# Investigating the sources and functions of Nerve Growth Factor in pancreatic islet development

by

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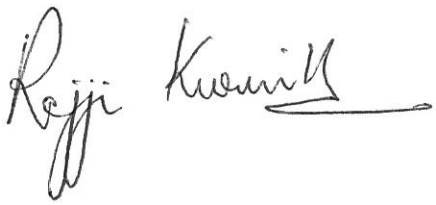
# Certificate

This is to certify that this dissertation entitled “Investigating the sources and functions of Nerve Growth Factor in pancreatic islet development” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Vasudha Aher at Johns Hopkins University under the supervision of Rejji Kuruvilla, PhD, Department of Biology during the academic year 2021-2022



Supervisor’s signature

# Declaration

I hereby declare that the matter embodied in the report entitled “Investigating the sources and functions of Nerve Growth Factor in pancreatic islet development” are the results of the work carried out by me at the Department of Biology, Johns Hopkins University, under the supervision of Rejji Kuruvilla, PhD and the same has not been submitted elsewhere for any other degree.

Student’s signature

# Abstract

Sympathetic innervation is critical for the proper development and functioning of its target tissues. Sympathetic innervation of an organ is dependent on target-derived neurotrophic cues such as NGF which drive the final stages of target innervation. In the pancreas, NGF exerts indirect and direct effects on islet development and function. During development, target-derived NGF recruits sympathetic nerves to the pancreas. Loss of sympathetic innervation impairs islet architecture and functional maturation. In adult islets, NGF signalling in beta cells directly augments glucose-stimulated insulin secretion. However, the sources of NGF and their respective roles in the developing pancreas remain unclear. Here, we show that vascular mural cells are the primary source of NGF at postnatal day 7, a time period that is critical for functional maturation of islets, and sympathetic innervation. We developed a tamoxifen delivery protocol to inducibly delete NGF from mural cells during early and late postnatal development and examined the effects on pancreatic islet development and sympathetic innervation. Early NGF deletion in neonates resulted in modest defects in islet architecture and organization, and an increased number of TH+ endocrine cells. Surprisingly, sympathetic innervation to islets was not lost after NGF deletion. Late postnatal NGF deletion did not have any major effects on islet innervation. This study highlights vascular mural cells as a previously under-studied source of NGF in the developing pancreas and demonstrates their relevance in pancreatic islet development.

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# Acknowledgements

This thesis stands on the shoulders of many giants, and so do I. I would like to express my heartfelt gratitude to every person who has supported me on this journey. Although I am unable to mention everyone here, I am immensely thankful for all of you.

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Working with a new model system, especially one that bites, was certainly a challenge but it was one that was overcome with relative ease, largely because of my two mentors in the lab – Joselyn and Raniki. They have been extremely patient in teaching me all the techniques I needed for my project. My conversations with them have shaped the course of this project more than anything else has, and I am very grateful for their help and guidance.

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# Chapter 1: Introduction

## Development of the sympathetic nervous system

The sympathetic nervous system (SNS) is a branch of the peripheral nervous system that is instrumental in maintaining the basal physiological state in mammals. Sympathetic nerves innervate various target tissues in the periphery to regulate physiological processes such as cardiac output, glucose metabolism and body temperature to maintain homeostasis in normal conditions as well as mediate the body's response to external stressors (Glebova and Ginty, 2005). This regulation is dependent on the proper establishment of neuronal contacts with target tissues during embryonic and postnatal development. Improper sympathetic innervation during development and sympathetic dysfunction are often associated with disease states such as diabetes, hypertension and peripheral neuropathies (Scott-Solomon et al., 2021).

The SNS is composed of preganglionic and postganglionic neurons, which are synaptically connected and organized in series. Preganglionic neurons have cell bodies that reside in the spinal cord and send axonal projections to postganglionic neurons that reside in either the prevertebral or paravertebral sympathetic ganglia, which then extend axons to their targets in the periphery. The establishment of sympathetic circuits during development has been well-characterized in mice and is known to rely on target-derived guidance cues such as neurotrophins. Neurotrophins are a family of growth factor molecules secreted by cells which are involved in axon guidance and elongation, neuronal survival, dendrite growth and establishment of synapses. Primary members of this family that are involved in sympathetic development include NGF (nerve growth factor), BDNF (Brain-derived Neurotrophic Factor), NT-3 (Neurotrophin-3) and NT-4/5 (Neurotrophin-4/5) (Skaper, 2012), which function at different stages across development to have specific effects on sympathetic innervation.

Postganglionic sympathetic neurons are derived from neural crest cells which give rise to neuroblasts that coalesce to form the sympathetic ganglia (Glebova and Ginty 2005). Sympathetic axon extension begins as early as E12.5 in rodents, while the ganglia are still forming (Rubin, 1985). Although the precise molecular mechanisms leading to axon initiation have not been established, local autocrine

signalling via HGF (Hepatocyte Growth Factor) has been implicated in the process (Yang et al., 1998). Following this, proximal axon extension occurs along the developing arterial vasculature and is directed by the guidance cues artemin and NT-3, which are secreted by vascular smooth muscles along the arteries (Honma et al., 2002; Scarisbrick et al., 1993).

Sympathetic innervation of the final targets begins around E15.5 in mice and continues for 3-4 weeks after birth, and it is largely driven by target-derived NGF (Scott-Solomon et al., 2021). NGF signals through its corresponding receptor TrkA which is present on the cell surface of neurons. The NGF-TrkA complex is internalized and retrogradely transported in the neuron as a signalling endosome, where it activates key signalling pathways such as the MAPK (Mitogen Activated Protein Kinase), PLCγ (Phospholipase C gamma) and PI3K (Phosphotidylinositide-3 Kinase) pathways, in addition to activating the transcriptional factor CREB which leads to large-scale transcriptional changes within the cell (Zweifel et al., 2005). Evidence from recently published literature also suggests that NGF signalling modulates axonal protein synthesis by inducing specific targeting of mRNAs to distal axons as well as facilitating localized post-translational modifications of proteins (Andreassi et al., 2010; Scott-Solomon and Kuruvilla, 2020). NGF is canonically believed to be involved in innervation only after axons have reached the targets, with minimal involvement in the earlier steps of axonal extension along the arterial vasculature. However, this idea has been challenged by a recent study which showed that local synthesis of NGF by vascular smooth muscle cells (vSMCs) along coronary veins is essential for distal axon extension during innervation of the heart, suggesting that vasculature-derived NGF could be needed for distal extension and could guide the pattern of innervation in certain organs (Nam et al., 2013).

Patterns of sympathetic innervation are also extensively shaped by cell death and axonal pruning after target innervation. Around half of sympathetic neurons undergo apoptosis around the time their axons reach their targets, a process that is driven by competition for limiting amounts of NGF produced by target tissues. It is believed that such large-scale elimination modifies the extent of innervation to suit the specific needs of the target organ. Correspondingly, larger targets secrete more NGF and overexpression of NGF drives hyperinnervation (Edwards et al., 1989; Korsching and Thoenen, 1983; Shelton and Reichardt, 1984). NGF signalling also promotes

expression of pro-death signals in certain neurons which act on neighbouring neurons to induce apoptosis (Deppmann et al., 2008). Additionally, excessive axon collaterals undergo pruning during later postnatal stages in a manner independent of neuronal loss. Studies in the postnatal rat eye show that this is an activity-dependent and neurotrophin-dependent mechanism wherein “winning” axons secrete BDNF which acts via its low-affinity receptor p75 to cause retraction of proximal “losing” axons (Singh et al., 2008). Thus, neurotrophin signalling is critical in establishing and refining the final pattern of sympathetic innervation in tissues.

Although there exists a large amount of research describing the roles of target tissues on neuronal development, very little work has explored the reciprocal effects of sympathetic innervation on its targets. The endocrine pancreas, composed of the islets of Langerhans, is an important target of sympathetic innervation. Sympathetic neurons form close associations with endocrine cells during development and continue to innervate them throughout adulthood. Abnormal pancreatic development is known to lead to metabolic disorders or increase the propensity for metabolic dysfunction in adult life. Thus, the endocrine pancreas serves as a valuable model to study the developmental effects of sympathetic innervation on target tissues.

**Pancreatic development**

The pancreas is a key organ involved in metabolism in mammals. It has both exocrine and endocrine functions whose activity is independent of each other. The exocrine pancreas is composed of acinar cells that secrete digestive enzymes via pancreatic ducts into the duodenum. The endocrine pancreas is composed of clusters of cells called the islets of Langerhans which synthesize and secrete hormones to regulate glucose metabolism. In mice, islets are primarily composed of insulin-producing beta cells which constitute 60-80% of the islet and form its core. Insulin release, triggered by increased blood glucose, acts on the liver and skeletal muscles to reduce gluconeogenesis and glycogenolysis and stimulates uptake of glucose by the liver, skeletal muscles and adipocytes, thereby collectively reducing the blood glucose level. It also induces the uptake of nutrients and triglyceride synthesis in adipocytes. Alpha cells, which produce the hormone glucagon, and delta cells, which produce the hormone somatostatin, are present in smaller numbers and at the

periphery of the islets. Glucagon acts in a manner opposite to insulin and increases blood glucose levels in hypoglycaemic conditions by stimulating glycogenolysis and gluconeogenesis in the liver. Somatostatin acts in a local paracrine manner to modulate the function of alpha and beta cells. Sympathetic activity usually potentiates glucagon secretion and downregulates insulin secretion to increase blood glucose levels. Pancreatic polypeptide cells and epsilon cells, which are not directly involved in glucose metabolism, make up the remainder of the endocrine cells of the pancreas (Noguchi and Huising, 2019; Shih et al., 2013).

