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# The Nuclear Localization Sequence and C-Terminus of Parathyroid Hormone-Related Protein Regulate Normal Pancreatic Islet Development and Function

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## Abstract

Parathyroid hormone-related protein (PTHrP) is a pleiotropic hormone essential for morphogenesis, tissue differentiation, as well as cell regulation and function. PTHrP is expressed by pancreatic beta cells which are responsible for insulin secretion. Previous studies have reported that N-terminal PTHrP stimulated proliferation in beta cells in rodents. We have developed a knockin mouse model (PTHrP Δ/Δ) lacking the C-terminal and nuclear localization sequence (NLS) of PTHrP. These mice die at ~day 5, are severely stunted in growth, weigh 54% less than control mice at day 1 –2 and eventually fail to grow. PTHrP Δ/Δ mice are also hypoinsulinemic and hypoglycemic yet have nutrient intake proportional to size. To characterize the pancreatic islets in these mice, islets (~10–20) were isolated from 2–5 day-old-mice using collagenase digestion. Islets from PTHrP Δ/Δ mice were smaller in size but secreted more insulin than littermate controls. PTHrP Δ/Δ and control mice islets were exposed to various glucose

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concentrations and intracellular calcium, the trigger for insulin release, was elevated for glucose concentrations of 8–20 mM. Immunofluorescence staining showed less glucagon-stained area in islets from PTHrP Δ/Δ mice (~250 $\mu m^2$) compared to islets from control mice (~900 $\mu m^2$), and ELISA confirmed there was reduced glucagon content. These data collectively demonstrate increased insulin secretion and reduced glucagon at the islet level, which may contribute to the observed hypoglycemia and early death in PTHrP Δ/Δ mice. Thus, the C-terminus and NLS of PTHrP are crucial to life, including regulation of glucose homeostasis and islet function.

**Keywords**

Insulin; Glucagon; PTHrP; Islet

## Introduction

The autocrine and paracrine roles of parathyroid hormone-related protein (PTHrP) have been studied in many tissues. In the mammary glands, PTHrP mediates the differentiation of mesenchymal tissue into specialized mammary tissue [1,2]. PTHrP and the parathyroid hormone receptor (PTH1R) are expressed in osteoblasts [3] and increase renal cell proliferation [4]. PTHrP also plays a role in the development of skin [5], pancreas [6], cartilage [7], brain [8] and heart [9], and PTHrP is found in almost all organs/tissue in the human body [10].

Apart from its autocrine/paracrine functions, PTHrP has intracrine roles that are mediated primarily through its nuclear localization sequence (NLS). PTHrP consists of an N-terminal (PTHrP 1–36), mid-region (PTHrP 35–66), NLS (PTHrP 67–94) and a C-terminal region (PTHrP 107–139). PTHrP and parathyroid hormone (PTH) are highly homologous at their N-terminus. They both bind to the PTH1R with similar affinity, and therefore activate the same signal transduction cascade [3,11]. The NLS of PTHrP interacts with the nuclear transport receptor importin-beta to facilitate translocation of PTHrP to the nucleus [12]. Nuclear PTHrP has been shown to be responsible for enhancing cell proliferation and preventing apoptosis in colon carcinoma cells [13], breast cancer cells [14] and osteoblastic cells [15].

Several mouse models have been developed to study the role of PTHrP in development. Knockout of full-length PTHrP is lethal, resulting in death by asphyxia at birth due to complete ossification of the rib cage [16]. Two separate mouse models were created that lacked the NLS and C-terminus of PTHrP (PTHrP KI) [17,18]. Unlike the PTHrP null strain, neonates from both models do not die at birth but have severely stunted growth and die prematurely. The *Pthrp ki* mouse model lacking PTHrP 88–137 die at 2–3 weeks of age and have signs of premature aging [18]. The PTHrP knockin (PTHrP Δ/Δ) mouse model lacks PTHrP 67–137, has an average lifespan of 5 days or less [17]. This is the model used for the current investigation. It has been reported that the global knockin mice have pathology of multiple body systems, including impaired hematopoiesis, altered skeletal development, lack of body fat, and hypoglycemia. The mice have severely stunted growth and early death [17].

Hypoglycemia can be caused by high insulin concentrations. The endocrine pancreas consists of the Islets of Langerhans, which contain alpha, beta and other endocrine cells. Alpha cells produce glucagon while beta cells are responsible for insulin production. PTHrP was discovered to be expressed in beta cells in 1990 by Drucker et al who investigated the presence of PTHrP in multiple normal and neoplastic endocrine tissues [19]. The first transgenic mouse model used to study the effect of PTHrP in islet cell proliferation and insulin secretion was developed in 1995 [6]. The mouse model (RIP-PTHrP), which overexpressed full-length PTHrP in the pancreatic islets, was hypoglycemic and hyperinsulinemic. Specifically, the role of the N-terminal PTHrP in the endocrine pancreas has been investigated [20,21] and it was discovered that N-terminal PTHrP increased beta-cell proliferation and insulin secretion. N-terminal PTHrP is homologous to parathyroid hormone (PTH) and PTH has been shown to increase intracellular calcium in pancreatic islets through a G protein-coupled receptor, most likely the PTH1 receptor [22]. This shows that islets are a target of PTH or n-terminal PTHrP, however, the islet cells do not express PTH.

The effects of the N-terminal of PTHrP are well studied, but there has been no study on the role of the NLS or C-terminal PTHrP on pancreatic islet function. Here we present evidence that the absence of the NLS and C-terminus of PTHrP modifies insulin secretion, maturation of islets, and glucagon production. Because the mouse model used for this study was generated as a global knockin with multiple body systems affected, it is possible that altered body systems external to the pancreas had effects on the islets cells. However, it has been shown that PTHrP can directly affect islet function [6,20,21], so it is likely that at least some of the observed effects are due to direct action on pancreatic islets. Separating islet-specific mechanisms of this truncated protein from other pathologies in this animal model will be the focus of future work. The present study provides a foundation for studying the effects of the NLS and c-terminal PTHrP on islet function.

## Methods

### Mice

PTHrP Δ/Δ mice were created by deleting exon 4 of PTHrP and replacing it with exon 4 lacking PTHrP 67–136. This was carried out using Tgo DNA polymerase and a 3-plasmid system on a C57BL/6 background. Successful knock in was confirmed by RT-PCR and sequencing in multiple tissues such as bone, skin, liver, kidney, lungs, and placenta and also by western blotting and immunohistochemistry [17]. Genotyping and sexing were performed on mice after birth using primer set 1 listed in Table 1. RNA was isolated from the pancreas of PTHrP Δ/Δ, heterozygous and wild-type mice using published protocol [23]. cDNA was transcribed using Invitrogen superscript IV reverse transcriptase (Waltham, Massachusetts). PCR was conducted using primer set 2 (table 1) to ensure that there was deletion of PTHrP 67–139 in pancreatic tissue (Figure 1C). Heterozygous and wild-type mice served as controls. No biological differences were found between the islets in heterozygous (PTHrP Δ/+) and wild-type (PTHrP +/+) mice. PTHrP Δ/Δ mice and controls were euthanized between 1 to 3 days by decapitation. All breeding and animal experiments were approved by the Ohio University Animal Care and Use Committee (IACUC number: 19-H-024)

### Blood Glucose and Stomach Weight Measurements

Blood glucose was measured post-decapitation using the McKerson™ blood glucose strip and meter (Lot number: 960302, Las Colinas, Texas). Stomach weights were measured after dissection and percentage stomach weight to total body weight for each pup was calculated (not shown).

