Paracrine Signaling in the Pancreatic Islet: Mechanisms for Macrophage Maintenance of Tissue Homeostasis

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PARACRINE SIGNALING IN THE PANCREATIC ISLET: MECHANISMS FOR MACROPHAGE MAINTENANCE OF TISSUE HOMEOSTASIS

By

Jonathan Weitz

A DISSERTATION

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PARACRINE SIGNALING IN THE PANCREATIC ISLET: MECHANISMS FOR MACROPHAGE MAINTENANCE OF TISSUE HOMEOSTASIS

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Dysfunction or death of the islet’s endocrine cells leads to diabetes, a devastating disease affecting millions worldwide. Islet hormones secreted from endocrine cells are responsible for maintenance of blood glucose levels. However, hormone secretion from beta cells also involves the co-release of pro-inflammatory molecules such as ATP and serotonin. Thus, as a by-product of hormone secretion, islet endocrine cells may activate and interact with the local population of immune cells. The immune system mediates inflammatory responses, but in many tissues, immune cells also provide a homeostatic function. Resident macrophages in the liver (Kupffer Cells), and brain (microglia), for instance, fulfill local roles in immune suppression and tissue repair. These findings suggest a sensitive crosstalk between the local microenvironment and their unique tissue macrophages. These signaling networks have yet to be determined in the pancreatic islet. This thesis project explores these signaling networks. Our data indicate that islet resident macrophages sense locally released ATP by beta cells during high glucose stimulation. We show that the activation of resident macrophages by beta cells through paracrine ATP signaling can be blocked directly by purinergic antagonists. In response to ATP islet macrophages have altered expression profile of matrix remodeling genes. Therefore, islet macrophages act as a local sensor of beta cell activity, and adjust their function accordingly.
This work is dedicated to:

To my parents, who kill bugs big and small.
ACKNOWLEDGEMENTS

I will first like to thank my mom, who is the person who inspired me most to pursue a career in science. In every step of my life she was there to develop my interests as a researcher. As a child, she would always come into my elementary school during science class and do basic experiments to get the kids excited about science. From miniature volcanos to sugary, crystal rock formations she always made science fun. As a high school student, all of the homework and tutoring sessions with other students. Finally, as a college student she introduced me to what it was like to be in a lab and encouraged me to join a research lab at the University of California Riverside. Some college students wander aimlessly and are nervous to go into the real world, but I was hooked on doing research for the rest of my life.

My dad is the kindest human being that I know. All he wants to do is be your friend and talk about whatever is happening in the world. He is literally exploding with love and only can get it out in the form of giving hugs and kisses to others. Although he can be embarrassing, I always knew that I had his support in whatever I wanted to do. I believe that I have had success because, I have learned from him to approach life and work always with a good attitude.

It is important to have a balanced life to maintain happiness. The two people that contribute to this balance are my boss at home; Vivi, and in the lab, Alejo. I think Vivi is the person who understands me the best in the entire world. She knows when I am happy, she knows when I am anxious, and she knows in these situations a cold corona will always help. The beauty of our relationship is that we wake up happy every day. This has not
changed once since I have met her. I have loved getting to know her. Each day I am more and more in love with her. She continues to impress me in all aspects of life.

Alejo is the kind of boss that everyone wants to have. He is intelligent, funny, fun, relaxed and probably the most interesting person that I have met. He can compete with the Dos Equis guy from the commercials. Just look at his accomplishments: Fluent in 6 languages, an artist, is a world-renowned professor, plays sports like a teenager, and although he gets older… his girlfriends’ magically stay the same age. On a more serious note, he has shaped my career as a scientist. When I entered his lab, I was naïve to think that I did not have much more to learn to become an independent scientist. I could develop logical experiments and perform them correctly. However, what Alejo has shown me that there is art in scientific writing and publishing. Alejo is a figure making artist. Every one of his papers has a unique feel, that you can immediately say, “oh… this is a Caicedo paper”. I am fortunate to have gained that knowledge from Alejo. I am also blessed to have Alejo as a friend. He has been the other half of my balanced life. The day only has twenty-four hours. I spend eight of it sleeping, eight at work, and eight with Vivi. I come to the lab every morning, because I am genuinely happy to be here.

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LIST OF ABBREVIATIONS

3G 3 mmol/l glucose
16G 16 mmol/l glucose
[Ca^{2+}]_i Intracellular free Ca^{2+} concentration
ΔF/F Change in fluorescence over fluorescence baseline
ACH Acetylcholine
ADP Adenosine Diphosphate
Adr Adrenaline
ANOVA Analysis of variance
αSMA Alpha smooth muscle actin
ATP Adenosine Triphosphate
AU Arbitrary units
CGRP Calcitonin gene-related peptide
CHR2 Channelrhodopsin 2
CSF1R Colony stimulating factor 1 receptor
ELISA Enzyme-linked immunosorbent assay
GCaMP3 Genetically encoded calcium sensor
GFP Green fluorescent protein
GLP-1 Glucagon-like-peptide-1
GSIS Glucose stimulated insulin secretion
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
L-NAME L-nitro-arginine methyl ester
ET-1 Endothelin-1
MHC Major histocompatibility complex
NG2 Neural-glial antigen 2
NOD Non-obese diabetic
PBS Phosphate Buffer Solution
PDGF-Rβ Beta-type platelet-derived growth factor receptor
PDMS Polydimethylsiloxane
PFA Paraformaldehyde
RFO Red fluorescent protein
SD Standard deviation
SEM Standard error of mean
STZ Streptozotocin
T2D Type 2 diabetes
TH Tyrosine hydroxylase
TRITC  Tetramethylrhodamine
vAChT  Vesicular acetylcholine transferase
VNUT  Vesicular nucleotide transporter
CHAPTER 1. Introduction

1.1 The known biology of the resident macrophages of the pancreatic islet

Insulin and glucagon secretion from the pancreatic islet is crucial for regulating glucose metabolism. Dysfunction or destruction of the islet beta cell leads to type 1 and type 2 diabetes, a devastating disease that affects roughly 415 million people worldwide. Complications from diabetes lead to unregulated fluctuations in plasma glucose levels, which have potentially life-threatening effects. Therefore, a well-functioning islet is crucial for survival. Researchers have primarily focused on the beta cell in the diabetes field, because it is the only cell in the body which secretes insulin. However, hormone secretion from beta cells also involves the co-release of pro-inflammatory molecules such as ATP and serotonin (Detimary, Jonas, & Henquin, 1996; Jacques-Silva et al., 2010). Thus, as a by-product of hormone secretion, islet endocrine cells may activate and interact with the local population of immune cells that mainly comprises resident macrophages. This thesis focuses on paracrine signaling mechanisms, which contribute to maintain islet homeostasis with a special emphasis on the islet residence macrophage.

The role of the macrophages in the islet has been studied mainly in the context of immunological function, where it contributes to inflammation and disease progression. Islet macrophages express high levels of MHCs in NOD mice, and by presenting beta cell derived peptides, play a role in diabetogenic T-cell localization to the pancreatic islets (Calderon et al. 2014). In models of chronic inflammation such as high fat diet, immune cell infiltration was observed within the isles (Ehses, Ellingsgaard, Boni-Schinetzler, & Donath, 2009). The immune system mediates inflammatory responses, but in many tissues, immune cells also provide a homeostatic function. Islets in mice that lack islet
macrophages caused by mutations in the CSF-1 gene show decreased beta cell mass (Banai-Bouchareb et al., 2004). The mechanisms leading to this dysfunction were not explored, although it is now known that macrophages promote beta cell proliferation (M. Brissova et al., 2014). These findings suggest a sensitive crosstalk between beta cells and macrophages that promotes islet function during normal physiology and contributes to inflammation and islet destruction during disease. These signaling networks have yet to be determined. It is not known what stimuli resident macrophages respond to and how they contribute to islet physiology in the normal state and during pathogenesis. Without understanding the functional role of islet resident macrophages, our model of islet biology and diabetes is incomplete.