Pancreatic endocrine cells begin to acquire their fates and proliferate at embryonic day 12.5 (E12.5) in mice, while they are still part of the ductal epithelium. They then migrate out of the ductal epithelium to form clusters that later mature into islets. Two models have been proposed for the process by which mature islets are formed from endocrine cells. According to one model, endocrine cells migrate singly out of the ducts to coalesce and form islet clusters (Puri and Hebrok, 2007). An alternative model suggests that endocrine cells differentiate and proliferate contiguously, forming branched cord-like structures which undergo fission in neonates to form distinct clusters which mature into islets (Miller et al., 2009). Endocrine cell clusters begin to acquire the stereotypical architecture and circular shape of adult islets around postnatal day 6 (P6), and this process is usually completed around postnatal day 10 (P10). Endocrine cells continue to proliferate and migrate towards islet clusters after birth (Shih et al., 2013). Cells within islets proliferate as well over the postnatal period, leading to an increase in islet size. Neonatal islets are functionally immature, characterized by their inability to respond acutely with insulin secretion to a glucose stimulus. They usually complete their functional maturation around P10.

**The role of sympathetic innervation in pancreatic development**

Previous work in the Kuruvilla lab investigated the effects of sympathetic innervation on pancreatic development. *TH-Cre; TrkA$^{f/f}$* mice, which have the TrkA receptor removed from sympathetic neurons, and mice treated postnatally with 6-OHDA, a drug that ablates nerve terminals, were used to assess the effects of denervation on islet development. Sympathectomized mice had defects in islet cytoarchitecture and impaired functional maturation of islets. Denervated animals had

elevated blood glucose levels and they secreted less insulin than controls in response to glucose stimulation (Borden, 2013; Borden et al., 2013).

These experiments clearly demonstrate the importance of sympathetic innervation in the development and maturation of pancreatic islets, which in turn strongly depends on NGF-TrkA signalling within the innervating sympathetic neurons. Despite this, no studies have directly investigated the sources of NGF within the developing pancreas yet.

Several lines of evidence support the hypothesis that beta cells can produce NGF, which has led this to be the primary candidate in the field in the context of islet development and function. Multiple studies have shown the production of NGF in isolated and cultured beta cells by immunocytochemistry, RT-PCR and ELISA experiments (Houtz et al., 2016; Rosenbaum et al., 1998; Vidaltamayo et al., 2003). However, it is important to note that these experiments have been done in adult beta cells *in vitro*. Thus, NGF production could be either an artifact of the culture conditions or it could only be produced in adult beta cells. Only one study has looked at *in vivo* synthesis of NGF in the pancreas – however, the study was done in rats and used an indirect approach by staining for proNGF, the precursor of NGF (Cabrera-Vásquez et al., 2009). No studies have yet identified beta cells as a source of NGF specifically during mouse pancreatic development.

Interestingly, *Pdx1-Cre; NGF$^{f/f}$* mice, which have NGF knocked out embryonically from all pancreatic progenitor cells, show normal responses when challenged with glucose (Houtz et al., 2016). Pdx1 expression begins early in embryonic development and Pdx1+ cells give rise to the endocrine, exocrine and ductal cells of the pancreas (Shih et al., 2013). Thus, *Pdx1-Cre; NGF$^{f/f}$* animals would be expected to have early pancreas-wide deletion of NGF from these cells. Since loss of sympathetic innervation leads to clear defects in glucose metabolism, it appears that loss of NGF from these cell populations may not be a requirement for sympathetic innervation of the pancreas. Given this, we hypothesize that another cell type might be acting as a primary source of NGF in the developing pancreas – vascular mural cells.

Vascular mural cells are contractile vascular cells that surround the endothelial cells of blood vessels. Depending on their morphology and location, they are

categorized into two classes - vascular smooth muscle cells (vSMCs) that are associated with the larger blood vessels and pericytes that are associated with capillaries (Armulik et al., 2011). Various lines of emerging evidence point towards a role of vascular mural cells in the development of pancreatic islets and the maintenance of their function. Neonatal pericytes support beta cell proliferation and functional maturation in islets, in part through the secretion of Bone Morphogenetic Protein 4 (BMP4) (Epshtein et al., 2017; Sakhneny et al., 2021a). Pericytes also support the islet niche in adult animals by secreting islet basement membrane factors that are crucial for supporting beta cell function (Sakhneny et al., 2021b; Sasson et al., 2016). These cells are largely derived from the pancreatic mesenchyme during development (Harari et al., 2019) which plays an important role in the expansion of endocrine progenitor cells in the developing pancreas (Landsman et al., 2011). Together, this evidence strongly supports a developmental role for vascular mural cells in the pancreas.

Work from the Kuruvilla lab has shown that pericytes and vSMCs in the pancreas secrete NGF in response to glucose stimulation which signals through TrkA receptors on beta cells and promotes insulin secretion. They are also the only known source of NGF in the adult pancreas *in vivo* (Houtz et al., 2016). Pericytes and vSMCs are also known to secrete neurotrophins in response to stressors such as hypoxia in the brain and soft tissue trauma (Ishitsuka et al., 2012; Lee et al., 2021). Thus, they are a promising candidate for a source of NGF in the developing pancreas.

In this study, we aim to elucidate the sources of NGF and the role they play in the postnatal development of the pancreas, with a specific emphasis on vascular mural cells. We focused on this period since the final pattern of sympathetic innervation to the pancreas is not completely specified at birth and continues to be refined over the postnatal period. Sympathetic innervation peaks around P6 and the final pattern of innervation is established by P21 (Borden, 2013). This process of establishment and refinement of sympathetic innervation is known to depend on NGF; thus, it is useful to study the effects of NGF deletion at this stage. Additionally, functional maturation of islets and endocrine cell proliferation continues throughout the postnatal period. We used the *NGF$^{LacZ}$* mouse line, a reporter line for NGF, to investigate the cell types producing NGF. We found that at postnatal day 7 (P7), a critical period during neonatal pancreatic development, vascular mural cells were the only cells expressing NGF in the pancreas. To study the impact of loss of NGF from mural cells at different

developmental timepoints, we used a tamoxifen-inducible Cre line (*PDGFRβ-P2A-CreER$^{T2}$*, henceforth referred to as *PDGFRβ-CreER$^{T2}$*) in combination with a floxed NGF line (*NGF$^{f/f}$*) to conditionally and temporally knockout NGF from mural cells from early and later postnatal stages. P10 was chosen to assess the effects of early NGF deletion because islets acquire the architectural and functional properties of mature islets by P10. P15 was chosen to analyse the effects of later NGF deletion because the pattern of sympathetic innervation resembles the pattern seen in adult animals by this stage (Burris and Hebrok, 2007). To achieve this, we developed and optimized two tamoxifen administration paradigms for neonatal mouse pups using a reporter mouse line generated by crossing *PDGFRβ-CreER$^{T2}$* mice (Cuervo et al., 2017) with *R26R-EYFP* mice (Srinivas et al., 2001). We then applied this paradigm to *PDGFRβ-CreER$^{T2}$;NGF$^{f/f}$* mice and assessed islet development and sympathetic innervation using immunohistochemistry. Deletion of NGF in the early postnatal period resulted in generally smaller and more elongated islets, with the exception of a few islets that were extremely large and misshapen. Contrary to what we expected, early NGF deletion did not cause loss of sympathetic innervation in islets. NGF deletion during the later postnatal period had no effect on islet innervation. These findings demonstrate that vascular mural cells are a predominant source of NGF in postnatal pancreatic development and suggest a role for vasculature-derived NGF in islet development and innervation during postnatal development in mice.

# Chapter 2: Materials and Methods

## Mice

*NGF$^{LacZ}$* (Liu et al., 2012) and *NGF$^{f/f}$* mice (Müller et al., 2012) were generous gifts from Dr David Ginty (Harvard Medical School) and Dr Liliana Minichiello (University of Oxford), respectively. *PDGFRβ-P2A-CreER$^{T2}$* mice (Cuervo et al., 2017) and *R26R-EYFP* mice (Srinivas et al., 2001) were purchased from Jackson Laboratories. All procedures relating to animal care and treatment conformed to institutional and NIH guidelines.

## Antibodies

The following primary antibodies were used: mouse anti-insulin (1:2000, Sigma-Aldrich I2018), guinea pig anti-glucagon (1:500), rabbit anti-somatostatin (1:500, Sigma HP019472), chicken anti-beta-galactosidase (1:100, Millipore AB3403-I), rat anti-PECAM (CD31) (1:200, BD Biosciences 550274), mouse anti-SMA (1:500, Sigma-Aldrich A2547), rabbit anti-NG2 (1:200, Millipore AB5320), rabbit anti-TH (1:500, Millipore AB152) and chicken anti-GFP (1:500, Abcam AB13970). Fluorescent secondary antibodies used were Alexafluor-488,-546, or -647-conjugated and used at 1:200 (Invitrogen).