### Islet Isolation

Neonatal islets were isolated as previously described [24]. Briefly, upon decapitation of the mice, the pancreas was removed from the body cavity and 0.5 ml of 1 mg/ml collagenase solution (C9263, Sigma Aldrich, St Louis, Missouri) was injected into the pancreas lobes in a 2 ml tube containing 0.5 ml cold Hank's balanced salt solution (HBSS). The pancreas with collagenase solution was incubated in a 37°C water bath for 70–90 seconds with intermittent shaking. After digestion of the pancreas, the pancreas and collagenase solution was diluted with 1 ml HBSS containing 10% FBS (R&D Systems, Minneapolis, Minnesota). The digested pancreata were centrifuged at 304 × g for 1 minute and the supernatant was discarded. The pellet was resuspended in 1 ml of HBSS + FBS solution and the procedure was repeated twice. The digested pancreas mixtures were transferred to a 25 ml tube containing 2 ml HBSS. Four ml of Histopaque-1077 (Sigma Aldrich, St Louis, Missouri) was gradually added to the mixture to create a gradient. The 25 ml tube was centrifuged at 226 × g for 16 minutes and the mid layer containing the islets was pipetted (about 6 ml) into a new 25 ml tube. HBSS + FBS solution (9 ml) was added to the mixture and centrifuged at 304 × g for 2 minutes. The supernatant was discarded and the pellet of islets was resuspended twice in 8 ml of HBSS + FBS solution to further remove the Histopaque. Finally, the pellet after discarding the supernatant was suspended in RPMI 1640 media containing 10% FBS and 1% penicillin/streptomycin (Gibco, Ontario, Canada) in a 6-well non-tissue culture-treated plate and incubated at 37°C with 5% of CO2. After 1 hour, the islets were cleaned by pipetting to remove the acinar tissue. Islets were placed in a new plate and left to recover overnight in RPMI 1640 medium with 11 mM glucose (Invitrogen, Waltham, Massachusetts), 10 % FBS and 1% penicillin/streptomycin. Islets were incubated in a 5% CO2 incubator at 37°C. It is important to note that because of the young age of the mice involved, this method yielded a small number of islets, which limits the available tissue for experiments.

### Insulin and Glucagon Secretion/Content

Ten islets per well were incubated in non-tissue culture treated12-well plates and used to measure glucagon and insulin secretion. Because of the limited tissue available from the lethal mice strain, size matching between PTHrP Δ/Δ and control mice islets was not possible. However, insulin secretion were normalized to islet area as measured using ImageJ [25]. Media was collected after overnight islet incubation, centrifuged at 9,838 X g for 5 minutes, and insulin concentrations in the supernatant were measured by enzyme-linked immunosorbent assay (ALPCO Insulin ELISA kit 80-INSHU-E01.1, Salem, New Hampshire).

For measurement of insulin content, acid ethanol extracts were prepared using 10 islets. Extraction buffer consisted of 75% ethanol and 1.5% HCl in distilled water. Extraction

buffer (50 μL) was added to islets in a 1.5 ml tube on ice and vortexed intermittently for 2 hours. The extract was centrifuged at 9,838 X g for 5 minutes and the supernatant was collected and immediately used to measure insulin content or stored at −80°C. The same ELISA kit used for secretion was used to measure insulin and content. All groups had samples from three or more PTHrP Δ/Δ and control mice before analysis.

Glucagon content from the pancreas was carried out in a similar way. Instead of islets, the pancreases were harvested, weighed and homogenized before acid-ethanol extraction was carried out overnight with gentle rotation at 4°C. The supernatant was collected after incubation and glucagon was measured using an ELISA (Mercodia Glucagon ELISA kit 10–1281-01, Winston Salem, North Carolina). The weight of pancreases was used to normalize glucagon content for each pup.

## Calcium Imaging

Islet cell Intracellular calcium concentrations were measured using a perfusion system and fura-2 AM dye as described previously [26]. For each experiment, one group of islets was stained with cell tracker red (CTR) to distinguish islets from control and PTHrP Δ/Δ mice. A six-step calcium response to glucose was measured. Islet were exposed to increasing glucose concentrations (0, 4, 8, 12, 16 and 20 mM). The experiment lasted 60 minutes with 10 minutes for each glucose concentration. To observe calcium response after altering potassium channel activity, 3 mM glucose for 10 minutes, 250 μm diazoxide + 3 mM glucose for 15 minutes, 11 mM glucose for 5 minutes and 250 μm tolbutamide + 11 mM glucose for 10 minutes were used to stimulate the islets in the chamber.

## Immunofluorescence

Pancreases were collected from pups after dissection and fixed in 10% formalin overnight. Pancreases were embedded in paraffin and cut into 5 μm sections and stained with hematoxylin and eosin (H&E) to identify islets. The area of islets was measured as described previously [27] and the experiment was repeated with pancreases from at least 4 different mice.

Insulin and glucagon immunofluorescence[27] were also carried out on 5 μm pancreas sections using anti-insulin antibody (ab7842, Abcam, Cambridge, UK)) and anti-glucagon antibody (ab10988, Abcam, Cambridge, UK). Secondary antibodies conjugated to Alexa488 and Alexa594 were used to visualize insulin and glucagon positive cells, respectively. A Nikon Microphot-SA fluorescent microscope was used to photograph islets and the total glucagon and insulin cell areas were measured using ImageJ as described previously[27]. Insulin and glucagon area was normalized to total islet area and the remaining islet area was labeled as “other cell area”, which includes other endocrine cells, such as delta and epilson cells.

## Statistical Analysis

Results were presented as mean ± standard deviation. Unpaired t-tests were carried out on each data set and results were considered statistically significant when $p<0.05$. Graphs

were plotted and statistical analyses carried out with Prism 8.02 (GraphPad, San Diego, California).

## Results

### PTHrP Δ/Δ mice have altered energy physiology

Figure 1A shows the PTHrP sequence present in the PTHrP Δ/Δ mice as well as the portions of the sequence that are missing. Results of PCR on pancreatic tissue showed that deletion of PTHrP 67–139 was present in the pancreas (Figure 1 B). As previously reported[17], we observed that PTHrP Δ/Δ mice were much smaller than control mice (Figure 1C) and lacked normal weight gain (Figure 1D). We measured blood glucose concentrations of both PTHrP Δ/Δ and control mice from days 1–4 of age. There was a steady increase in blood glucose in the control pups, but this did not occur in the PTHrP Δ/Δ mice. Blood glucose concentrations in PTHrP Δ/Δ mice were significantly lower than controls on postnatal days 3 and 5 (Figure 1E). At day 5, PTHrP Δ/Δ mice had blood glucose concentrations of ~55 mg/dl, which was 50% lower than controls ($p<0.01$). We also observed based on stomach weight measurements that PTHrP Δ/Δ mice pups were able to suckle milk adequately. Stomach weight of PTHrP Δ/Δ mice when normalized to body weight was not significantly different to controls (data not shown), and all stomachs contained milk.

### PTHrP Δ/Δ mice had smaller islets

We measured the cross-sectional area of islets identified by H&E-staining of pancreatic sections from PTHrP Δ/Δ and control mice (Figure 2A & B). The islets from PTHrP Δ/Δ mice were significantly smaller than the islets from control mice ($p<0.05$). Figure 2C shows that mean islet area of PTHrP Δ/Δ mice was 45% (1,177 ± 155 $\mu m^2$) the mean area of the control islets (2,615 ± 215 $\mu m^2$). We also normalized each individual islet area to the pancreas area and the PTHrP (Figure 2D) Δ/Δ islets were significantly smaller after normalization ($p=0.012$). Grouping the islets based on their size showed that majority of the islets from the PTHrP Δ/Δ mice had areas of less than 1000 $\mu m^2$ with almost no islets greater than 5000 $\mu m^2$ (Figure 2E) and this difference was statistically significant for each of the groups. Similarly, upon isolation, islets were photographed (Figures 3A–B) and their area measured using image J. As expected, the islets from the PTHrP Δ/Δ mice were significantly smaller ($p<0.05$) than the control mice (Figure 3C).