The study of islet macrophages is a relatively novel field, when compared to other tissue resident immune cells. Microglia were first described by Franz Nissl in 1880, where he was able to see that leukocytes of the brain were histologically related to macrophages. In 1920 Pio del Rio Hortega named these macrophage-like cells “microglia”. In the pancreatic islet, an antigen presenting cell (APC) was found in 1989 by Hemmo Drexhage, nearly 100 years after Franz Nissl. It is now clear that pancreatic islets contain their own unique resident macrophages. Islet resident macrophages have been recently characterized based on embryonic origin and classical immunological markers: LyzM+, F4/80+, CD206-, CD11c+ (Calderon et al., 2015; Yin et al., 2012). The islet resident macrophages originate from the definitive hematopoiesis, which develops from the fetal liver (E11.5-16.5). These cells do not undergo a high rate of turnover with blood cells. Additionally, they are long lived cells and have a low level of self-replication. Islet macrophages express a wide variety of transcripts that are typical of “activated” macrophages such as Tnfa and Il1b. In the
event of macrophage disruption by lethal radiation, donor stem cells can replace islet macrophages, with similar cluster of differentiation profiles under the steady state (Calderon et al., 2015). This evidence suggests that the local microenvironment is capable of patterning the phenotype and function of the local resident macrophages.

1.2 An experimental platform to investigate paracrine signaling in the local islet microenvironment in situ

Traditionally, islet biologists have studied macrophage behavior in isolated islets, by an enzymatic digestion process using collagenase (Lacy & Kostianovsky, 1967). This technique breaks all networks between the islet and acinar tissue. We propose a new experimental platform in which the islet microenvironment is preserved in a semi-intact preparation. Using a model of living pancreatic tissue slices as described by (Marciniak et al., 2014), we are able to preserve all of the cell-to-cell interactions in the pancreatic islet and islet-acinar network. We have adapted this model using living slices to investigate local tissue resident macrophages.

Since the discovery that GFP could be used as a genetic tool (Heim, Cubitt, & Tsien, 1995), there has been a surge of new available tools to label specific cell types, as well as fluorescent reporters to assay for biological function. To specifically label islet tissue macrophages we generated transgenic mice using a cre-lox system (Csf1r-Cre mice crossed with ROSA-GCaMP3 mice) to express the genetically encoded Ca\(^{2+}\) reporter GCaMP3 in macrophages. Increases in cytoplasmic Ca\(^{2+}\) levels can be measured as increases in GFP fluorescence intensity. Calcium ions (Ca\(^{2+}\)) act as secondary messengers important for many cell signaling processes. Increases in cytoplasmic Ca\(^{2+}\) levels indicate cell activation, which can lead to secretion. The role of Ca\(^{2+}\) in immune cell function has
been shown to be biologically important in macrophages. \( \text{Ca}^{2+} \) levels influence phagocytic activity and are modulated by ATP, a “find-me” signal that regulates clearance of apoptotic cells (Elliott et al., 2009). By recording \( \text{Ca}^{2+} \) signaling in resident macrophages we can determine which stimuli activate these specialized immune cells.

The main advantage of this technique is that we are able to preserve living tissue in a semi-intact state. It is now clear that the local environment drives selection and function of tissue-specific macrophage identities. Once removed from their local tissues, the transcriptome of resident macrophages reverts to a generic macrophage identity, rather than staying as specialized tissue identity (Gosselin et al., 2014). These observations have led to the idea that macrophages specifically are highly plastic cells, and they fulfill the needs of their local tissue niche. Macrophage populations are currently classified by their classical (M1) or alternative (M2) activation state. Although these classifications exist, it is clear that not all macrophages can fit within these two categories. There is a continuum between these activation states that more accurately describes macrophage populations in their native conditions. Culturing tissue macrophages can change their gene expression in as little as six hours (Gosselin et al., 2017). If the signals maintaining macrophage polarization are lost, by extraction and culturing techniques, we may lose the ability to correctly study macrophage physiology. Therefore, the living tissue slices may provide an ideal tool for the study of islet macrophage function.

1.3 The mechanisms in which islet macrophages support islet function and glucose homeostasis are unknown

In other tissues, resident macrophages adopt highly specific phenotypes (e.g. microglia, alveolar macrophages, Kupffer cells) to maintain tissue integrity and
homeostasis. They do this by fulfilling the needs of their local tissue niche. The local resident macrophages of the brain, microglia, have been shown to support normal brain function by actively engulf synaptic material and play a role in synaptic pruning (Paolicelli et al., 2011). However, islet macrophage function has not been studied systematically.

Current investigations have focused in models of inflammation, autoimmunity, since macrophages have been shown to play a clear role in the pathogenesis of diabetes by presenting beta cell antigens to T-cells (Vomund et al., 2015), however, relatively few groups have looked at the homeostatic role these local macrophages fulfill. The most convincing evidence so far that islet macrophages support beta cell health has been from studies from (Banaei-Bouchareb et al., 2004), which mice that lack islet macrophages caused by mutations in the CSF-1 gene show altered beta cell mass. The authors were unable to identify the specific signals from macrophages, which cause changes islet morphology and beta cell mass. The task to identify the specific signals which affect morphology and beta cell mass is understandably laborious, as there are an infinite number of possible secreted molecules by islet macrophage which influence islet structure and function.

1.4 Cholinergic innervation of islet macrophages

The beta cell is a unique cell, in that it secretes high levels of ATP (10 µM) during insulin secretion (Detimary et al., 1997). Macrophages are well known sensors of purinergic signals (Alonso-Torre et al., 1993). It is now known that beta cells are able to communicate with islet macrophages through ATP during beta cell activation (Weitz et al., 2017). The amount of ATP released during beta cell stimulation is sufficient to activate inflammatory responses in other tissues (Melani et al., 2005), however intriguingly, there
is no inflammation in the islet during normal physiology. This raises the question; if ATP levels are so high, why is there no inflammation in the islet? It is possible that other paracrine signals are maintaining macrophages “quiescent” to elevation of ATP levels during high glucose stimulation.

In recent publications, activation of nicotinic receptors by acetylcholine has been shown to inhibit macrophages Ca\(^{2+}\) responses to ATP stimulation (Nemethova et al., 2013). The β2 and α7 nicotinic receptor subtypes were implied to be responsible for the reduction of Ca\(^{2+}\) responses. In addition to Ca\(^{2+}\) responses, nicotine is capable of inhibiting ATP dependent IL-1 release in alveolar macrophages through activation of nicotinic receptors (Hecker et al., 2015).