## Tamoxifen preparation and injections

Tamoxifen solution was prepared by dissolving 10 mg of tamoxifen in 1 ml of corn oil (vehicle) in a light-protected tube and leaving it to rotate for 1 hour at 65ºC. This solution was used as stock and stored at 4ºC up to a week. Dilutions of either 1 mg/ml or 2 mg/ml were prepared for injections in mice. For early postnatal knockouts, 50 µl of 1 mg/ml tamoxifen solution was injected intragastrically (into the milk spot) from P1 to P3. For the late postnatal knockouts, 50 µl of 2 mg/ml tamoxifen solution was injected intraperitoneally from P7 to P10. Time of injection was largely kept similar on all days to ensure tamoxifen levels in animals stayed consistent and no variability was introduced. Since tamoxifen is light-sensitive, littermates were always injected at the same time and with the same solution to prevent possible variation caused by changes in tamoxifen content of the solution.

## Mating strategy for genetic knockouts

To knock out NGF from vascular mural cells at specific timepoints, the *PDGFRβ-CreER$^{T2}$* mouse line was used, which expresses Cre recombinase in pericytes and vSMCs in a tamoxifen-inducible manner. These mice were crossed with *NGF$^{f/f}$* mice, in which both copies of NGF are flanked by LoxP sites, to generate animals that were heterozygous for both the Cre locus and the floxed NGF gene (*PDGFRβ-CreER$^{T2}$; NGF$^{+/f}$*). These animals were then crossed with *NGF$^{f/f}$* animals to generate PDGFRβ-CreER$^{T2}$; *NGF$^{f/f}$* mice for knockouts and *NGF$^{f/f}$* mice as control animals in the same litter. Heterozygous knockout animals (*PDGFRβ-CreER$^{T2}$; NGF$^{+/f}$*) were also obtained from these litters but they were not included in our analysis. *PDGFRβ-CreER$^{T2}$; NGF$^{f/f}$* mice from the F2 generation were crossed with *NGF$^{f/f}$* mice to get animals for knockout analyses.

## Tissue processing and immunohistochemistry

Upon dissection, pancreata were fixed in 4% paraformaldehyde solution overnight at 4ºC. They were then washed with PBS for an hour and cryoprotected with 30% sucrose for 24-72 hours (until the tissue sank, depending on the size of the tissue). Tissues were then embedded with OCT and sectioned for immunostaining or stored at -80ºC. For morphometric analysis of islets, 10 µm-thick sections were taken every 200 µm to ensure representation of the entire tissue in our dataset. To analyse sympathetic innervation and blood vessels in NGF knockout mice and *NGF$^{LacZ}$* mice, 30 µm-thick or 50 µm-thick sections were stained and imaged. Slides were stored at -20ºC.

For immunostaining, slides were washed in PBS (10 µm) or 0.3% Triton X-100 solution in PBS (30 µm, 50 µm). Slides were then permeabilized with 1% Triton X-100 solution and incubated with blocking solution for an hour. The blocking buffer for thin sections (10 µm) was composed of 0.1% Triton X-100, 5% goat serum and 3% bovine serum albumin in PBS. For thick sections (30 µm, 50 µm), blocking buffer was composed of 0.3% Triton X-100, 10% goat serum and 3% bovine serum albumin in PBS. After blocking, slides were incubated with primary antibodies overnight at 4ºC. They were

washed and incubated with secondary antibodies for an hour on the following day and then mounted with DAPI.

**Image analysis**

Image analysis was performed using the ImageJ software. Co-localization of markers was assessed by merging pseudocoloured images. Parameters such as islet area, islet circularity and area covered by the TH channel were quantified in NGF knockout animals. Islet circularity was measured using the ImageJ software's shape descriptors tool, which calculates it using the equation: circularity index=4π(area/perimeter2) with '1' and '0' denoting a perfect circle and an increasingly elongated polygon, respectively. An islet was considered to have increased core localization of alpha cells if it had at least three alpha cells more than two cell layers deep in the islet. Islet innervation was quantified by calculating the percentage of islet area covered by TH fluorescence, using the Area fraction feature in ImageJ.

# CHAPTER 3: RESULTS

**Establishing genotyping protocols**

I spent the first few weeks of my thesis project optimizing the genotyping protocols for different mouse lines in order to set up crosses between them to obtain the desired genotypes for analyses. New genotyping protocols were established for the following lines:

*PDGFRβ-CreER$^{T2}$* mice:

The *PDGFRβ-CreER$^{T2}$* line was used for temporal deletion of genes of interest in vascular mural cells using tamoxifen induction. Genotyping for the wildtype PDGFRβ allele and the modified locus with a creER$^{T2}$ insert (referred to here as the mutant allele) was performed using a protocol previously used in the lab. PCR results from DNA samples often showed neither a wild-type nor a mutant gene band – a result that is logically inconsistent (Fig 1A). Therefore, I systematically tested and optimized various parts of the genotyping protocol to obtain reliable results.

First, I tested the DNA samples using the NanoDrop apparatus to ensure DNA quality and optimal DNA concentrations. I used the 260/280 ratio, which is the ratio of the absorbance at 260 nm and 280 nm, to measure the purity of the DNA. Samples with 260/280 ratios less than 1.8 were considered to be of poor quality. Since a few samples showed poor quality, the DNA extraction protocol was slightly modified to improve the quality of DNA. The purified DNA pellet was washed with cold 70% ethanol and vortex mixing was used to resuspend the pellet instead of pipetting. This improved the quality of the DNA samples; however, genotyping results still remained variable.

Then, I ruled out other possible causes such as ineffective reagents and primers by using different aliquots. Upon analysing the PCR protocol, I found that the annealing temperature in the original PCR protocol was too high. To solve this, I implemented a touchdown PCR protocol that cycles through a range of annealing temperatures (65ºC to 60ºC, with a 0.5ºC decrease in every cycle) to optimize primer annealing, which led to a dramatic increase in the clarity and consistency of results (Fig 1B).

*R26R-EYFP* mice:

The *R26R-EYFP* mouse line was used in conjunction with the *PDGFRβ-CreER*$^{T2}$ line to report Cre-induced recombination. The EYFP protein is expressed only in the cells that express the Cre recombinase, since the floxed STOP sequence that blocks EYFP expression is removed by the Cre enzyme. I optimized the genotyping protocol for this line as well since the original protocol showed inconsistent PCR results. After multiple trials with wild-type DNA controls, I found that the original protocol occasionally resulted in the wild-type DNA showing a band which could not be clearly distinguished from the EYFP allele (Fig 1C), which had confounded previous experiments and led to false positive results. The original protocol used the Taq DNA Polymerase and Standard Taq buffer from New England Biolabs as reagents for the PCR reaction. I switched to using the GoTaq Green Master Mix in the new genotyping protocol, which resulted in consistent results with no false positive bands shown by the wild-type DNA samples. Therefore, this protocol was used henceforth to genotype the R26R-EYFP line.

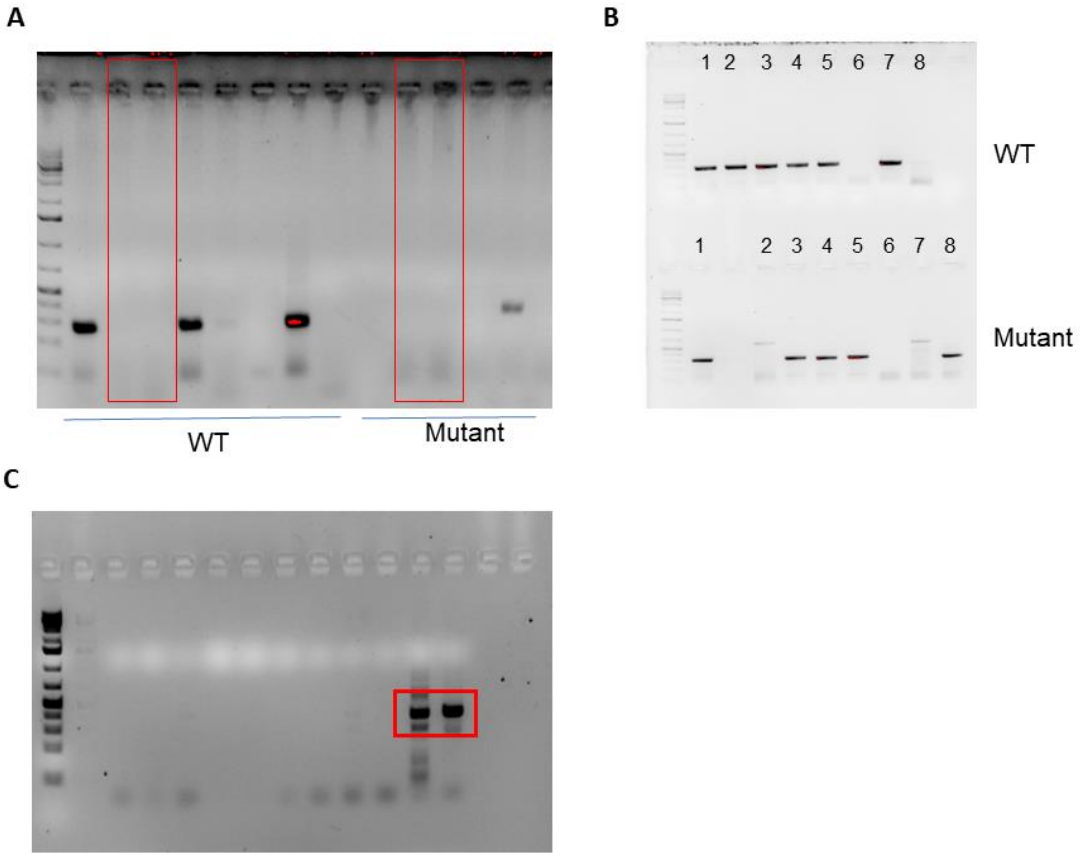

**Figure 1. Optimizing PCR-based genotyping protocols for *PDGFRβ-CreER$^{T2}$* and *R26R-EYFP* mice**

(A) The previous protocol in use for *PDGFRβ-CreER$^{T2}$* showed inconsistent results, with some samples showing a band neither for the WT allele nor the mutant allele (highlighted with red boxes)

(B) New protocol after troubleshooting shows clear, consistent results with homozygous animals showing either a band for the WT allele or a band for the mutant allele, and heterozygous animals showing both bands. 1-5: DNA samples from experimental animals, 6: No DNA control, 7: WT DNA control, 8: Mutant DNA control

(C) Previously used genotyping protocol for *R26R-EYFP* gave false positive results, with WT DNA samples occasionally showing a band very close to the mutant band instead of no band.