### PTHrP Δ/Δ mice islets secreted more insulin in vitro despite their smaller size

Plasma insulin concentration was measured from 3 mice from each group. As shown in Figure 3D, plasma insulin concentration for PTHrP Δ/Δ mice was approximately half the control group ($p<0.1$). Although the p-value $P = 0.096$ was not significant for this observation due to a small sample size, our earlier work reported a statistically significant similar ~50% reduction in serum insulin in PTHrP Δ/Δ mice compared to wildtype or heterozygous controls [17]. The observed hypoglycemia in these mice is thus not due to high circulating levels of insulin. However, insulin secretion from isolated islets during overnight incubation was significantly greater for islets from PTHrP Δ/Δ mice ($p<0.05$) compared to control mice (Figure 3E), and this effect was more pronounced when the data were

normalized to islet area in both control and PTHrP Δ/Δ mice (Figure 3F). Insulin content was not statistically different (Figure 3G) even when normalized to islet area (Figure 3H).

### PTHrP Δ/Δ mice have a greater calcium response to glucose

Calcium influx is a requisite step to insulin secretion, and therefore a useful marker for glucose-stimulated insulin secretion. Islets from PTHrP Δ/Δ mice had higher intracellular calcium concentrations than controls (figure 4A). Calcium levels did not differ significantly in basal glucose (0–4mM) in islets from 2-day-old mice From 8–20 mM glucose, there was a spike in the first phase response of the islets, and the fura-2-ratio value for calcium in the PTHrP Δ/Δ mice was ~1.5-fold higher than controls (figure 4B–4C). Although PTHrP Δ/Δ mice rarely lived passed 5 days, we were able to measure the calcium response in one 9-day-old PTHrP Δ/Δ mouse and control mouse. As neonatal islets mature, their calcium responses become more responsive to glucose by maintaining low intracellular ionized calcium in low glucose, and elevated ionized calcium in high glucose[24]. Figure 5A shows that islets from a PN Day 9 control mouse maintained low calcium levels in low glucose (0–4 mM) and produced a sharp first phase spike at 8mM glucose, which is consistent with the glucose threshold in mature islets. We were also able to obtain islets from one PTHrP Δ/Δ that survived to postnatal Day 9. Islets from this Day 9 PTHrP Δ/Δ mouse maintained elevated calcium in low glucose but were unable to mount a large increase in calcium in higher glucose. Islets from the 9-day-old PTHrP Δ/Δ mouse also produced a spike response in 4mM glucose that was absent in controls. As shown in Figure 5B, the difference in first phase responses from 4 to 8mM glucose is substantial (control: 0.115 +/− 0.07, N=7 vs. PTHrP KI: 0.47 +/−.02 fura-2 ratio 340/3480nm, N=5, P=.00016). Between 0 and 4 mM glucose, at which calcium should remain low, the increase in islet calcium for PTHrP Δ/Δ mouse was 4-fold greater than for controls (Figure 5C).

We next tested the the $K_{ATP}$-channel-dependent pathway using diazoxide and tolbutamide. Diazoxide effectively opens the ATP-gated potassium channels while tolbutamide closes them. Closure of the ATP-gated potassium channels causes an influx of calcium which leads to increase in insulin secretion. As shown in Figure 6A–B, intracellular calcium levels were not significantly different between islets from PTHrP Δ/Δ mice and controls which is consistent with observations in Figure 4A. Exposing the islets to diazoxide decreased the calcium response in islets from both PTHrP Δ/Δ mice and control mice (figures 6A–B) to nearly identical levels (p-value =0.96). Islets from PTHrP Δ/Δ mice resumed elevated calcium response with 11mM glucose stimulation as expected, based on the data in Figure 6. The elevation was sustained when the islets were treated with 11 mM glucose + tolbutamide which closes the cell membrane potassium channels and depolarizes the beta cells. These findings suggest that islets from PTHrP Δ/Δ mice have an enhanced capacity to take up calcium and thus to secrete insulin through the triggering ($K_{ATP}$-channel-dependent) pathway [28].

### PTHrP Δ/Δ mice had less glucagon than control mice

As shown in Figure 7A, we used immunohistochemistry to examine islets for the presence of glucagon-positive alpha cells (red) and insulin-positive beta cells (green). The PTHrP Δ/Δ mice had fewer glucagon-positive cells in the pancreases as measured

by immunofluorescence (Figure 7B). In some PTHrP Δ/Δ sections, no glucagon-positive cells were identified and most islets from these mice had a glucagon area less than 500 $\mu m^2$. In contrast, glucagon-positive areas in control mice ranged between 800–1200 $\mu m^2$. As seen in Figure 6C, the area of insulin-positive cells trended downward ($p<1.5$). Each point in the analysis represents the average area of insulin or glucagon-positive cells in all islets analyzed from one mouse (n=6 mice). These results were reflected in figure 7D where insulin and glucagon area were normalized to the total islet area. PTHrP Δ/Δ mice had larger non- beta and alpha cell areas compared to the control mice. We also measured glucagon content from pancreases (Figure 8A) and normalized the data with the weight of pancreases (Figure 8B). There was significantly lower glucagon content in the PTHrP Δ/Δ mice pancreases ($p<0.05$). We attempted to measure glucagon content directly from isolated islets, however, glucagon values were below the limit of detection of the assay. Overall, our findings demonstrated that PTHrP signaling is important to production of glucagon and secretion of insulin by pancreatic islets. The results also indicate that the NLS of PTHrP contributes to these biological effects.

## Discussion

This study demonstrates a novel role for the (NLS) and C-terminus of PTHrP in glucose metabolism and islet function. The NLS and C-terminus of PTHrP are important for survival as mice lacking these portions of PTHrP fail to grow and die between 3 days to 2 weeks of life [16,17]. Although previous studies have reported the effect of full-length PTHrP or N-terminus PTHrP on beta-cell proliferation[20], regeneration[21], protection[29], and insulin secretion [6], there has been no study on the role of the NLS and C-terminus of PTHrP in islet function. Our data show that lack of the NLS and C-terminus of PTHrP not only slows somatic growth of the mice but also prevents the maturation of internal tissues such as the Islets of Langerhans in the pancreas. We present evidence for the first time that islets from PTHrP Δ/Δ mice differ from control mice in their size, their glucagon content, and their ability to secrete insulin in response to glucose stimulation, resulting in key differences in glucose metabolism. Our laboratory has deleted the C-terminal region of PTHrP alone from mice and the mice were phenotypically similar to wild-type mice (data not shown). Therefore, we suggest that the effects of the PTHrP knockin mice are predominantly due to the midregion of PTHrP containing the NLS. We discuss each of the novel observations in the following sections.

### Cytosolic Calcium and Insulin Secretion

The cytoplasmic calcium response of islets is a good measure of islet activity and can be used to understand aberrant insulin secretion. Glucose transporters in the cell membrane of beta cells transport glucose, which is processed through glycolysis and oxidative phosphorylation to release ATP. The subsequent increase in ATP/ADP causes the ATP-gated potassium (KATP) ion channels in beta cells to close and this results in opening of calcium ion channels. Influx of calcium ions occurs in the beta cells and as intracellular ionized calcium levels rise, insulin is secreted. Our lab recently reported the calcium responses of islets from 0, 4 and 12 day old mice [24]. The findings from this study revealed that, unlike adult islets, neonatal islets are more sensitive to lower glucose concentrations, such as 4

mM glucose. Using similar analytic methods, we showed differences in the intracellular calcium response of islets from PTHrP Δ/Δ compared to control islets at day 2 of age. Islets from PTHrP Δ/Δ mice had a significant increase in calcium response at higher glucose concentrations. This increase would account for the increase in insulin secretion observed when the islets were incubated overnight in media containing 11mM glucose.

To further test for differences in the glucose-stimulated insulin secretion pathway, we examined the maximum and minimum responses of the KATP-channel dependent pathway with calcium as a reporter. The roles of diazoxide and tolbutamide in altering potassium ion channels have been studied extensively [30–37]. In our tests, the opening of all KATP channels with diazoxide reduced intracellular calcium to similar levels in both genotypes. Islets from PTHrP Δ/Δ mice had an increased calcium response to 11mM glucose, suggesting hypersensitivity of the islets from PTHrP Δ/Δ mice to normal and higher glucose concentrations. However, additional stimulation with tolbutamide to close all KATP channels resulted in a significantly greater increase in calcium in islets from PTHrP Δ/Δ mice compared to controls. This suggests enhanced insulin secretion through the KATP-channel-dependent pathway in PTHrP Δ/Δ mice and explains why islets from PTHrP Δ/Δ mice continued to show elevated calcium levels at 8, 12, 16, and 20 mM glucose. The cause of this enhanced capacity will require further investigation.