Cholinergic anti-inflammatory mechanisms have been well described in other tissues, however it has not yet been described in the pancreatic islet. Currently there numerous FDA approved cholinergic therapeutic medications to increase acetylcholine levels in the body including Galatamine (Razadyne), Donepezil (Aricept), and Rivastigmine (Exelon). These drugs are currently used to treat other inflammatory diseases such as rheumatoid arthritis and Alzheimer’s disease. If we are able to determine that cholinergic nerves are capable of supporting islet homeostasis, by blocking islet macrophage inflammation, we may be able to repurpose FDA approved drugs, which increase acetylcholine levels for new clinical indications to treat diabetes.

1.5 Working model and hypothesis

Since macrophages have been shown to promote angiogenesis, proliferation, and affect beta cell mass, we hypothesized that the macrophages must be communicating with other cell types in the islet microenvironment. We aimed to identify communication
networks in the islet, by finding the local signals in the islet microenvironment that can activate macrophages (Figure 1-1). Our long-term goal is to understand the contribution of resident macrophages in normal islet function, and how their dysregulation leads to diabetes progression.

Our results show that islet macrophages (1) have robust Ca\textsuperscript{2+} responses to ATP, (2) sense beta cell activity via purinergic signals (ATP or ADP), and (3) express a unique repertoire of purinergic receptors. Macrophages are well known sensors of purinergic signals (Alonso-Torre, Alvarez, Montero, Sanchez, & Garcia-Sancho, 1993). We thus hypothesized that islet macrophages detect purinergic signals derived from endocrine cells to gauge the level of beta cell activity and adjust their homeostatic support accordingly. We postulate during diabetes pathogenesis, overworking beta cells cause ATP levels to increase, thereby activating a different subset of purinergic receptors, leading to a pro-inflammatory response in macrophages, as they do in other organs such as the gut in Crohn’s disease (Rybaczyk et al., 2009). Additionally, excess glycemic load can induce neuropathy (Iadecola et al., 1997). We hypothesized that the loss of cholinergic innervation leads to a destabilization of pro-inflammatory ATP levels within the islet. We found that (1) cholinergic nerves directly contact islet macrophages. Moreover, the majority of macrophages are highly contacted in the pancreatic islet (roughly 70%). Additionally, (2) specific cholinergic stimulation of islet macrophages dampens macrophage functional Ca\textsuperscript{2+} responses to ATP in living pancreatic slices. We postulate that acetylcholine, is released from local cholinergic nerves and inhibits immune responses in islet resident macrophages. However, during diabetes, there are compounding effects where beta cells produce elevated
levels of pro-inflammatory signals (ATP), as well as lose neuronal anti-inflammatory compensatory mechanisms to maintain homeostasis.

1.6 Summary of goals

The goal of this thesis was to identify the local signals that regulate islet resident macrophages function, and how dysregulation leads to diabetes progression. In this thesis, I have developed the technological platforms needed to investigate macrophages in situ without disruption of the local islet microenvironment using an adaptation of a technique to study real time ex vivo imaging of living pancreatic slices (Marciniak et al. 2014).

1) Establish a model to investigate macrophage physiology in living tissue slices
2) Physiological characterization of the islet resident macrophages
3) Identify the molecular signals derived from the pancreatic islet which shape the islet resident macrophage

**Figure 1-1:** Paracrine signaling networks between resident macrophages and the islet microenvironment.
CHAPTER 2: Materials and methods

2.1 Mouse models

F1 mice for Ca\(^{2+}\) imaging experiments were made by crossing Rosa-GCaMP3 mice (JAX labs, B6, stock number 014538) with mice expressing Cre recombinase in myeloid cell-specific promoters; LysM-cre (JAX labs, B6, stock number 004781), Csf1r-cre (JAX labs, FVB, stock number 021024), and Cx3cr1-cre mice (JAX labs, B6, stock number 025524). F1 offspring expressing GCaMP3 in macrophages (≥ 3 months old of both sexes) were sacrificed, and pancreatic tissue slices were processed using the protocol as described below.

2.2 Immunohistochemistry

Mice were anesthetized, exsanguinated and perfused with 4% paraformaldehyde. Blocks of mouse pancreas (0.5 cm\(^3\)) were postfixed in 4% paraformaldehyde, cryoprotected (30% sucrose), and tissue sections (40 μm) cut on a cryostat. After permeabilization (PBS-Triton X-100 0.3%), sections were incubated in blocking solution (Biogenex, San Ramon, CA). Primary antibodies were diluted in blocking solution. To visualize macrophages we used antibodies against Iba1 (Wako Chemicals USA, Richmond, VA), F4/80 (1:200 Abcam, Cambridge, MA cat No. ab6640), and CD206 (1:100 Biolegend San Diego, CA cat No. 141721). We performed immunostaining for GFP (1:200 Abcam, Cambridge, MA cat No. ab6658) to visualize GFP expression under the control of the myeloid promoters (see JAX labs mice below). Cell nuclei were stained with DAPI. Slides were mounted with ProLong Anti Fade (Invitrogen).
2.3 Preparation of living pancreatic tissue slices

Tissue slices were prepared from 15 young adult mice (25-30 g) as described (Marciniak et al., 2014). Mice were anesthetized with isofluorane (2%) and euthanized by cervical dislocation to prepare for pancreatic duct injection of low gelling temperature agarose (1.2%, Sigma Aldrich cat. no 39346-81-1, dissolved in HEPES-buffered solution as described below, without BSA). Syringes (5 ml) were filled with agarose solution, and a 30-gauge needle was used to inject in the common bile duct. After injection, tissue blocks were cut, imbedded, and left to solidify (4°C). Living slices were then cut (150 µm) on a vibroslicer (Leica 1000s). Slices were incubated in HEPES-buffered solution (125 mmol/l NaCl, 5.9 mmol/l KCl, 2.56 mmol/l CaCl₂, 1 mmol/l MgCl₂, 25 mmol/l HEPES, 0.1% BSA, pH 7.4).

2.4 Ca²⁺ imaging of living pancreatic tissue slices

We generated mice for [Ca²⁺]ᵢ imaging by using F1 mice crossed from mice expressing Cre recombinase in myeloid cell-specific promoters with mice expressing the floxed Ca²⁺ indicator GCaMP3 (see above). Mice (≥ 3 months old of both sexes) were sacrificed, and pancreatic tissue slices were processed as described above. Living tissue slices containing GCaMP3 labeled macrophages were placed in a perfusion chamber and immersed in HEPES-buffered solution. Glucose was added to the buffered solution to give a basal glucose concentration of 3 mmol/l, unless otherwise specified. All stimuli were bath applied. Throughout the study we used the nonhydrolysable ATP agonist ATP₇S (Tocris Biosciences, Bristol, UK). Antagonists were allowed to equilibrate with receptors for 5 min before stimulation with an agonist. For [Ca²⁺]ᵢ imaging, a Z stack of ~15-30 confocal images was acquired every 8 s using a Leica SP5 confocal laser-scanning microscope.
[Ca\textsuperscript{2+}]\textsubscript{i} responses in pancreatic macrophages were quantified as the areas under the curve of individual traces of GCaMP3 fluorescence intensity during the application of stimuli. To be included in the analyses, [Ca\textsuperscript{2+}]\textsubscript{i} responses had to be reproducible in $\geq 3$ pancreatic slices. We further analyzed and quantified pseudopodia movement and velocity using the ImageJ plugin MTrackJ. For immunohistochemical staining after [Ca\textsuperscript{2+}]\textsubscript{i} imaging.