**Characterization of NGF expression in the postnatal pancreas**

Since it is yet unknown which cells produce NGF in the neonatal pancreas, I first sought to characterize its expression. Antibodies for NGF that were previously tested in the lab did not show clear labelling in immunofluorescence assays; hence, I chose to use the reporter mouse line *NGF$^{LacZ}$* which has a LacZ gene knocked into the endogenous NGF locus and thus expresses the enzyme beta-galactosidase instead of NGF (Liu et al., 2012). I originally planned on examining expression at postnatal stages P0, P7, P14 and P21 to understand the pattern of NGF expression across the postnatal maturation period. However, due to issues with breeding of the line and ensuing time constraints, I could only characterize the P7 timepoint. Immunostaining for beta-galactosidase and markers for islet cell types was used to localize NGF expression.

At P7, the vasculature is the only source of NGF in the pancreas (Fig 2A, B). Surprisingly, islet endocrine cells, particularly beta cells, do not show any expression at this stage. Since the mouse pancreas can express endogenous mammalian beta-galactosidase, staining was done on WT samples as a control to verify the antibody's

accuracy. WT controls do not show any beta-galactosidase staining (Fig 2C, D), confirming that the antibody is specific to the protein expressed by the reporter allele. To further examine which vascular cells are involved in NGF synthesis, immunostaining was done for endothelial cells using the marker, Platelet Endothelial Cell Adhesion Molecule (PECAM1) and vascular mural cells using the marker, nerve glial antigen 2 (NG2). NG2 marks the mural cells present on capillaries (pericytes) as well as those on the larger blood vessels (vascular smooth muscle cells). Beta-galactosidase clearly co-localizes with NG2 and not with PECAM (Fig 2E-I), which shows that mural cells are the predominant source of NGF in the pancreatic vasculature. Smooth muscle actin (SMA) was used as a secondary marker for vascular smooth muscle cells and similarly co-localized with beta-galactosidase (Fig 2J).

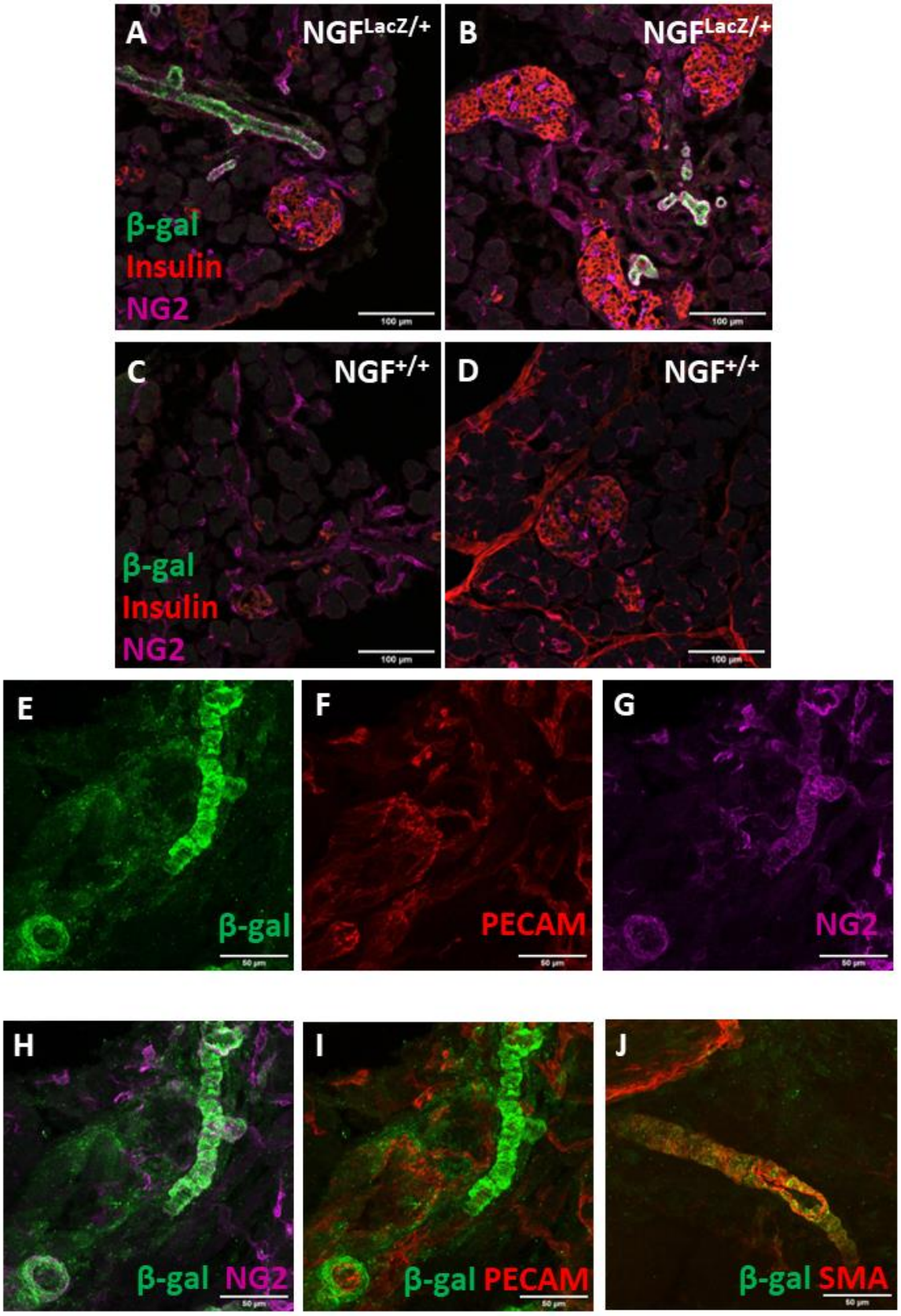
A
NGF LacZ/+
β-gal
Insulin
NG2
100 μm
B
NGF LacZ/+
100 μm
C
NGF +/+
β-gal
Insulin
NG2
100 μm
D
NGF +/+
100 μm
E
β-gal
50 μm
F
PECAM
50 μm
G
NG2
50 μm
H
β-gal NG2
50 μm
I
β-gal PECAM
50 μm
J
β-gal SMA
50 μm

**Figure 2. NGF is expressed in vascular mural cells in the mouse pancreas at P7**

(A,B) The vasculature marked with NG2 shows beta-galactosidase expression in *NGF*$^{LacZ/+}$ mice. Islets do not show any beta-galactosidase expression. Scale bar denotes 100 µm.

(C,D) *NGF*$^{+/+}$ mice do not show any staining for beta-galactosidase, which confirms that the antibody used is specific to the protein expressed by the reporter gene construct. Scale bar denotes 100 µm.

(E-I) Vasculature was stained with PECAM and NG2 along with beta-galactosidase in *NGF*$^{LacZ/+}$ mice to check which vascular cells synthesize NGF. Beta-galactosidase clearly co-localizes with NG2 and not with PECAM, showing that vascular mural cells make NGF, not endothelial cells. Scale bar denotes 50 µm.

(J) SMA staining was used to visualize NGF expression by vSMCs on the macrovasculature, to further validate the results from NG2 staining. SMA co-localizes with beta-galactosidase, confirming the results from NG2 staining. Scale bar denotes 50 µm.

**Developing tamoxifen administration paradigms for inducible genetic labelling or gene deletion**

Since the *PDGFRβ-CreER*$^{T2}$ mouse line is a tamoxifen-inducible line, a tamoxifen administration paradigm had to be established for the postnatal deletion of NGF. To visualize recombination, I used *R26R-EYFP* mice which contains an Enhanced Yellow Fluorescent Protein (EYFP) sequence under the ubiquitous Rosa26 promoter. Expression of EYFP is blocked by a LoxP-flanked STOP sequence upstream of the gene. When crossed with *PDGFRβ-CreER*$^{T2}$ mice, EYFP expression is induced in vascular mural cells upon tamoxifen administration. Thus, the *PDGFRβ-CreER*$^{T2}$*; R26R-EYFP* mice can be used to verify whether the tamoxifen paradigm works and can also be used to validate whether Cre expression is specific to the cells of interest.