**The conundrum of hypoglycemia and hypoinsulinemia—**The difference in insulin secretion observed in islets of the PTHrP Δ/Δ mice in vivo and in vitro was striking. Previous studies on the role of PTHrP in insulin secretion have focused mainly on plasma insulin levels and not on the islets themselves [20,21,29]. In this study, we showed that the islets functioned differently in vivo compared to in vitro. This may be a result of the hypoglycemia observed in the PTHrP Δ/Δ mice. Insulin secretion is stimulated by increased blood glucose. In the absence of such increase as seen in the PTHrP Δ/Δ mice, insulin secretion is reduced, causing lower plasma insulin levels.

An alternative explanation relates to how the in vitro studies of insulin secretion were conducted. After the islets were isolated, they were placed in media containing 11mM glucose (~200 mg/dL), which is standard practice[38]. However, the PTHrP mice from which these islets were isolated had blood glucose levels about half that of control mice. The effects of exposing islets to higher glucose than accustomed to is well documented [39–41]. Beta cells in islets become hyper-stimulated after exposure to higher glucose concentrations and maintain increased insulin secretion triggered by an increase in cytosolic calcium concentration. We speculate that moving the islets of the PTHrP Δ/Δ mice from a hypoglycemic to a normoglycemic condition led to hyperexcitation of the islets, resulting in increased insulin secretion when compared to the islets from control mice that were originally in normoglycemic conditions. One additional possibility, although unlikely, is that substantial cell death in isolated islets contributed preferentially to insulin secretion during overnight culture. Because these islets showed distinct calcium responses to glucose stimulation and showed to outward signs of poor health or cellular debris in culture, it is very likely that the observed insulin is due to secretion, not lysing.

The most plausible explanation for this phenomenon may lie in the glucose counter-regulatory response to hypoglycemia [42]. Glucagon is an important counter-regulatory hormone to drive glucose above hypoglycemic levels. As we have shown, pancreases from PTHrP Δ/Δ mice have diminished glucagon content, which suggests abnormal development of this counter-regulatory system in the knockin mice and an inability to respond to low blood glucose.

**Stunted growth and maturation of islets—**Islets from PTHrP Δ/Δ mice had smaller areas compared to islets from the control mice. Although not shown, islet area when normalized to pancreas area remained lower in the PTHrP Δ/Δ mice, implying that the differences in islet area seen are not as a result of reduced pancreas mass. Smaller isolated islets from rodents[43] and humans[44,45] have been shown to secrete more insulin than their larger counterpart. This may explain in part the increased insulin secretion observed in the islets from the PTHrP Δ/Δ mice.

Islets from neonatal mice continue to mature throughout the first few days of life[46]. At 2 days of age, there is an increase in the overall calcium response of the islets from the PTHrP Δ/Δ mice but no other obvious indications that maturation is altered. The islets from 9-day old mouse provided additional insight into the role of the NLS and C-terminus of PTHrP in islet development. Unlike islets from the control mouse, islets from 9-day old PTHrP Δ/Δ mice remained responsive to 4 mM glucose. This responsiveness to lower glucose concentration is usually observed in islets from 0 and 4-day-old mice [24]. Although these data suggest that the absence of the PTHrP NLS and C-terminus may impair glucose sensing and the maturation of islets, we must emphasize that these data were collected from a single mouse that survived to 9 days, which is rare due to the lethal nature of PTHrP Δ/Δ mice. Any conclusion at this time would thus be speculative.

**A possible role for PTHrP in glucagon-producing alpha cells—**The immunofluorescence data and islet glucagon content revealed that PTHrP Δ/Δ mice had reduced alpha cells as well as less glucagon synthesis. This is the first study to document a relationship between PTHrP and glucagon-producing alpha cells. Glucagon triggers the breakdown of glycogen to release glucose in hypoglycemic conditions. As discussed above, a reduction in glucagon-producing cells may have contributed to the hypoglycemia observed in PTHrP Δ/Δ mice and subsequent low plasma insulin. Serum PTHrP and glucose concentrations have been shown to be correlated in patients with Type 2 diabetes [47]. It is possible that the NLS and C-terminus of PTHrP play a role in maintaining glucose homeostasis by regulating differentiation of alpha cells from pancreatic progenitor cells. Future plans include using in vitro methods, such as islet cell lines and isolated islets from adult mice, to elucidate the role of PTHrP in glucagon production and secretion as well as the cytoplasmic regulatory pathways.

**Conclusions—**Understanding the regulation of pancreatic beta cells is important for investigating the pathogenesis of type 2 diabetes and discovering new therapeutics. When serum PTHrP and insulin concentrations were measured in type 2 diabetes and control patients [47], the patients with type 2 diabetes had higher serum PTHrP concentrations than control patients. In a separate study, PTHrP was shown to be secreted with insulin

after glucose stimulation [48]. Upon administering glucose, the serum PTHrP and insulin levels rose simultaneously, and this suggests that PTHrP may play a paracrine/autocrine role in the islet to enhance insulin secretion. Indeed, our collective observations of increased calcium levels, increased capacity of the KATP-channel dependent pathway, and reduced counter-regulatory glucagon content all point to increased insulin secretion and signaling in vitro. Findings from our study therefore show that the relationship between PTHrP and insulin secretion is not mediated by the N-terminus of PTHrP alone but the NLS and C-terminus of PTHrP have an important role in this relationship.

We have shown for the first time a distinct role for the NLS and C-terminus of PTHrP in pancreatic islet maturation, glucose sensing and glucagon production. Neonatal islets differ from adult islets in their glucose sensitivity as well as gene expression [49]. Although islets from 21-day old mice are yet to attain a fully mature state [50], development of islets is progressive such that islets from a new born mice differ from islets from 4-day-old mice [24]. We recognize that the truncation of the PTHrP gene in the PTHrP Δ/Δ mouse model was not islet specific and there were multiple pathologies in the mice. For this reason, we cannot confirm that the NLS and C-terminus of PTHrP support normal islet development solely through islet-mediated mechanisms. However, Vasavada et al. overexpressed PTHrP in pancreatic islets using a rat insulin promoter, and enhanced insulin secretion [6], as well as, beta-cell protection [29] was reported. The results in this study demonstrate that PTHrP NLS and C-terminus are important for normal function of islets and may be required for the development, of both alpha and beta cells. Further studies will be conducted using beta cell lines as well as isolated islets to investigate directly, the effects of these PTHrP domains on islet function. Experiments to assess liver function will also be carried out since insulin and glucagon directly act on the liver.