### 2.5 Confocal imaging

Confocal images (pinhole = airy 1) of randomly selected islets were acquired on a Leica SP5 confocal laser-scanning microscope with 40x magnification (NA = 0.8). Macrophages were reconstructed in Z-stacks of 15-30 confocal images (step size = 2.5-4.0 $\mu$m) and analyzed using ImageJ. Using confocal images, we established the location of macrophages within islets (endocrine) or in acinar regions (exocrine). To prevent bias, we used an automated method in ImageJ to segment the different pancreas regions based on DAPI staining before determining macrophage position.

### 2.6 Flow cytometry

Islet macrophages were sorted based on the viable, GFP+ and F4/80+ labeled cells. For non-macrophage internal controls, islet cells were also sorted based on the viable GFP-, F4/80- population. Islets were isolated by injecting collagenase buffer (20 mg/50 ml) into the common bile duct. The islets were subsequently hand-picked and dissociated into individual cells using trypsin. Dissociated islet cells were washed and placed into Universal Blocking Buffer solution (Biogenex, San Ramon, CA). The staining was performed with F4/80 (1:200 Abcam, Cambridge, MA cat No. ab6640) and CD206 (1:100 Biolegend San Diego, CA cat No. 141721). Myeloid-GCaMP3 mice were used to identify GFP
macrophages. DAPI was used to identify viability. FACS buffer contained PBS and 1% FBS. Populations were isolated and quantified by flow cytometric cell sorting (FACS).

2.7 RT-PCR

RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA), and cDNA was prepared using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) from FACS sorted islet macrophages and non-macrophage internal controls. cDNA products were pre-amplified 10 cycles using the TaqMan pre-amp master mix (Applied Biosystems). PCR reactions were run using the TaqMan gene expression assays (Applied Biosystems) in a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative quantification of gene expression was done based on the equation

$$\text{relative quantification} = 2^{-\Delta Ct} \times 1,000,000$$

where $\Delta Ct$ is the difference between the threshold cycle (Ct) value (number of cycles at which amplification for a gene reaches a threshold) of the target gene and the threshold cycle value of the ubiquitous housekeeping gene 18s. For fold change comparisons, we used the delta delta Ct method, calculated by

$$= 2^{\Delta\Delta Ct}.$$ 

To ensure for consistent qPCR results, 18s normalised samples were compared to that of GAPDH normalised samples. Fold change differences were less than a 1.2-fold between groups when normalised to 18s versus GAPDH.

2.8 Measurement of blood vessel diameter

Living pancreatic slices of NG2-GCaMP3 mice were placed on a coverslip in an imaging chamber (Warner instruments, Hamden, CT, USA) for imaging on a Leica TCS SP5 upright laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Slices were continuously perfused with HEPES-buffered solution containing 3 mM glucose and confocal images were acquired with LAS AF software (Leica Microsystems).
using a 40X water immersion objective (NA 0.8). We used a resonance scanner for fast image acquisition to produce time-lapse recordings spanning 50-100 μm of the slice (z-step: 5-10 μm, stack of ten confocal images with a size of 512 × 512 pixels) at 5 sec resolution (XYZT imaging). GCaMP3 fluorescence was excited at 488 nm and emission detected at 510–550 nm, DyLight 594 labeled tomato lectin was excited at 594 nm and emission detected at 610-650 nm.

We recorded changes in GCaMP3 fluorescence and blood vessel diameter induced by adrenaline, noradrenaline, endothelin-1, tyramine, L-nitro-arginine methyl ester (L-NAME), kainate, calcitonin gene-related peptide (CGRP), ATP, adenosine or 5′-(N-Ethylcarboxamido)adenosine powder (NECA) in basal glucose solution (3 mM), or by high glucose (16 mM) alone, with theophylline or A1 receptor antagonist PSB36. All chemicals were from Sigma, except kainate, CGRP and PSB36 that were from Tocris.

2.9 Transplantation into the anterior chamber of the eye and in vivo imaging

Mouse islet isolation was performed using collagenase digestion followed by purification with histopaque. Approximately 20 islets isolated from young NG2-GCaMP3 mice (6-8 weeks old) were aspirated into a 27G eye cannula connected to a 1 ml Hamilton syringe via a 0.4 mm polyethylene tubing. Young NG2-GCaMP3 mice (6-8 weeks old; n = 5 mice) were anesthetized with ~2% isoflurane and the eyes were kept humidified (ophthalmologic eye drops) to avoid drying of the cornea. Under a stereomicroscope, the cornea was punctured close to the sclera at the bottom part of the eye with a 31G insulin needle and a small radial incision of approximately the size of the eye cannula (~ 0.5 mm) was made. The blunt eye cannula was then gently inserted through this incision and the islets slowly injected into the anterior chamber, where they settle on the iris. After
injection, the cannula was slowly withdrawn to avoid islets from flowing back through the incision. The mouse was left lying on the side before awakening. Mice were then put back in the cages and monitored until full recovery, and observed daily thereafter.

Analgesia was achieved after surgical procedures with buprenorphine (0.05-0.1 mg/kg s.c.).

Mice were imaged 3-4 months after transplantation after 5 consecutive i.p. injections of tamoxifen (see above). Imaging of islets *in vivo* in the anterior chamber of transplanted animals was performed as previously reported (Speier et al., 2008). Briefly, mice were anesthetized with ~2% isoflurane air mixture, placed on a heating pad and the head restrained with a headholder. The eyelid was carefully pulled back and the eye gently supported for fluorescence confocal imaging on an upright laser-scanning confocal microscope (Leica TCS SP5) using long distance water-dipping lenses (Leica HXC APO 20x 0.5 W), using PBS as immersion liquid. Blood vessels were labeled by tail vein injection of 150,000 Da Dextran (FITC or TRITC conjugated (Sigma)). FITC and GCaMP3 were excited at 488 nm and emission light was collected between 500-550 nm; TRITC was excited at 561 nm and emission light collected at 570 nm. Reflected light was imaged by illumination at 633 nm and collection between 630-639 nm. We used a resonance scanner for fast image acquisition to produce time-lapse recordings of blood flowing through islet capillaries on confocal mode with a size of 512 × 512 pixels at 68 msec resolution (XYT imaging).

For functional hyperemia experiments, glucose was injected i.p. (2 g/kg body weight, stock 50% dextrose solution) in anesthetized mice and glycemia measured using a portable glucometer (Contour). The sympathomimetic phenylephrine was administered
as eye drops (two drops; phenylephrine hydrochloride solution 2.5%; Alcon Laboratories, Forth Worth, TX) and imaging resumed 5 min later. The pulse and respiration of the animal was measured to ensure that the drug had not entered the systemic circulation.

Data analysis was performed with ImageJ. For quantification of blood vessel diameter, we drew a line on the blood vessel and used the “plot profile” function to determine the vessel borders. Vessel diameter was calculated by subtracting these 2 position values. For quantification of blood flow, we manually counted the number of red blood cells (identified as black shadows crossing the vessel lumen) in different vessels and normalized it for an average vessel length of 50 µm.