To begin with, I tested a tamoxifen delivery protocol developed by Alexis Ceasrine, a previous graduate student in the lab, which involved injecting 20 µl of 10 mg/ml tamoxifen solution subcutaneously into neonatal mice at postnatal days P0 and P1. However, after day 1 of injections, the entire litter was found dead. Due to such a high rate of mortality, I had to develop and test new protocols.

In the second protocol I tested, 20 µl of 5 mg/ml tamoxifen solution, which is half of the previous dose, was administered subcutaneously at P0 and P1. 6 out of 8 pups survived the first injection; 2 out of 6 survived the second. None of the surviving pups were positive for both Cre and EYFP and hence could not be used for further analysis. Since mortality was high in very young pups, I tested the same dosage in older animals by injecting it subcutaneously from P7 to P10. All of the pups survived this round of injections. Pancreata were dissected for analysis at P15. Immunostaining was done for YFP and NG2 and the extent of colocalization was assessed. Partial recombination was observed in all *PDGFRβ-CreER$^{T2}$; R26R-EYFP* animals, with some but not all NG2+ cells expressing YFP (Fig 3A). However, this method was abandoned due to the logistical problem of accurately injecting such a small volume of the tamoxifen solution with the syringes usually used for animal injections.

Then, I modified and adapted a protocol developed for targeting endothelial cells in the neonatal vasculature (Pitulescu et al., 2010) and tested two versions of it, described below. To induce recombination soon after birth, 50 µl of 1 mg/ml solution was administered from P1 to P3 via intragastric injection. Pancreata were dissected for analysis at P10 and stained for NG2 and YFP. Robust reporter expression was observed, with most NG2+ cells showing YFP expression (Fig 3B). To check whether there is leaky Cre recombinase expression in the *PDGFRβ-CreER$^{T2}$* animals, the vehicle (corn oil) was injected using the same paradigm in *PDGFRβ-CreER$^{T2}$;R26R-EYFP* animals. Vehicle-injected animals showed no YFP expression (Fig 3C), thus confirming that the *PDGFRβ-CreER$^{T2}$* expresses Cre recombinase only upon tamoxifen induction.

A similar regimen was tested in the older pups by injecting 50 µl of 2 mg/ml solution intraperitoneally from P7 to P10 and dissecting pancreata at P15. No mortality was observed and the extent of recombination was variable across animals, with one animal showing more recombination than the other (Fig 3D,E). These two paradigms,

termed “early” and “late” respectively, were used henceforth with the *PDGFRβ-CreER$^{T2}$; NGF$^{f/f}$* animals to delete NGF from vascular mural cells (Fig 4). All tested protocols and their results have been summarized as Table 1.

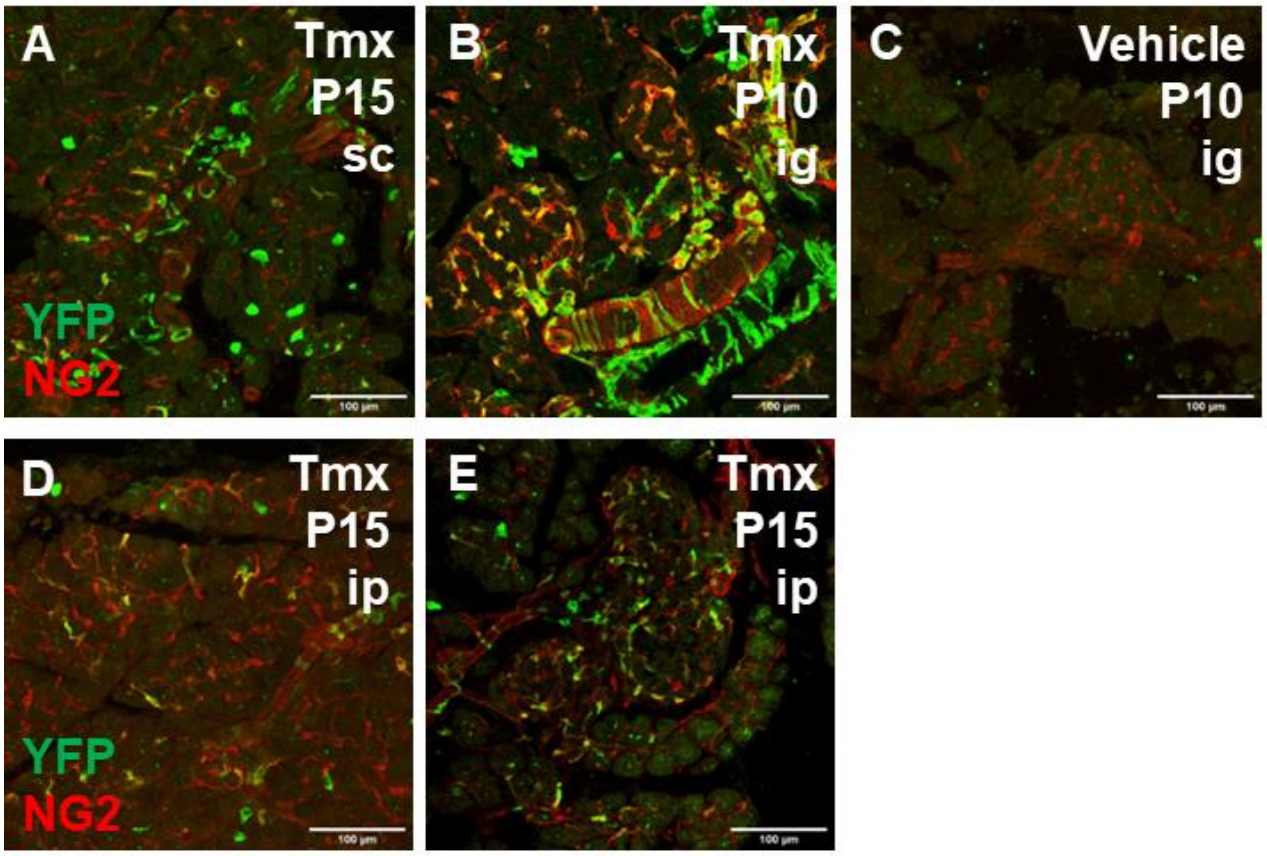


**Figure 3. Establishment of tamoxifen administration regimens using *PDGFRβ-CreER$^{T2}$;R26R-EYFP* mice**

All scale bars denote 100 µm. EYFP expression is shown in green and NG2 expression is shown in red. The terms “sc”, “ig” and “ip” refer to the subcutaneous, intragastric and intraperitoneal modes of tamoxifen administration, respectively.

(A) 100 µg of tamoxifen was injected subcutaneously from P7 to P10 and pancreata were dissected at P15. Partial recombination was observed, with only some NG2+ cells expressing YFP (N=2).

(B) 50 µg of tamoxifen was injected subcutaneously from P1 to P3 and the sample was dissected at P10. Recombination was successful to a large extent – most NG2+ cells expressed YFP (N=1).

(C) Vehicle-injected control for the same injection paradigm did not show any expression of YFP (N=1).

(D,E) 100 µg of tamoxifen was injected intraperitoneally from P7 to P10 and pancreata were dissected at P15 (N=2). Recombination occurred to a variable degree in the animals, with one animal showing a moderate level of recombination (D) and the other showing a considerable extent of recombination (E).

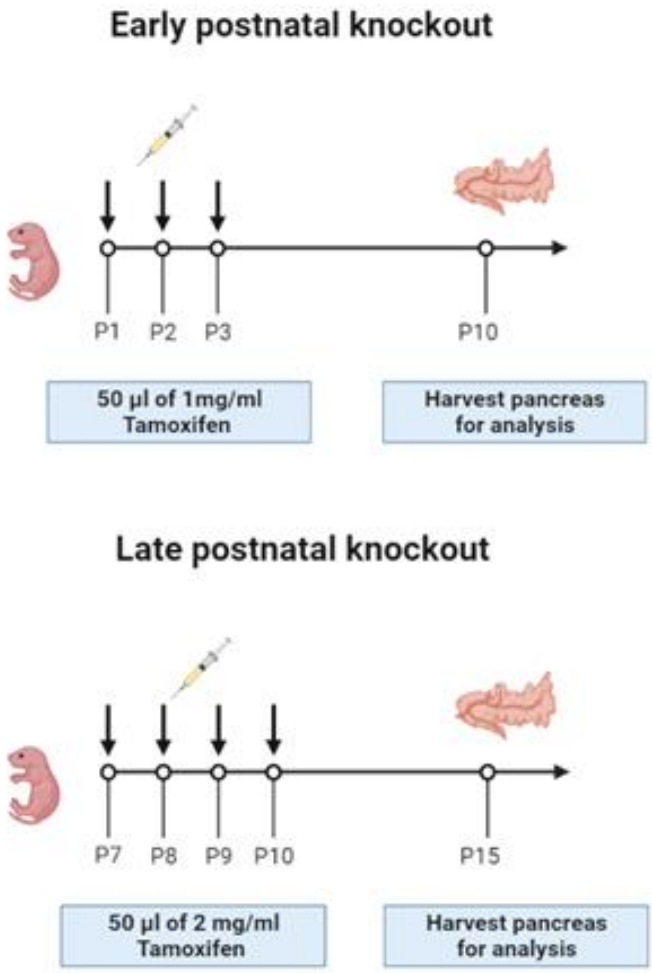


**Figure 4. Tamoxifen administration regimens that were used for NGF knockouts**

| | Volume injected | Total amount injected | Timepoints of administration | Mode of administration | Result |
|---|---|---|---|---|---|
| 1 | 20 µl of 10 mg/ml solution | 200 µg | P0 and P1 | Subcutaneous | The whole litter (7 pups) died |
| 2 | 20 µl of 5mg/ml solution | 100 µg | P0 and P1 | Subcutaneous | 6/8 pups survived 1st injection; 2/6 pups survived 2nd injection |
| 3 | 20 µl of 5 mg/ml solution | 100 µg | P7-P10 | Subcutaneous | All pups survived - partial recombination observed |
| 4 | 50 µl of 1 mg/ml solution | 50 µg | P1-P3 | Intragastric | Most pups survive – a large extent of recombination observed |
| 5 | 50 µl of 2 mg/ml solution | 100 µg | P7-P10 | Intraperitoneal | All pups survived – variable degree of recombination observed |

