acting in other tissues influenced pancreas morphology and β cell function. The ability of non-β cell leptin receptor action to influence β cell function is evident in mice with restored *Lepr* in brains of *db/db* mice. Over-expression of *Leprb* driven by synapsin 1 and neuron specific enolase promoters in *db/db* mice resulted in normalized islet morphology and

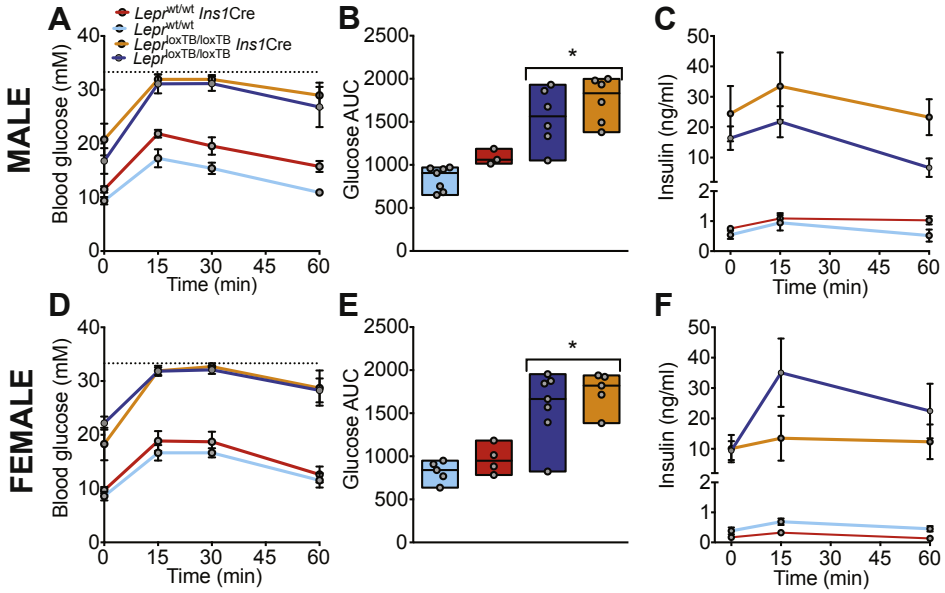

Figure 4: Male (A) and female (D) *Lepr*$^{wt/wt}$, *Lepr*$^{wt/wt}$ *Ins1*Cre, *Lepr*$^{loxTB/loxTB}$, and *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice between 7 and 12 weeks of age were fasted for 6 h to assess glucose tolerance by gavage of 40% glucose at a dose of 1.5 g glucose/kg body weight. Blood glucose was measured every 15 min and area under the curve was analyzed (B, E). Blood collected at 0, 15 and 60 min of the glucose tolerance test was used to measure plasma insulin levels (C, F). Values are presented as mean ± SEM in panels A, C, D, and F while values in B and E are presented individually with a line indicating the median. A one-way ANOVA with Tukey post hoc analysis was used to assess statistical significance (n = 3–7 per group).

partial correction of body weight [39]. These authors concluded that *Lepr* in the CNS is able to rescue the diabetic and obese phenotype of *db/db* mice. To prevent *Lepr* in non-β cells from influencing β cell function, we selectively re-expressed *Lepr* in β cells of mice lacking *Lepr* elsewhere. Our results suggest that *Lepr* in β cells is not sufficient to prevent hyperinsulinemia and glucose intolerance from occurring. It is likely that *Lepr* in the CNS and other islet cell populations are involved in inhibiting insulin secretion and preventing hyperplasia of β cells.

Previous studies have reported *Lepr* expression in β cells of human and rodent islets and cell lines [9,40–43]; however, in another study, non-detectable levels of *Lepr* were reported in β cells from dispersed islets using RT-qPCR [28]. More recently, studies using single-cell RNA-seq to determine the transcriptome profiles of human and murine islets revealed that the expression of *Lepr* was higher in δ cells than β cells [44,45]. Though insulin secretion and expression was reduced in isolated murine and human islets treated with leptin [8,10,12], this effect may have been a result of leptin acting on δ cells. Collectively, this suggests that despite restoration of *Lepr* in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice, the relatively low levels of *Lepr* in β cells may not be sufficient to provoke improvements in β cell function in the absence of leptin receptor signaling in other pancreatic islet populations.

The presence of hyperinsulinemia and islet hyperplasia despite restoration of *Lepr* in β cells of *Lepr*$^{loxTB/loxTB}$ mice may be due to hyperleptinemia that was present in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice. Plasma leptin levels were ~150-fold higher in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice compared to control mice. Hyperleptinemia has been previously observed in *Lepr*$^{loxTB/loxTB}$ [29], and in mice with disruption of *Lepr* in peripheral tissues [46]. This increase of plasma leptin levels was attributed to the disruption of *Lepr* in white adipose tissue (WAT), which led to inactivation of a negative feedback loop regulating leptin synthesis in WAT [46]. Indeed the presence of hyperleptinemia can cause leptin resistance [47]. Thus, despite restoration of *Lepr* in β cells, the chronically elevated leptin levels in *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice may have resulted in a state of leptin resistance in β cells of *Lepr*$^{loxTB/loxTB}$ *Ins1*Cre mice.

5. CONCLUSION

In summary, selective restoration of *Lepr* in pancreatic β cells of *Lepr* knockout mice does not prevent the development of hyperinsulinemia and β cell expansion. These findings suggest that leptin receptor signaling directly in β cells does not regulate β cell function. We propose that actions of leptin on β cells are secondary to action of leptin signaling in other tissues. Whether this involves a combination of leptin signaling in other islet cell populations and in the CNS warrants further study.

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CONFLICT OF INTEREST

None declared.

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