2.10 Data analyses and statistics

For quantification of [Ca^{2+}]_i, responses we calculated the areas under the curve of the fluorescence intensity traces of GCaMP3. Our criteria for accepting [Ca^{2+}]_i responses for analyses were (1) that responses could be elicited ≥ 2 times by the same stimulus and (2) the peak signal was ≥ 2 times the baseline fluctuation. Statistical comparisons were performed using Student’s t test or one-way ANOVA followed by multiple-comparison procedures with the Tukey or Dunnett’s tests. Data are shown as mean ± SEM.
CHAPTER 3: Mouse pancreatic islet macrophages use locally released ATP to monitor beta cell activity

To study physiological responses of pancreatic macrophages we prepared living pancreatic slices for \([\text{Ca}^{2+}]_i\) imaging (Figure 3-3b-3c). Calcium ions (\(\text{Ca}^{2+}\)) act as second messengers in several intracellular signaling pathways that are activated by surface receptor classes, including G protein-coupled surface receptors and receptor tyrosine kinases. Increases in \([\text{Ca}^{2+}]_i\) indicate cell activation. The role of \(\text{Ca}^{2+}\) in immune cell function has been shown to be biologically important and well characterized in resident macrophages such as microglia, alveolar macrophages, as well as in non-myeloid lymphocyte activity (Szalay et al., 2016; Westphalen et al., 2014).

3.1 Experimental strategy to study functional responses of pancreatic macrophages

_in situ_

To perform \([\text{Ca}^{2+}]_i\) imaging of macrophages in real time, we generated mouse models expressing the genetically encoded \(\text{Ca}^{2+}\) reporter GCaMP3 (GFP-calmodulin) selectively in myeloid cells. Using a cre-lox system, we crossed three different strains of mice expressing cre recombinase under myeloid specific promoter (Csf1r-Cre, Cx3r1-Cre, LyzM-Cre) with floxed GCaMP3 transgenic mice **Figure 3-3**. In these mouse strains, cre expression has also been reported in dendritic cells, granulocytes, and monocytes. Pancreas macrophages in Csf1r-GCaMP3 and Lyz-GCaMP3 mice were immunostained for the macrophage marker Iba1 and showed a high percentage (~90%) of co-localization with GCaMP3 (Fig. 3-3d, e). We did not use Cx3cr1-GCaMP3 mice in our functional experiments because many non-myeloid cells expressed GCaMP3 (Fig. 3-3e).
Isolating macrophages from their native environment can cause changes in their phenotype and does not allow studying their dynamics and interactions with the local environment in situ (Chamberlain, Holt-Casper, Gonzalez-Juarrero, & Grainger, 2015). By contrast, living pancreatic slices show minimal disruption of tissue integrity and preserve the islet cytoarchitecture within the acinar tissue (Huang, Rupnik, & Gaisano, 2011; Marciniak et al., 2014). We sought to establish that living tissue slices are suitable to characterize the physiology of macrophages in their native environment. We found that macrophages in pancreatic tissue slices showed a morphology and CD profile comparable to those of macrophages in the pancreas of mice perfused with fixative (Figure 3-1 and Figure 3-2). Based on these data, we conclude that the slicing procedure did not alter macrophage features.

**Figure 3-1** Immunohistochemistry for macrophage markers in pancreas sections from perfusion fixed mice show that islet macrophages can be distinguished from those in exocrine regions. a) Confocal image of a mouse pancreatic section showing
immunostaining for insulin (white), Iba1 (red), and CD206 (green). DAPI (blue) = nuclei. Left panel merged (Insulin, Iba1, CD206, DAPI), middle-left (Iba1), middle-right (CD206), right merged (Iba1, CD206). Dotted line delineates the endocrine/exocrine border. Scale bar, 50 µm. (b) Higher magnification of panel (a) showing the endocrine/exocrine border (dotted line) of the islet. Scale bar, 10 µm. (c) Quantification showing the percentage of Iba1 colocalization with F4/80, CD206, Csf1r, or LysM for macrophages in the endocrine regions (d) Quantification showing the percentage of Iba1 colocalization with F4/80, CD206, Csf1r, or LysM for macrophages in the exocrine regions. Colocalization and intensities (e-h) were calculated in > 60 macrophages pooled from 3-5 mice (*P < 0.05, Student’s t-test).

**Figure 3-2.** Immunohistochemistry for macrophage markers in fixed pancreas slices show that in living tissue slices from mouse pancreata, islet resident macrophages maintain their resident phenotype. (a) Living pancreatic slice (150 µm thick) cut using a vibroslicer.
Confocal image of a fixed mouse pancreatic slice showing immunostaining for Iba1 (red), CD206 (green), and DAPI (blue). Shown is a maximal projection of a Z-stack of 40 images. White dotted line represents the endocrine/exocrine border. Scale bars: 50 µm. (b) Higher magnification of the pancreatic slice shown in (a), of endocrine/exocrine border (dotted line). Scale bars, 20 µm. (c-e) Intensity levels of Csf1r (c), Iba1 (d), and CD206 (b) immunostaining of macrophages in endocrine (black symbols) versus exocrine tissue (grey symbols). Intensities were calculated in > 25 macrophages pooled from 3 pancreas slices (*P < 0.05, Student’s t-test).

After validating the transgenic mouse lines and the slicing procedure, we performed [Ca\textsuperscript{2+}]\textsubscript{i} imaging of macrophage responses to stimuli. GCaMP3-expressing macrophages could be discerned both in exocrine and endocrine regions in pancreatic slices Figure 3-3. Islets were identified based on their strong backscatter (Fig. 3-3f). Macrophages in endocrine and exocrine tissues responded to ATPyS (100 µM) with increases in [Ca\textsuperscript{2+}]\textsubscript{i}  (Fig. 3-3g, h).
Figure 3-3 [Ca^{2+}]_i imaging of pancreas macrophages in situ. **a)** Cre-lox model used to generate the [Ca^{2+}]_i indicator GCaMP3 under the control of myeloid specific promoters. **(b, c)** Living pancreatic tissue cut using a vibroslicer (b) and placed in a recording chamber (c). White stars denote location of a pancreatic islet. **(d)** Confocal image of a fixed pancreatic slice from a Csf1r-GCaMP3 mouse showing GCaMP3 (green) and Iba1 (red) immunostaining. DAPI (blue) = nuclei. Smaller boxes show zoomed image of an islet macrophage. Scale bars, 50 (left) and 10 µm (right). **(e)** Quantification of the proportion of Iba1 positive cells within the population of GCaMP3+ cells in slices (> 75 macrophages pooled from 4 sections in 3 mice per group). **(f)** Confocal image taken from a video recording during [Ca^{2+}]_i imaging of macrophages in a living slice from a GCaMP3+ mouse. Shown is a maximal projection of a Z-stack of ~40 images. The image shows islet backscatter (grey) and GCaMP3 labeled macrophages (green). **(g)** Three sequential higher magnification images of (f) showing the [Ca^{2+}]_i response in macrophage denoted with the letter ‘A’ (box) to stimulation with ATP (100 µM). Pseudocolor scale indicates level of fluorescence intensity. Time points are before (1), during (2), and after stimulation (3) with ATP. Scale bar, 10 µm. **(h)** Traces of [Ca^{2+}]_i responses in A-D macrophages in (f). Arrows
indicate time points before (1), during (2), and after stimulation (3) with ATP at which the images in (g) were taken.

After functional $[\text{Ca}^{2+}]_i$ imaging, we immunostained pancreatic slices for macrophage markers. With our approach, functionally characterized macrophages, easily tracked in the immunostained slices because they retained their GCaMP3 labeling, could be further classified based on their CD profile Figure 3-4.