**Table 1. Summary of tamoxifen administration regimens that were developed and tested**

**Analysis of early NGF knockouts**

To examine the effects of mural cell-derived NGF in early postnatal pancreatic development, the early knockout paradigm of tamoxifen administration was used with *PDGFRβ-CreER$^{T2}$; NGF$^{f/f}$* mice to remove NGF from pericytes and vSMCs (Fig 4). Tamoxifen-injected *NGF$^{f/f}$* mice were used as controls to rule out effects caused by

administration of tamoxifen alone. Pancreata from these animals were dissected at P10. Islet cytoarchitecture was assessed by immunostaining 10 µm-thick sections with glucagon, insulin and somatostatin to mark alpha cells, beta cells and delta cells respectively. Thicker sections (50 µm-thick) were immunostained with tyrosine hydroxylase (TH) and insulin to visualize islet innervation. TH is a rate-limiting enzyme involved in the synthesis of catecholamines dopamine, epinephrine and norepinephrine, and thus it acts as a marker of sympathetic neurons. Area covered by insulin was used as a measure for islet area.

Surprisingly, islets from the mutant animals did not show a decrease in sympathetic innervation compared to control islets. Islet innervation was quantified as the percentage of the islet area covered by the TH-positive sympathetic axons (Fig 5E). Sympathetic axons from mutant islets show brighter TH fluorescence as well (Fig 5A, B). Interestingly, knockout islets tend to have a higher amount of TH-expressing endocrine cells than control islets (Fig 5C, D).

By P10, control islets have largely acquired their canonical globular shape with alpha cells at the periphery and beta cells at the core. Since islet cells are still migrating and multiplying at this stage, not all islets show this stereotypical shape. Nevertheless, a large majority of control islets from our analysis conformed to this stereotypical architecture (Fig 6A). NGF knockout islets tend to have a more disorganized phenotype, with less circular and more elongated islets (Fig 6B). This phenotype was quantified using a circularity index, where 1 indicates a perfect circle and 0 indicates an elongated polygon (Fig 6C). Knockout animals also tend to have a larger proportion of islets where alpha cells are mislocalized to the core of the islet (Fig 6D). In general, islets from knockout animals tend to be smaller (Fig 6E-G); however, in certain cases, extremely large and misshapen islets are seen in the knockouts. (Fig 6H, I).

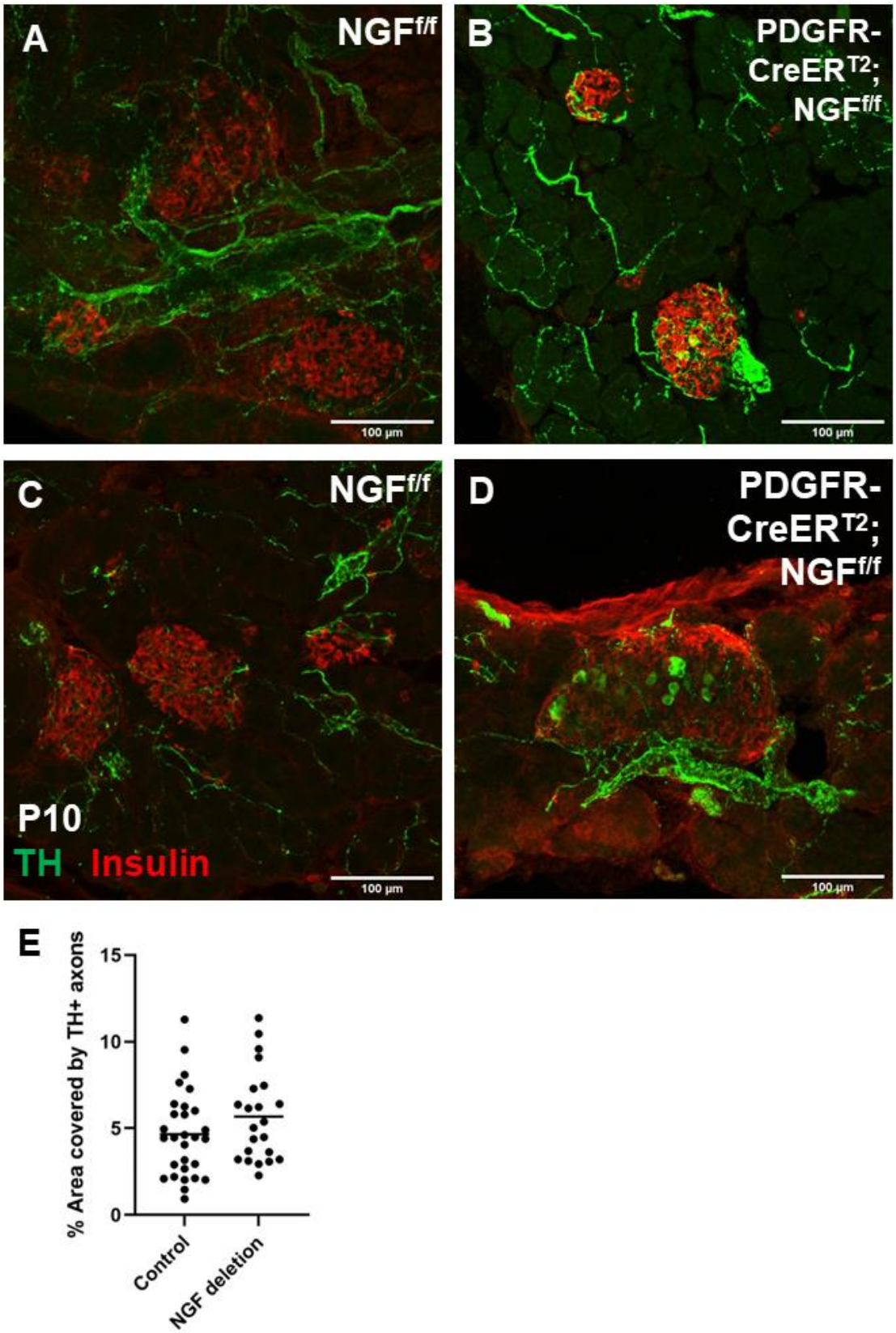


**Figure 5. TH staining of early NGF knockout islets reveals differences in TH intensity and TH+ endocrine cells**

All scale bars denote 100 μm.

(A, B) NGF knockout islets show brighter TH immunofluorescence than control islets (N=1 for each)

(C, D) NGF knockout islets have a greater number of TH+ endocrine cells (N=1 for each)

(E) The extent of sympathetic innervation was measured by calculating the percentage of islet area that is covered by TH fluorescence. Control and NGF knockout islets have comparable innervation.