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## Highlights

- Nuclear and C-terminus PTHrP are important for islet development and insulin secretion
- Nuclear and C-terminus PTHrP regulate normal calcium response of islets
- PTHrP Δ/Δ mice have less glucagon than control mice

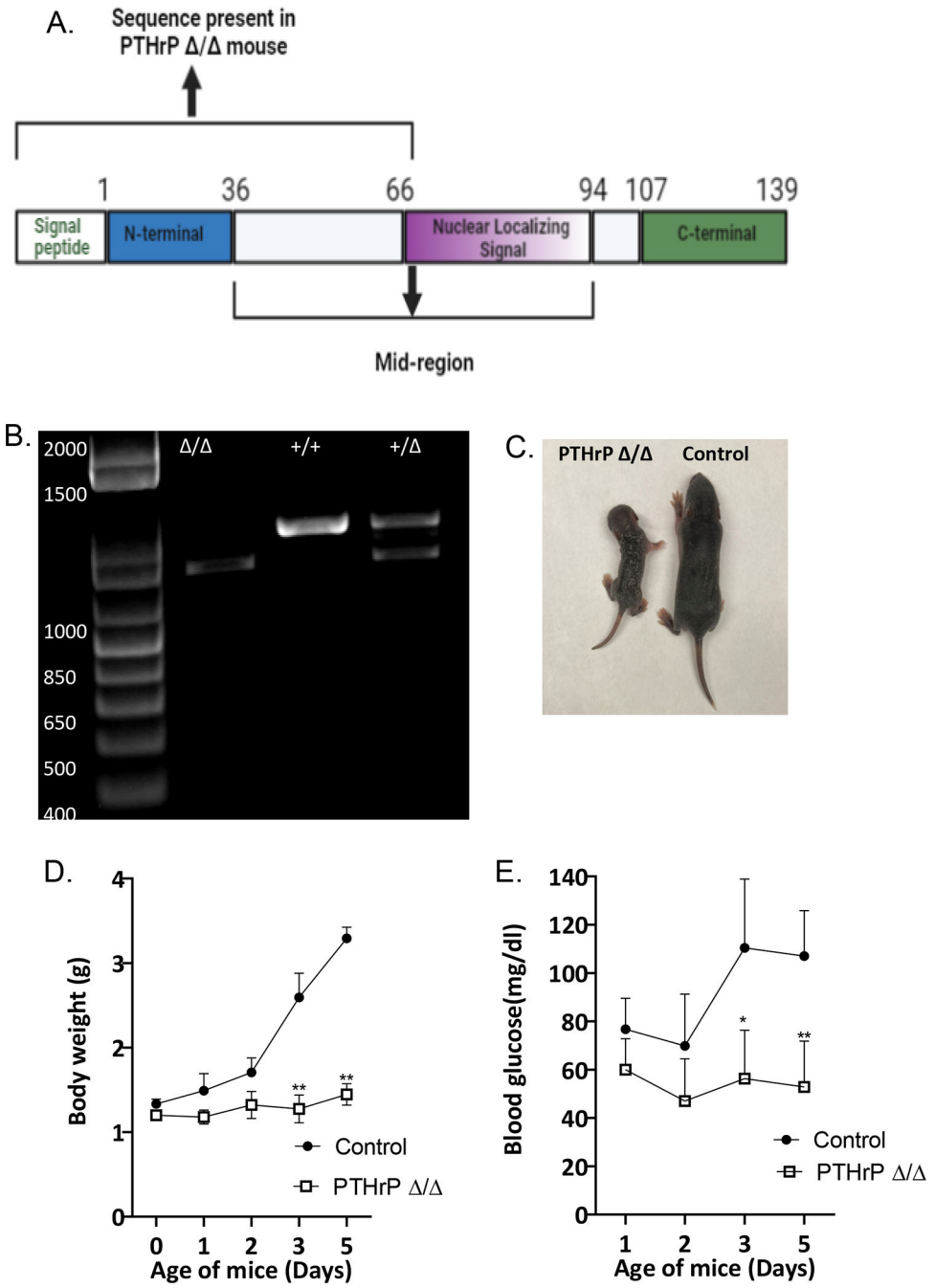


**Figure 1.**

PTHrP 67–139 is important for growth and normal glucose homeostasis in mice. (A) Structure of PTHrP; PTHrP Δ/Δ mice contain only PTHrP 1–66. (B) Gel image illustrates the absence of PTHrP 67–139 mRNA in the pancreatic tissue of PTHrP Δ/Δ mice (lower band is truncated PTHrP and upper band is full-length PTHrP. (C) PTHrP Δ/Δ mice are much smaller than control mice at day 3. (D) Growth rate shows that PTHrP Δ/Δ mice do not grow noticeably after birth (n>5 mice, **p<0.01). (E) PTHrP Δ/Δ mice are hypoglycemic. (n>5 mice, *p<0.05, **p<0.01).

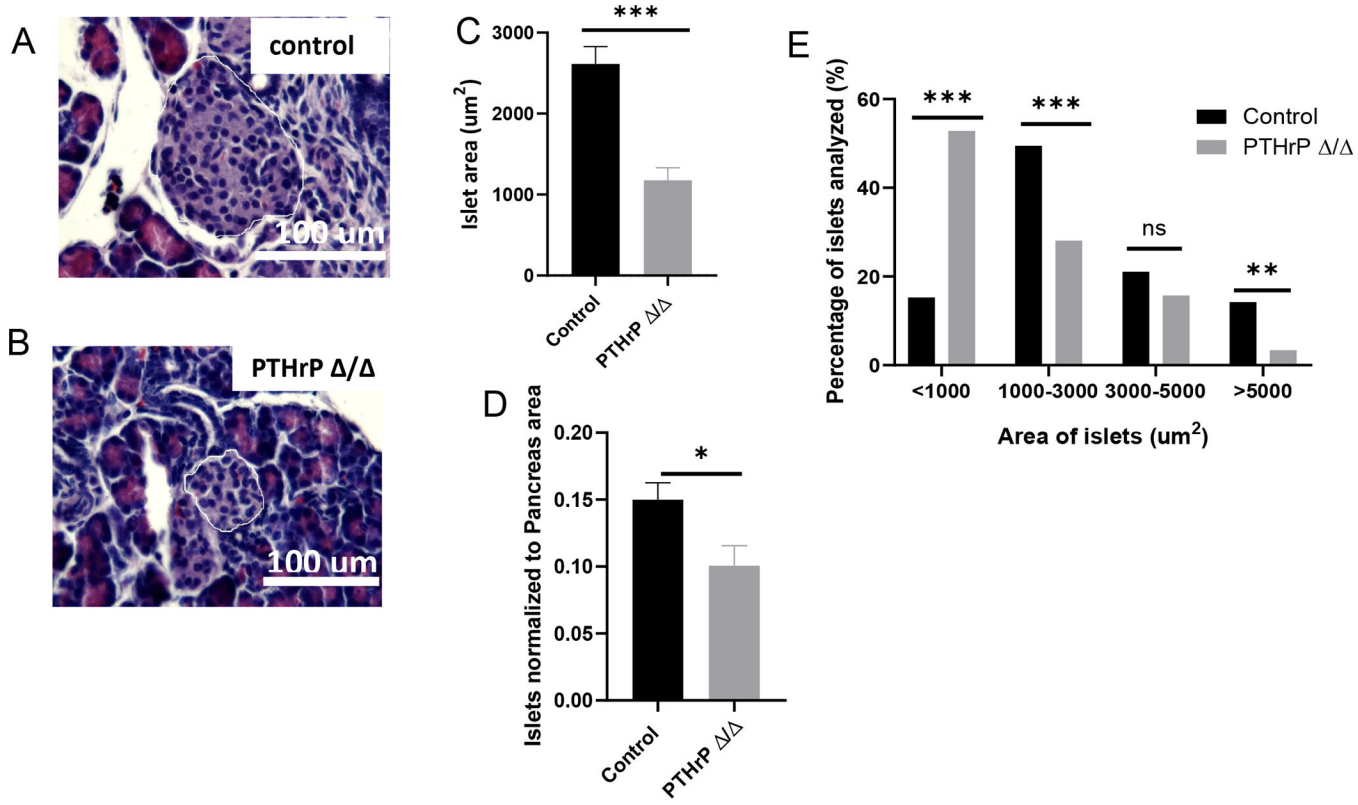


**Figure 2.**

Islets from PTHrP Δ/Δ mice are smaller than islets from control mice. (A-B) Hematoxylin and eosin images of islets from control (A) and PTHrP Δ/Δ mice (B). (C) Quantification of islet sizes using ImageJ showed that control islets were significantly larger than islets from PTHrP Δ/Δ mice (n=4 control, n=3 PTHrP Δ/Δ mice, *** p<0.00001). (D) Each individual islet area was normalized to pancreas area (*p=0.012). (E) Frequency distribution of islets based on size (n = 190 islets for control, n=89 islets for PTHrP Δ/Δ mice, fisher's exact test: *** p<0.001, **p<0.01)