**Figure 3-4:** Real time functional Ca$^{2+}$ imaging of macrophages in living pancreatic tissue slices allow physiological characterization, followed by immunohistochemical classification. (a) Confocal image of this same pancreatic slice after fixation and immunohistochemical staining for Iba1 (red), CD206 (cyan), and GCaMP3 (green). Scale bar, 50 µm. (b) Higher magnification image of a showing the immunostaining patterns of the four macrophages 1-4 that were characterized physiologically see Fig. 3-3f-h). Images are meant to illustrate the procedure and representative of $n > 15$ experiments. Rows show GCaMP3 (green, top), Iba1 (red, middle), and CD206 (cyan, bottom) immunostaining. Scale bar, 10 µm.
3.2 Pancreatic macrophages respond selectively to beta cell-derived stimuli

Pancreatic macrophages are exposed to molecules released from acinar, neural, or endocrine cells (Fig. 3-5a). To identify interactions between these cell types and pancreas macrophages, our strategy was to apply stimuli known to activate these cells and monitor increases in $[\text{Ca}^{2+}]_i$ in macrophages Figure 3-5.

**Figure 3-5** $[\text{Ca}^{2+}]_i$ responses of pancreas macrophages to tissue-specific stimulation. To selectively stimulate beta cells we raised the glucose concentration from 3 mM to 16 mM and found that it increased the frequency and amplitude of $[\text{Ca}^{2+}]_i$ responses in macrophages (Fig. 3-5b, c). Cell membrane depolarization with KCl (25 mM), which stimulates endocrine cells and neurons, produced similar $[\text{Ca}^{2+}]_i$ increases in macrophages (Fig. 3-5d). Macrophages in slices did not respond to application of acetylcholine (ACh, 100 µM), a neurotransmitter that stimulates pancreatic acinar cells and postganglionic
autonomic neurons (Fig. 3-5f). Macrophages responded strongly to capsaicin (10 µM, Fig. 3-5g), a stimulus for cells expressing TRPV1 channels (e.g. visceral sensory neurons and macrophages). However, when we exposed slices to substance P (SP, 10 µM), a molecule released by sensory nerves in the context of local axon reflexes (Lau, Wong, & Bhatia, 2005), [Ca^{2+}] did not increase in macrophages.

Our results suggest that pancreas macrophages responded mainly to activation of endocrine cells. We therefore examined the effects of the signaling molecules ATP, GABA and serotonin (5HT), which are known to be released by beta cells (Detimary et al., 1996); (Ekholm, Ericson, & Lundquist, 1971). These potential paracrine signals also activate macrophages in other organs (Inoue & Tsuda, 2012; Konno et al., 2012; Schwiebert & Zsembery, 2003). Exposure to GABA or 5HT did not produce increases in macrophage [Ca^{2+}]. By contrast, ATPyS (50 µM) elicited the strongest increases in [Ca^{2+}] (Fig. 3-5e).

### 3.3 Identification of functional purinergic receptors in pancreas macrophages

We identified ATP as a strong activator of islet and acinar macrophages. The P2 receptor antagonist suramin (20 µM) blocked [Ca^{2+}], responses in pancreatic macrophages to ATP (Fig. 3-6a), confirming that purinergic receptors were involved. Macrophages in the exocrine tissue showed larger [Ca^{2+}], responses to low levels of ATP (10 µM), while higher levels of ATP (1 mM) elicited [Ca^{2+}], responses in endocrine and exocrine macrophages equally (Fig. 3-6b, c). ADP (10 µM), a P2Y receptor agonist, MRS2693 (1 µM), a nucleotide with preference for P2Y6 receptors, and MRS2768 (1 µM), an agonist with preference for P2Y2 receptors, also stimulated [Ca^{2+}], responses in macrophages (Fig.
3-6). The P2Y receptor agonists elicited significant increases in the $[\text{Ca}^{2+}]_i$ responses in macrophages (Fig. 3-6e).

**Figure 3-6.** Islet resident macrophages express functional purinergic receptors. (a) Traces of $[\text{Ca}^{2+}]_i$ responses to ATP (100 $\mu$M) from Csf1r-GCaMP3 islet macrophages in pancreatic slices showing that responses were inhibited by suramin (20 $\mu$M), a broad P2 receptor antagonist. (b) $[\text{Ca}^{2+}]_i$ responses to increasing concentrations of ATP of individual macrophages in the islet (Endo, black) and exocrine regions (Exo, grey) in pancreatic slices from Csf1r-GCaMP3 mice. Vertical dotted lines denote start of stimulation. (c) Concentration response curves for $[\text{Ca}^{2+}]_i$ responses to ATP of macrophages in endocrine (black symbols) and exocrine regions (grey symbols). Responses were quantified as area
under the curve and were normalized as a percentage of the maximum response ($n = 9$ cells pooled from 9 slices from 4 mice). (d) Traces of $[\text{Ca}^{2+}]_i$, responses to ADP (10 µM) and MRS2693 (1 µM) in pancreatic slices from Csf1r-GCaMP3 mice. (e) Quantification of $[\text{Ca}^{2+}]_i$ responses to 3 mM glucose (3G), BzATP (10 µM), ADP (10 µM), MRS2768 (1 µM), and MRS2693 (1 µM). The dot plot shows the average area under the curve for $[\text{Ca}^{2+}]_i$ responses to various stimuli (black bar). [$n > 5$ macrophages from 2 slices from 2 - 3 mice; $^*P < 0.05$, ANOVA followed by Dunnett test for multiple comparisons to the baseline (3G)]. (f) Quantification of the mRNA levels of purinergic receptors expressed by sorted islet macrophages from ($n = 3$ independent isolations from > 3 mice).

To identify specific receptors expressed by islet macrophages, we performed RT-PCR on FACS sorted macrophages from isolated islets. FACS sorted islet macrophages maintained the same surface marker expression profile of that of frozen fixed tissue.

Figure 3-7. Csf1r-GCaMP3 islet macrophages CD marker profile using flow cytometry is consistent with islet macrophages from IHC frozen fixed tissue. (a) Flow cytometry
analysis of isolated islet macrophages from Csf1r-GCaMP3 mice. F4/80 positive cells (small black box) showed a discrete population from islet cell extract (left). F4/80 positive cells were identified as GFP (488-513) positive as well as CD206 (405-610) negative (right). (b) F4/80 and GFP (488-513) positive acinar macrophages in Csf1r-GCaMP3 mice include macrophage populations that were CD206 (405-610) positive and negative. 200 islets were pooled from pancreas tissues from $n = 3$ mice.

We found mRNA expression of genes encoding macrophages specific genes (csf1r) P2X receptors (p2x4, p2x7), P2Y receptors (p2y2, p2y6, p2y14) and the P1 receptor (adora3, Fig. 3-8f). Because other cells within the islet are known to respond to ATP, we compared purinergic receptor gene expression of islet macrophages with that the F4/80-islet cell population Figure 3-8.

![Graph showing mRNA expression](image)

**Figure 3-8** Islet resident islet macrophages are enriched for purinergic receptors compared to islet endocrine cell populations. Quantification of the mRNA shown as a difference in fold change between purinergic receptors expressed by islet macrophages and compared to that of endocrine cells sorted from isolated islets. $n = 3$ mice. Islet macrophages showed the largest differences in the mRNA expression of the *bona fide* macrophage gene Csfl1.
Islet endocrine cells showed enrichment for the beta cell gene *Ins*. Expression of purinergic genes of the P2X, P2Y and P1 receptor classes were identified. Quantification of the mRNA levels of purinergic receptors expressed by sorted islet macrophages from $n = 3$ independent isolations from $> 3$ mice.