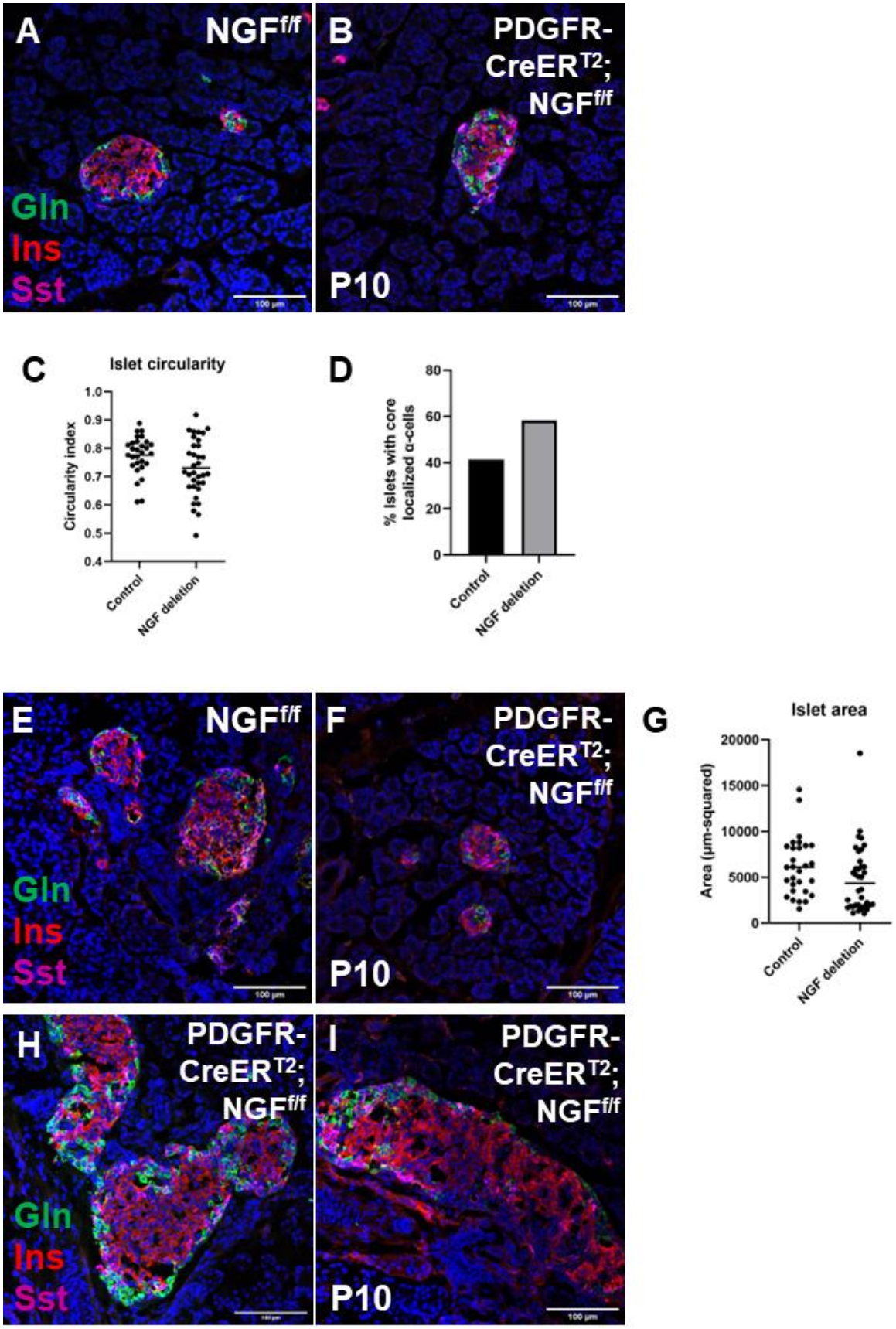


**Figure 6. Early postnatal NGF knockout causes disruptions in islet architecture.**

All scale bars denote 100 µm. Alpha cells are marked with glucagon (green), beta cells are marked with insulin (red), and delta cells are marked with somatostatin (magenta).

(A, B) Control islets have stereotypical circular shape and peripheral alpha cell localization. Knockout islets are more elongated and have more core-localized alpha cells. N=1 for control, N=2 for knockout.

(C) Mutant islets are less circular than control islets. Islet shape is quantified by circularity index where “1” denotes a perfect circle, and “0” denotes an elongated polygon. N=1 for each quantification, at least 30 islets per animal.

(D) Mutant animals have a greater proportion of islets with core-localized alpha cells. N=1 for each quantification, at least 20 islets per animal.

(E, F, G) Islets from knockout animals are smaller in general. N=1 for each quantification, at least 30 islets per animal.

(H, I) A few islets in knockout animals are abnormally large and misshapen. N=1 for control, N=2 for knockout.

**Analysis of late NGF knockouts**

The late knockout paradigm of tamoxifen administration was used to remove NGF from mural cells in *PDGFRβ-CreER$^{T2}$; NGF$^{f/f}$* animals. Tamoxifen-injected *NGF$^{f/f}$* mice were used as controls. Pancreata were dissected at P15 and sliced into 30 µm-thick sections, which were stained with TH and insulin to assess islet innervation. Due to time constraints and difficulties with immunostaining, islet cytoarchitecture could not be assessed for this timepoint.

Animals with NGF deletion appeared to have similar innervation within islets as well as outside islets as the controls (Fig 7A-D). Unlike the early knockout animals, they do not show any difference in TH fluorescence intensity either. However, there was variable antibody penetration in different sections of the same tissue section; therefore, immunostaining needs to be repeated on these to enable accurate quantification and analysis of the extent of innervation. No striking differences were seen in the population of TH+ endocrine cells either.

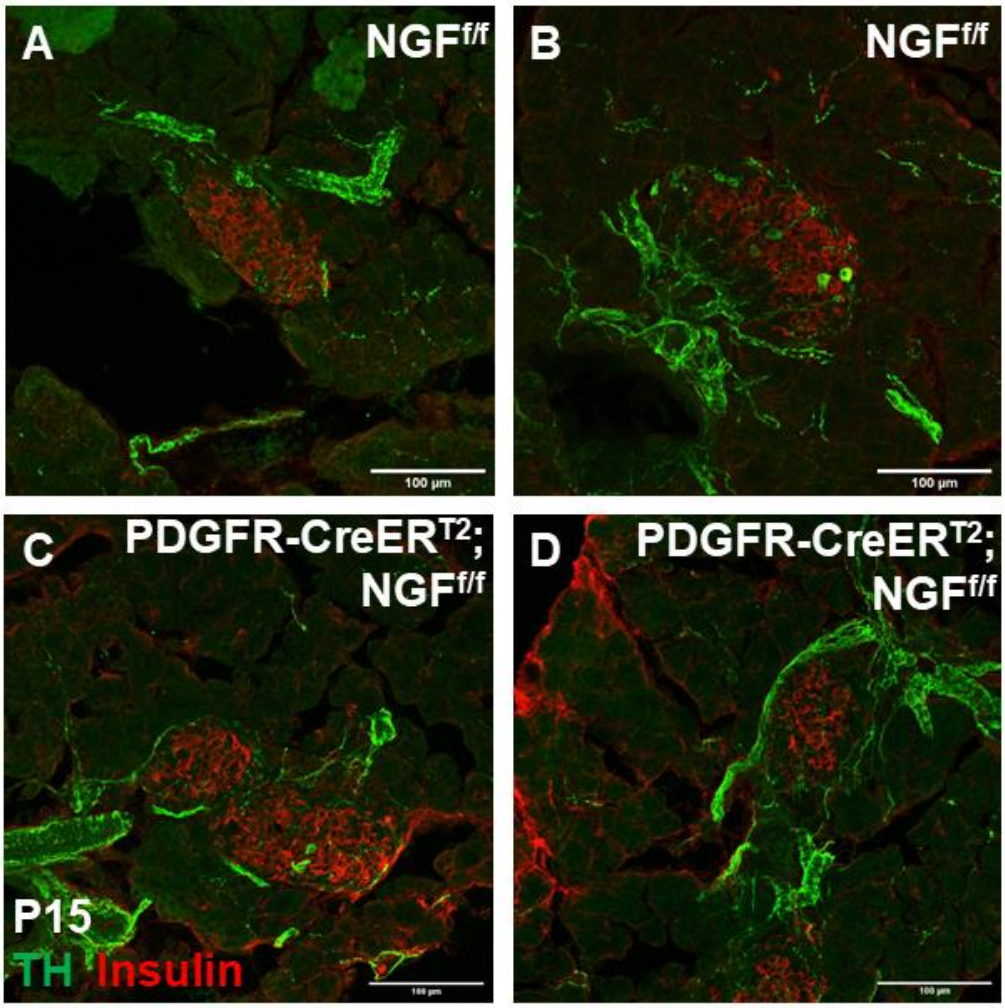


**Figure 7. Late postnatal NGF deletion does not cause changes in sympathetic innervation.**

(A, B) are tamoxifen-injected control islets (N=1) while (C, D) are islets from NGF knockout animals (N=2). Sympathetic axons are marked with TH (green) and islets are marked with insulin (red). TH staining looks largely identical across both sets. All scale bars denote 100 µm.

# CHAPTER 4: DISCUSSION

**Characterization of NGF expression in the postnatal pancreas**

Based on the analyses from the reporter mouse line *NGF$^{LacZ}$*, I found that at P7, vascular mural cells are the predominant source of NGF in the pancreas. This is a particularly surprising and interesting finding since the field has long believed that beta cells are the key producers of NGF in the developing pancreas. P7 is also an important stage to characterize NGF expression since sympathetic innervation to the pancreas peaks at P6 and gets refined over the later postnatal period until P21. Final innervation of target tissues and refinement of the pattern of innervation is critically dependent on NGF; NGF acts as a guidance cue to attract the distal axons and is important for survival of innervating neurons, while competition for limiting amounts of NGF drives axonal pruning and the establishment of the final pattern of innervation (Deppmann et al., 2008; Glebova and Ginty, 2005; Shelton and Reichardt, 1984). This makes P7 a particularly relevant time period to study the sources of NGF.

Vascular mural cells are known to express NGF *in vitro* (Creedon and Tuttle, 1991) and in adult animals, in situations of physiological stress such as brain hypoxia and soft tissue trauma (Ishitsuka et al., 2012; Lee et al., 2021). However, the role of mural cell-derived NGF during late stages of sympathetic development has not yet been investigated. The only known role of mural cell-derived NGF in the pancreas is its promotion of glucose-stimulated insulin secretion by boosting exocytosis of insulin granules in beta cells (Houtz et al., 2016). Given that islets do not show their acute response to glucose stimulation until their functional maturation after P10, it is particularly striking that mural cells are a major source of NGF before the stereotypical GSIS response begins. It suggests that mural cell-derived NGF might be developmentally important, apart from its physiological role in stimulating insulin secretion. This phenomenon could be driven by NGF's role in establishing innervation of the tissue. Interestingly, sympathetic association with pancreatic vasculature becomes more well-defined around P7, which could explain why NGF is strongly expressed in these cells at this particular time (Burris and Hebrok, 2007). Alternatively, it is possible that early NGF secretion by mural cells shapes the functional maturation of islets directly, independent of its role in islet innervation. Further analysis needs to be done at different timepoints to see how the dynamics of NGF expression change over development.