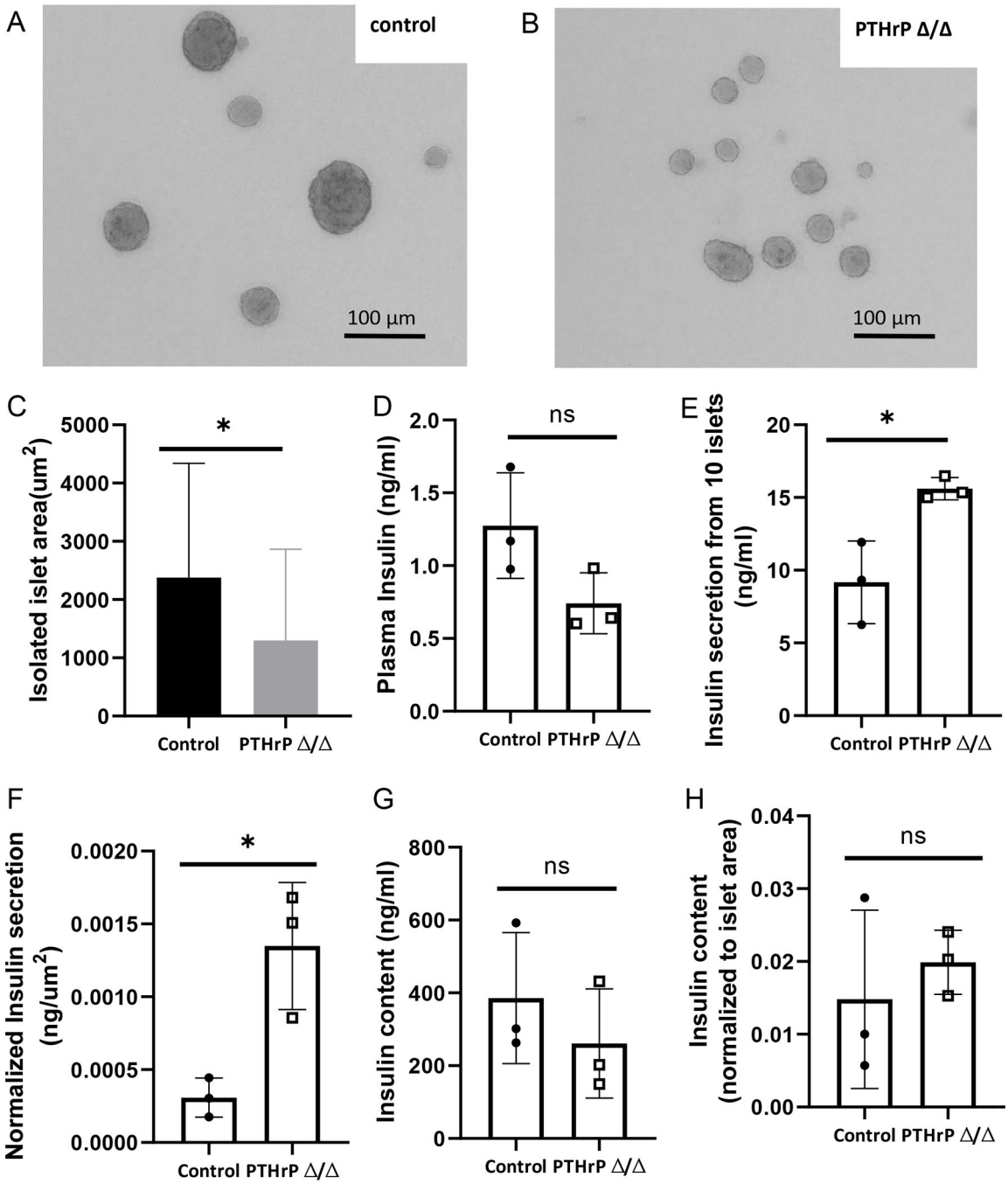


**Figure 3:**

Islets from PTHrP Δ/Δ mice secreted more insulin in vitro despite their smaller size. Representative images of isolated islets from (A) control and (B) PTHrP Δ/Δ mice. (C) Estimated islet area was calculated from perimeter traces of each islet (N>5 mice). (D) Plasma insulin concentrations were measured for 3 control or PTHrP Δ/Δ mice (p=0.092) (E) Overnight insulin secretion was measured from groups of 10 islets in conditioned media and (F) normalized to size of islets for all 3 mice. The groups of islets from PTHrP Δ/Δ mice secreted more insulin than islets from control mice (*p<0.05). (G) Insulin content

was measured after extraction with an acid-ethanol buffer and (H) insulin content was normalized to area of islets for all 3 mice.

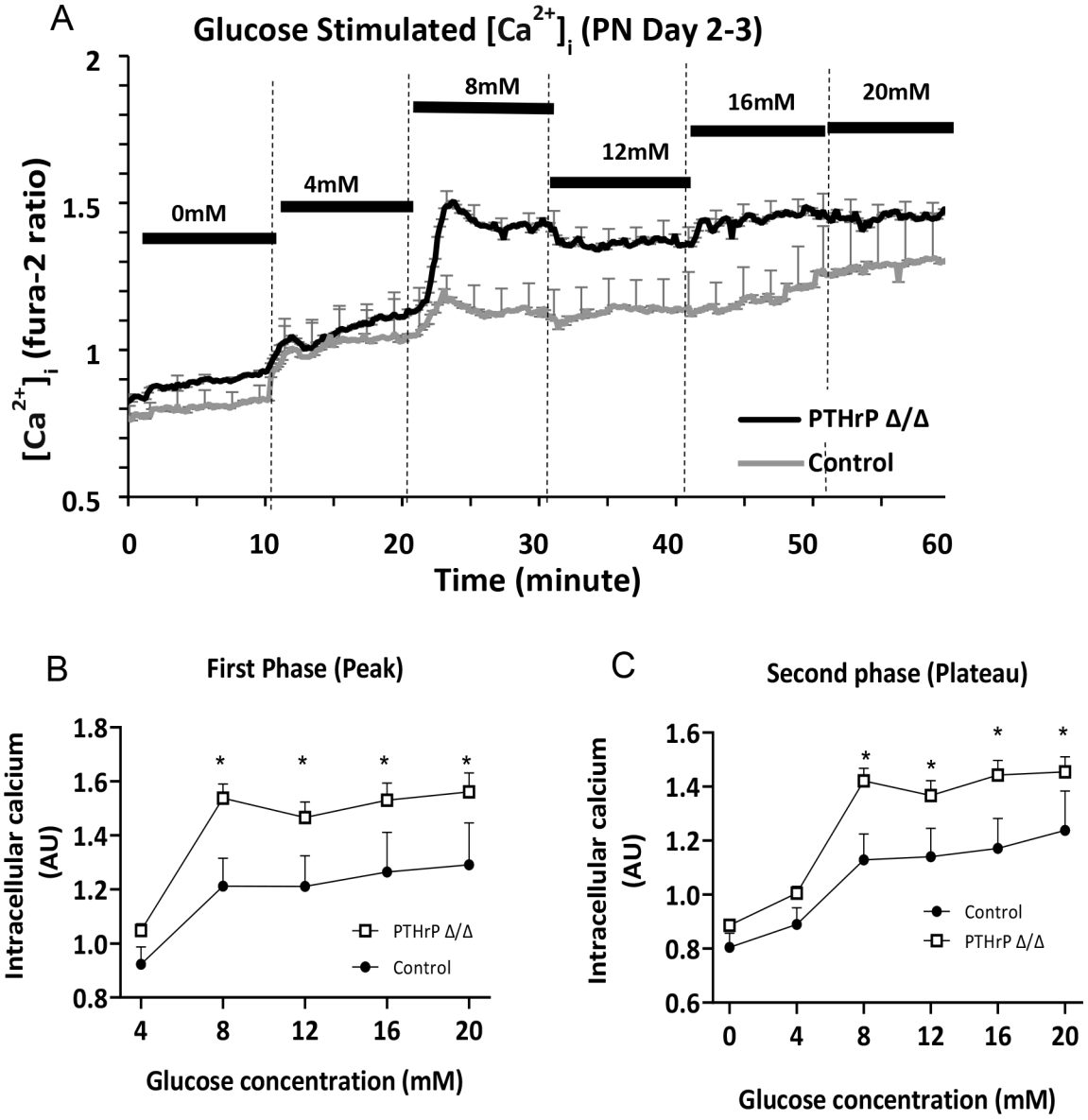


**Figure 4:**

PTHrP Δ/Δ islets had a higher intracellular ionized calcium response to glucose stimulation. Data was collected from 3 trials with n=30 islets. (A) Calcium response to six-step glucose stimulation: 0, 4, 8, 12, 16, 20 mM glucose measured using fura-2 dye showed higher intracellular calcium from 0 to20 mM glucose. From 8–20 mM glucose, the difference was statistically significant (*$p<0.05$). (B) First phase (peak calcium) was calculated as the maximum calcium level for each glucose concentration. (C) Second phase (plateau) was calculated as mean calcium level during a 5-minute time interval.