We found that most receptor genes were more highly expressed in macrophages, in particular $p2x4$ (9-fold), $p2x7$ (82-fold), $p2y2$ (52-fold), $p2y4$ (15-fold), $p2y6$ (670-fold), and the p1 receptor *adora2a* (13-fold). Only the expression of *adora1*, the gene for a P1 receptor known to be highly expressed in alpha cells (Hillaire-Buys, Bertrand, Gross, & Loubatieres-Mariani, 1987), was 10.5 fold higher in the islet (F4/80-) population. The results suggest that islet macrophages express high levels of purinergic receptor genes.

### 3.4 Acutely culturing islets with ATP alters macrophage gene expression

Pancreatic macrophages have been shown to modulate gene expression of cytokines and chemokines during diet alteration (Calderon et al., 2015). We identified functional purinergic receptors in islet macrophages and sought to identify the direct effect of acute purinergic stimulation on islet macrophage gene expression **Figure 10.** In FACS sorted islet macrophages, we found mRNA expression of genes from encoding for matrix metalloproteinase (*Mmp2*) and genes characteristic of both M1 (*Tnfa, Il1b*) and M2 (*Tgfb, Il6*) macrophage polarization (Fig. 3-9a). Upon acute ATP (50 µM) stimulation (16 hours) we found *Mmp2* to be significantly down regulated when compared to the control islet culture medium (5.5 mM glucose RPMI + supplements) treatment. The purinergic receptor inhibitor suramin blocked the effect of ATP (Fig. 3-9b). *Il6* gene expression was increased compared to controls, however the results were not significant. There was no observable
change in the macrophage specific gene \textit{Csf1r} (Fig. 3-9c), nor other genes identified in the basal state (Fig. 3-9a).

\textbf{Figure 3-9.} Culturing islets with ATP (16 h) alters macrophage gene expression from the basal state. \textbf{(a)} mRNA expression from FACS sorted islet macrophages. \textbf{(b)} Changes in \textit{Mmp2}, \textit{Il6}, and \textit{Csf1r} gene expression compared to that in control islet culture medium, presented as fold change (mean ± SEM, \(n = 3\) independent islet preparations, with islets pooled from 5 mice per preparation; *\(P < 0.05\) One-sample t-test to a hypothetical value of 1).

\textbf{3.5 ATP induces pseudopodia movement of pancreatic macrophages}

In many tissues, macrophages display characteristic movements of their fine processes to scan the microenvironment (Nimmerjahn, Kirchhoff, & Helmchen, 2005). Because islet macrophages showed strong responses to ATP, we reasoned that these increases in \([\text{Ca}^{2+}]_i\) could lead to changes in dynamic behavior. Therefore, in parallel to measurements of \([\text{Ca}^{2+}]_i\), we recorded movement of macrophage pseudopodia \textbf{Figure 3-10}. 

\begin{figure}[h] 
\centering 
\includegraphics[width=\textwidth]{figure3-9.png} 
\caption{Culturing islets with ATP (16 h) alters macrophage gene expression from the basal state. \textbf{(a)} mRNA expression from FACS sorted islet macrophages. \textbf{(b)} Changes in \textit{Mmp2}, \textit{Il6}, and \textit{Csf1r} gene expression compared to that in control islet culture medium, presented as fold change (mean ± SEM, \(n = 3\) independent islet preparations, with islets pooled from 5 mice per preparation; *\(P < 0.05\) One-sample t-test to a hypothetical value of 1).} 
\end{figure}
Figure 3-10. ATP stimulates movement of macrophage pseudopodia. (a) Sequential images of a macrophage taken during 3 mM glucose (labeled green) and with ATP (50 µM, labeled red). Merged image shows extended macrophage pseudopodia (red). Scale bar, 10 µm. Images were acquired at the time points denoted by the green and red arrows in (b) showing pancreatic macrophages Ca$^{2+}$ responses to ATP (50 µM, grey symbols) with pseudopodia process movement (black symbols). (c) Quantification of the velocity of macrophage processes during 3 mM, 16 mM, ATP, and ADP stimulation. Macrophage pseudopodia showed increased velocity during ATP stimulation ($n > 7$ macrophages from $n = 3$ mice, box-and-whisker plots; *P < 0.05 Student’s t-test).

Modest increases in pseudopodia movement were observed in ADP (10 µM), but no observable changes in movement were seen during high glucose stimulation.
Stimulation with ATP (50 µM) elicited a sharp [Ca^{2+}]_{i} response and simultaneously increased the velocity of pseudopodia movement of macrophages (Fig. 3-10b, c). Thus, pancreas macrophages respond to ATP by increasing the extent and rate of microenvironment scanning. Macrophage motility, however, may not be increased by the lower levels of ATP present during physiological stimulation of beta cells.

3.6 Pancreatic macrophages respond to endogenous ATP released from beta cells

ATP levels are generally higher inside the cell, and increased extracellular ATP generally reflects cell disruption. However, ATP can be released physiologically into the extracellular space through various mechanisms, including exocytosis. In the islet microenvironment, islet macrophages are exposed to ATP because it is co-released with insulin from beta cells (Detimary et al., 1996). Within the mouse pancreas, islets are uniquely competent to release ATP because they express the vesicular nucleotide transporter VNUT (Geisler et al., 2013). Concentrations of ATP can rise above 25 µM around stimulated beta cells (Hazama, Hayashi, & Okada, 1998), which matches the EC_{50} of the observed macrophage responses to ATP (15-30 µM, Fig. 3-6c). We therefore hypothesized that macrophages sense local changes in ATP concentration during activation of beta cells.

To test this hypothesis we raised the glucose concentration from 3 mM (3G) to 16 mM (16G) and challenged the [Ca^{2+}]_{i} responses in macrophages with suramin (20 µM) Figure 3-11. After suramin washout, activity in macrophages increased again to later subside after return to low glucose concentrations (Fig. 3-11a). Suramin inhibited the increased activity during 16G quantitatively (Fig. 3-11b), indicating that purinergic receptors were mediating the macrophage responses to beta cell activation with increased
glucose concentration. By contrast, exocrine macrophages showed modest, not significant increases during 16 mM glucose stimulation (Fig 3-11c).