It is important to note that NGF expression was studied using a genetic reporter line and not by directly assessing endogenous NGF expression. This approach has its limitations – it depends on the ability of the reporter allele to accurately reflect endogenous expression. Low levels of promoter activity could lead to levels of beta-galactosidase that are too low to be detected. Since LacZ expression was assessed using immunohistochemistry, this approach also depends on the antibody's sensitivity to beta-galactosidase. Thus, it is possible that our results do not accurately reflect true NGF expression by underestimating the amount produced by some cell types.

**Optimization of tamoxifen regimens – rationale and implications for methodology**

Although NGF largely affects the later stages of sympathetic innervation, it is also an essential survival factor for sympathetic neurons in early development. As such, early deletion of NGF can lead to neuronal loss and make it difficult to examine its role specifically in the final stages of innervation. Additionally, since sympathetic nerves use the vasculature as a scaffold to track along and innervate nerves, early deletion of NGF from vascular cells might impede distal axon extension at a wide scale, making it difficult to assess its role specifically in the pancreas. Therefore, I chose to use a tamoxifen-inducible CreER$^{T2}$ system to remove NGF from vascular cells specifically during the postnatal period. This specific window of time was chosen since sympathetic innervation of the pancreas is very plastic across this period – it increases up to P6 and is gradually refined over adolescence until P21, which makes this period the ideal time to study NGF's effects. P10 was chosen as a time point to assess the effects of early postnatal deletion since islets begin to mature and acquire their stereotypical globular shape at this time, which makes analysing islet architecture easier. P15 was chosen to assess the later knockouts as this is when sympathetic innervation of islets begins to resemble the pattern seen in adult islets (Burris and Hebrok, 2007).

Tamoxifen induction is notoriously difficult in neonatal mice since they are particularly susceptible to death by overdose. Merely scaling down dosing by weight is ineffective and still leads to a large extent of lethality. This has proven to be a hurdle in doing inducible genetic knockouts in early postnatal development. Therefore, I

developed new protocols for inducing recombination at different time periods of postnatal development by refining pre-existing protocols to suit our specific developmental analyses. The established protocols lead to a reasonable degree of recombination; however, the extent of recombination seems to vary across animals injected at the same time with the same solution. This is an important caveat to keep in mind when considering the results from the NGF knockouts. Additionally, we used the reporter line *R26R-EYFP* to evaluate recombination instead of directly checking for NGF expression since immunostaining for NGF often yields unreliable results. However, different floxed genes may not recombine to the same extent with the same Cre construct, which is another important caveat to consider while assessing the data from the knockouts.

**Effects of early postnatal NGF knockout from mural cells resemble those caused by sympathetic denervation**

Cytoarchitectural analyses of islet architecture showed interesting changes in the early postnatal NGF knockout mice. Islet area is generally lower in the knockouts, which is in line with the known role of NGF in providing trophic support to beta cells. However, certain islets in the knockout animals are abnormally large and elongated. Alpha cell localization to the core, a phenomenon observed in denervated animals, is also increased in these knockouts. Additionally, mutant islets are less circular than controls, like those in denervated animals, although it is yet unclear whether this difference is significant. In general, islet architecture is affected in a manner similar to the disruption caused by denervation. However, the effects are comparatively less striking, which could either be a reflection of incomplete recombination due to variability in tamoxifen induction or an indication of other compensatory sources of NGF in the tissue.

Postnatal expansion and fission of islets is a known mechanism for the increase in islet numbers and endocrine cell numbers required after birth. Previous work has showed that NCAM, a cell adhesion molecule, is downregulated in denervated islets (Borden, 2013). NCAM is also known to regulate integrin-dependent cell migration (Schmid and Maness, 2008). This could possibly explain the extremely large islets in mutants – perhaps, endocrine cells proliferate but fail to migrate out to form new islets

due to changes in NCAM expression, leading to large, misshapen islets. It would be interesting to look at expression levels of adhesion molecules such as NCAM which show reduced expression in islets of denervated animals.

Surprisingly, sympathetic innervation of islets from knockout animals was not decreased compared to controls. This is an unexpected result since NGF is known to be required for the establishment and maintenance of innervation, especially in late embryonic and postnatal stages. It is possible that our induction protocol did not sufficiently delete NGF and the change we observed is not statistically significant. These experiments need to be replicated in more animals to verify the consistency of the results. Quantitative PCR or *in situ* hybridization can be used to assess the extent of NGF deletion. Alternatively, mural cell-derived NGF could be dispensable in the pancreas in the postnatal period. Innervation of certain tissues has been shown to be refractory to loss of NGF, and while NGF is needed for pancreatic innervation during later embryonic stages, it is possible that vascular NGF is not required to maintain innervation after birth (Glebova, 2004). It is also possible that other sources of NGF compensate for the decrease in NGF synthesized by mural cells. Similarly, other ligands might signal through TrkA to compensate for the loss of NGF. For example, Neurotrophin-3 (NT-3) has been shown to bind to TrkA and signal through it during development (Davies et al., 1995). This could explain why *TH-Cre; TrkA$^{f/f}$* mice show a drastic loss of innervation but NGF deletion does not lead to similar changes.

TH fluorescence intensity is higher in the sympathetic axons of knockout animals. The implications of this are unclear – it could either mean that TH synthesis is upregulated or that axons form thicker nerve bundles. TH upregulation by existing neurons could be a compensatory response to accommodate for a loss of nerve fibres and the neurotransmitters derived from them (Acheson et al., 1980). On the other hand, formation of thicker bundles of axons could indicate a defect in the ability of individual axons to branch out and form a finer synaptic network. Indeed, it is also possible that our imaging technique is not sensitive enough to visualize the finer axonal network, which could result in underestimation of the extent of islet innervation in control animals.

Knockout islets show an increase in TH+ endocrine cells, similar to islets from *TH-Cre; TrkA$^{f/f}$* denervated animals. This is particularly interesting since TH has been

suggested as a marker of stress or senescence in beta cells; upregulation of TH in beta cells has been observed in obesity and pregnancy which are situations of metabolic stress and are known to attenuate beta cell function (Teitelman et al., 1988). Thus, this could indicate that islets in knockout animals are under metabolic stress.

**Future Directions**

The work presented in this thesis has laid the groundwork for a study on the relationship between NGF and pancreatic development and innervation. Future experiments could focus on the effects of embryonic deletion of NGF from vascular mural cells. Preliminary results from crosses of *Myh11-Cre; NGF$^{f/f}$* mice show that these animals can survive upto P10, which makes the mice a promising tool to study the effects of early knockout of NGF from mural cells. It could overcome the variability caused by the use of tamoxifen to induce recombination and result in more widespread NGF deletion, which might lead to a stronger phenotype than the one we observed. Assessing sympathetic innervation in older *PDGFRβ-CreER$^{T2}$; NGF$^{f/f}$* animals could show the effects of loss of mural cell-derived NGF over long periods of time. Additionally, using whole-mount imaging to image the entire tissue, instead of immunostaining of tissue sections, would provide a more complete view of the pancreas and make our analyses unbiased. It would also be interesting to assess islet architecture and innervation of *Pdx1-Cre; NGF$^{f/f}$* mice to see if early deletion of NGF from pancreatic progenitors would have any effect on islet development and innervation. Previous work done in the lab showed that the effects of denervation can largely be attributed to the loss of beta-adrenergic signalling. Therefore, it would be very interesting to see if administration of agonists or antagonists of beta-adrenergic receptors would rescue the effects caused by NGF loss. This could help elucidate whether NGF has a role on islet development independent of its role in islet innervation.

Characterizing NGF expression at different embryonic and postnatal stages and comparing it to the extent of sympathetic innervation at those timepoints could provide insight into how NGF expression affects innervation. Since the vasculature is also directly innervated by sympathetic nerves, it would be interesting to see if there is any change in the amount of innervation vSMCs and pericytes receive. Not much is

known about the nature of the synaptic contacts sympathetic axons form with their targets in the pancreas. Ultrastructural analysis of these synapses would be valuable in understanding the nature of the contacts formed between sympathetic nerves and islet cells.

Mural cell-derived NGF is needed by mature islets to acutely respond to a glucose stimulus with insulin secretion. This complicates the ability to perform functional assays in knockout animals since an inability to respond to the stimulus cannot be solely attributed to a developmental defect. However, using islets isolated from these animals and supplementing the cultures with added NGF could possibly account for the ongoing need for NGF in insulin secretion. If islets from knockout animals fail to respond appropriately even when stimulated with NGF *in vitro*, it would suggest that islets from knockout animals are functionally immature which would lead to defective metabolism in adult animals. Additionally, transcriptomic analyses of islets could be performed, which may reveal changes in molecular profiles of endocrine cells following loss of NGF. Changes in levels of known molecular markers of mature islets could indicate whether NGF deletion has an effect on the functional maturation of islets.

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