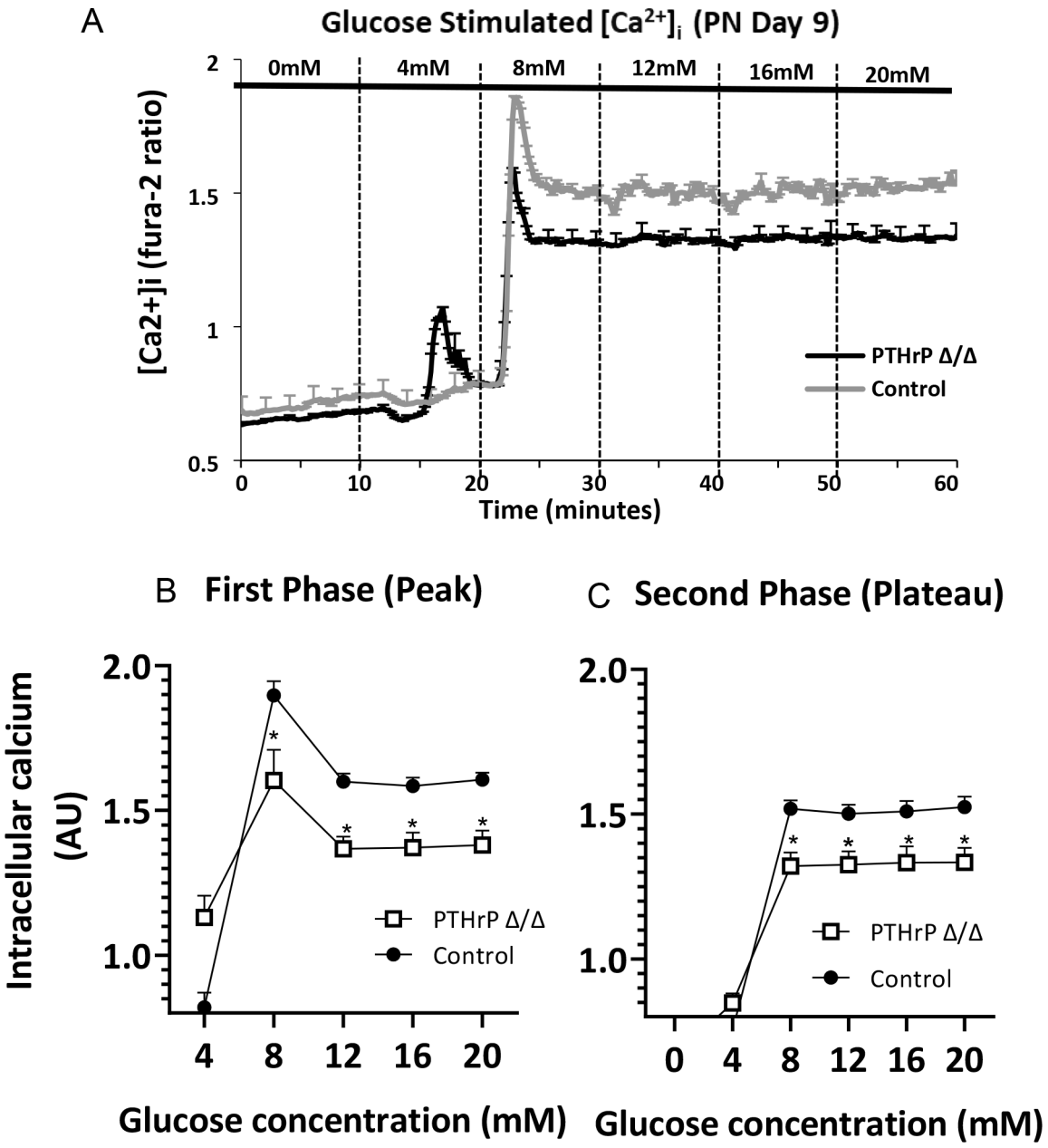


**Figure 5:**

(A) Calcium response of islets from a 9-day old PTHrP Δ/Δ mice showed hyperactivity at 4mM glucose unlike islets from control mice. B) First phase (peak calcium) was calculated as the maximum calcium level for each glucose concentration. (C) Second phase (plateau) was calculated as mean calcium level during a 5-minute time interval.

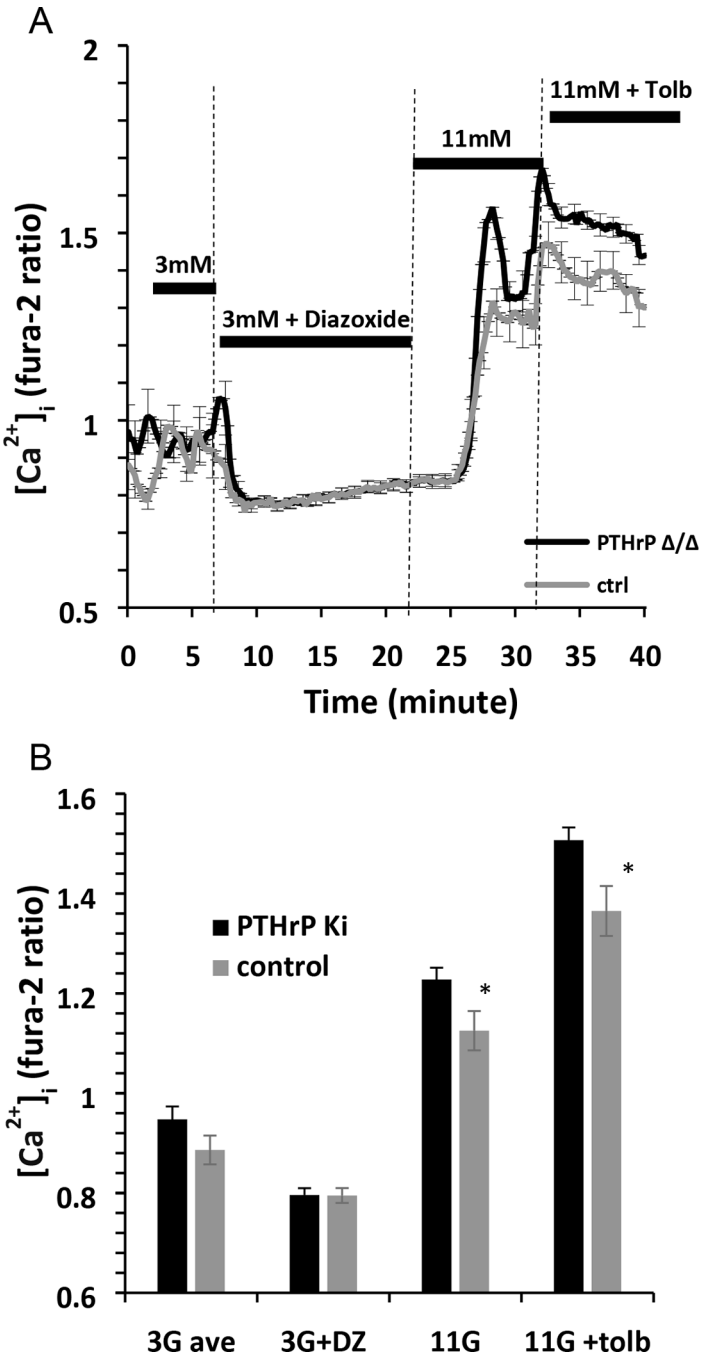


**Figure 6:**

PTHrP Δ/Δ islets maintained higher glucose sensitivity even after altering potassium channels with diazoxide and tolbutamide. (A) Line graph and (B) Bar graph show calcium response to 3 mM glucose, 3 mM glucose + diazoxide, 11 mM glucose and 11 mM glucose+ Tolbutamide. Upon stimulation with diazoxide, calcium response diminishes to basal levels. However, PTHrP Δ/Δ islets resumed increased calcium response upon stimulation with 11mM glucose and tolbutamide. *p<0.05, n=18.

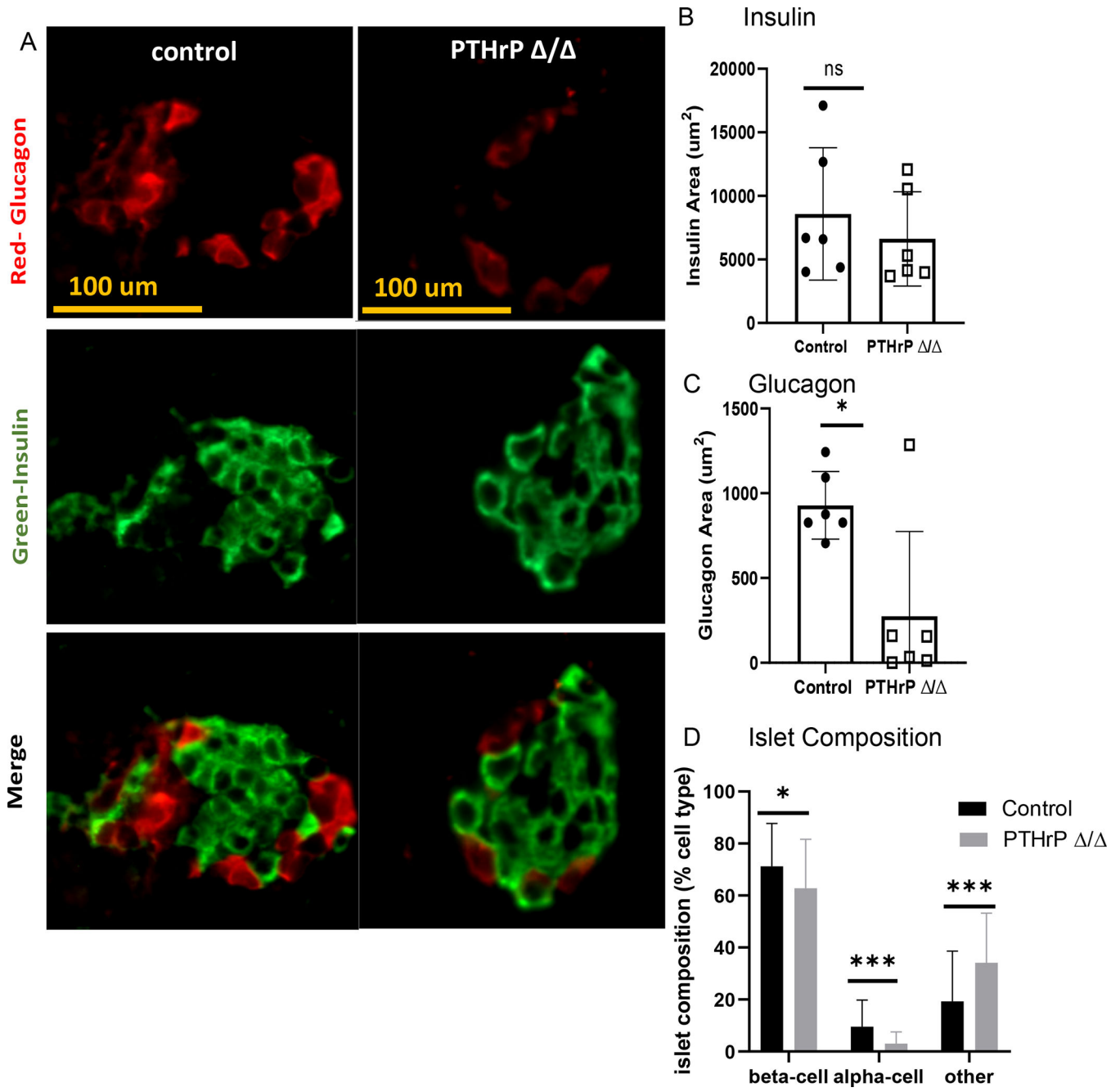


**Figure 7:**

PTHrP Δ/Δ mice have less glucagon than control mice. (A) Representative immunofluorescence images of islets from control and PTHrP Δ/Δ mice (red- glucagon, green – insulin). (B) Quantification showed no significant difference in insulin area between PTHrP Δ/Δ mice and control mice. (C) Quantification showed significantly less glucagon area in PTHrP Δ/Δ mice. Each point is the average glucagon or insulin area per mouse (n=6 mice, *p<0.05). (D) Alpha and beta cell area was normalized to total islet area to obtain percentage islet mass for each cell type. (n=6, *p<0.05, ***p<0.001)

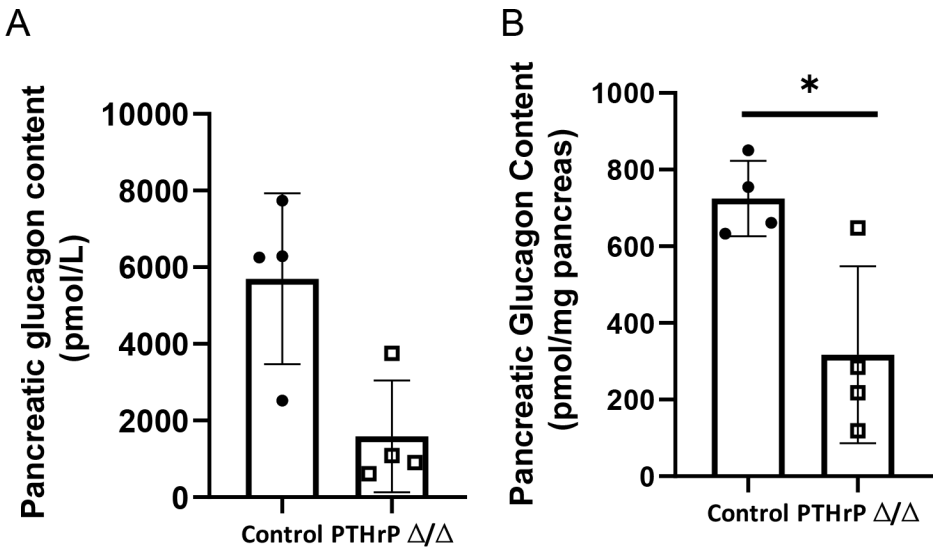


**Figure 8:**

PTHrP Δ/Δ mice have less glucagon content in the pancreas). (A) Glucagon content was measured from pancreas (n=4) and the data was (B) normalized to pancreas weight ($p<0.05$.

**Table 1**

Primer sequences for genotyping PTHrP Δ/Δ mice.

| | |
|---|---|
| mPTHrP 1 F | 5’-AGATCCACACAGCCGAAATCAGA-3’ |
| mPTHrP 1 R | 5’-AGCCCTGCTGAACACAGTGAACA - 3’ |
| mPTHrP 2 F | 5’ -AGTGTCCTGGTATTCCTGCTC- 3’ |
| mPTHrP 2 R | 5’- ATGCAGTAGCTGATGTTCAGACAC-3’ |