![Graphs and images](image-url)

**Figure 3-11.** Pancreatic macrophages sense endogenous ATP released from insulin secreting beta cells. (a) Spontaneous activity in pancreatic macrophages increased when the glucose concentration was raised from 3 mM to 16 mM to stimulate beta cells. Spontaneous activity was inhibited by suramin (20 µM). Vertical, dotted lines denote exchange of solutions. (b, c) Quantification of Ca^{2+} increases (area under the curve) of Ca^{2+}...
traces as shown in a in response to 3G, 16G or 16G with suramin (20 µM) \( n = 4-20 \) cells pooled from 3 pancreatic slices from 3 mice in macrophages from pancreatic islets (b) and from exocrine macrophages (Exo in c); *P < 0.05, ANOVA followed by Tukey test for multiple comparisons. (d) Activity in pancreatic macrophages induced by increasing the glucose concentration from 3 mM to 16 mM was inhibited by nifedipine (10 µM). (e) Quantification of Ca\(^{2+}\) increases (area under the curve) of Ca\(^{2+}\) traces as shown in (d) in response to 3G, 16G, 16G with nifedipine (10 µM), and 16G with ATP (50 µM) and nifedipine (10 µM) \( n = 6-12 \) cells pooled from 3 pancreatic slices from 3 Csf1r-GCaMP3 mice; *P < 0.05, ANOVA followed by Tukey test for multiple comparisons. (f) A quantification of Ca\(^{2+}\) increases (area under the curve) in response to exchanging solutions between 3G, KCl (25 mM), and KCl (25 mM) with suramin (20 µM) \( n = 9 \) cells pooled from 3 pancreatic slices from 2 mice; *P < 0.05, ANOVA followed by Tukey test for multiple comparisons. (g) Spontaneous activity in pancreatic macrophages did not increase when the glucose concentration was raised from 3 mM to 16 mM in pancreatic slices devoid of islets. Macrophages became activated during ATP (50 µM) stimulation. (h) Quantification of Ca\(^{2+}\) increases (area under the curve) of Ca\(^{2+}\) traces as shown in g \( n = 14 \) cells pooled from 3 pancreatic slices from 3 mice; *P < 0.05, ANOVA followed by Tukey test for multiple comparisons. (i) Proposed model of macrophage communication with beta cells.

To uncouple the direct effect of glucose on pancreatic macrophages and to pinpoint the beta cell as the source of increased spontaneous activity, we used the L-type voltage gated Ca\(^{2+}\) channel blocker nifedipine, a known inhibitor of beta cell secretion (Yang & Berggren, 2006). Application of nifedipine (10 µM) blocked [Ca\(^{2+}\)], responses elicited by
16 mM glucose concentration (Fig. 3-11d), without affecting ATP sensitivity in islet macrophages (Fig. 3-11e). KCl depolarization induced increases in macrophage \([\text{Ca}^{2+}]_i\), but not in the presence of suramin (Fig. 3-11f). These results indicate that ATP released endogenously from beta cells stimulates pancreas macrophages. There was no change in macrophage \([\text{Ca}^{2+}]_i\) responses during 16 mM glucose stimulation in pancreatic slices devoid of islets (Fig. 3-11g, h). From these data, we propose a model for communication between pancreatic islets and their resident macrophages in which islet resident macrophages respond to ATP and other purines released from beta cells under physiological stimulation (Fig. 3-11i).

### 3.7 Summary and discussion of findings

Our results demonstrate that pancreatic islet macrophages express a complement of purinergic receptors that detect endogenous levels of interstitial ATP. While extracellular ATP generally reflects cell disruption, we established here that macrophages respond to ATP secreted from beta cells. ATP can therefore be considered a bona fide paracrine signal the beta cell uses to communicate with resident macrophages.

Functional imaging in living slices allowed us to study local interactions between islet endocrine cells, local neurons, acinar cells, and resident macrophages. While \(\text{Ca}^{2+}\) imaging of macrophages is commonly performed in other tissues (Davalos et al., 2005; Moghimi & Patel, 1990; Westphalen et al., 2014), the same had not been attempted in the pancreas. Immune cell infiltration and movement has been monitored in the native pancreas (Lee, Spalding, Ben-Josef, Wang, & Simeone, 2010; Martinic & von Herrath, 2008; Turvey et al., 2005), but given the low spatial resolution and limited access to local drug application it is unlikely that these in vivo approaches allow the physiological
characterization and pharmacological interrogation of macrophages in situ we conducted here.

Using our approach, we determined that islet macrophages respond to activation of endocrine cells, but not to stimulation of acinar cells. Macrophage responses to endocrine cell activation with high glucose concentrations were mediated by ATP receptors. It is very likely that most ATP released under these conditions is derived from beta cells because: (a) beta cells of the mouse islet are the predominant cell type responding to high glucose; (b) in pancreas slices that did not contain islets, acinar macrophages did not respond to high glucose concentration; (c) beta cells release ATP from insulin granules (Braun et al., 2007; Detimary et al., 1996; MacDonald, Braun, Galvanovskis, & Rorsman, 2006); and (d) beta cells are the only pancreatic cells that express the vesicular nucleotide transporter VNUT (Geisler et al., 2013), making them uniquely competent to release stored ATP. Because ATP release is tightly coupled to insulin secretion, extracellular ATP levels can be considered a proxy for beta cell secretory activity. ATP signaling thus converts islet macrophages into local sensors of the beta cell’s hormonal output.

ATP may trigger important physiological processes in islet macrophages. Indeed, purinergic signaling has been shown to be essential for macrophage function, including chemotaxis (Kronlage et al., 2010), phagocytosis (Koizumi et al., 2007), and cytokine production (Perregaux, McNiff, Laliberte, Conklyn, & Gabel, 2000). We determined that ATP induces increases in $[Ca^{2+}]$, and movement of macrophage pseudopodia, but otherwise it remains unclear what secretory responses are triggered in islet macrophages when exposed to ATP. Recent studies suggest that macrophages play a trophic role in beta cell proliferation, but the results were obtained under extreme conditions which involved
recruited macrophages (M. Brissova et al., 2014; Criscimanna, Coudriet, Gittes, Piganelli, & Esni, 2014; Riley et al., 2015).

In addition to proliferation factors, local release of pro-inflammatory cytokines such as IL-1 \( \beta \) have been shown to modulate insulin secretion (Comens, Wolf, Unanue, Lacy, & McDaniel, 1987) and is sufficient to damage human pancreatic islets (Arnush et al., 1998). IL-1 \( \beta \) release from islet macrophages may act in a feedback loop to regulate insulin secretion. In our limited study, we did not detect significant differences in cytokine gene expression under acute treatment of ATP or high glucose. However, we did detect a significant downregulation of Mmp2. It will be important to determine if the purinergic axis between beta cells and resident macrophages regulates local inflammation (e.g. IL-1 \( \beta \) (Comens et al., 1987)), tissue integrity (e.g Mmp2 (C. Liu et al., 2014)), as well as trophic support (e.g. TGF\( \beta \) (Xiao & Gittes, 2015), IL-6 (Ellingsgaard et al., 2011),) during physiological and pathological states.

To fulfill their homeostatic role, macrophages must monitor the local microenvironment to detect changes in tissue characteristics such as cell number and composition and the acidity and osmolarity of interstitial fluids (Chovatiya & Medzhitov, 2014). Our results suggest that resident macrophages use ATP receptors and probably TRPV1 receptors to respectively monitor beta cell activity and the acidity of the interstitial milieu in the islet. Islet macrophages have been shown to be key players in autoimmune destruction of the islet caused by type 1 diabetes (Appels et al., 1989; Burkart & Kolb, 1996; Ferris et al., 2014; Hildner et al., 2008; Jansen et al., 1994; Kolb et al., 1990; Thornley et al., 2016; Vomund et al., 2015), and play a role in producing inflammation during type 2 diabetes (Donath, Dalmas, Sauter, & Boni-Schnetzler, 2013; Eguchi &
Manabe, 2013). Here we show that islet macrophages monitor and respond to local environmental cues not only during extreme challenges such as inflammation and autoimmunity, but also during less dramatic alterations in tissue physiology. Sensing the microenvironment helps the macrophage gauge its production of factors that promote islet tissue stability.
CHAPTER 4: Parasympathetic nerves are a source of anti-inflammatory signals that dampen the pro-inflammatory environment in the mouse pancreatic islet

In tissues such as the brain and liver, resident macrophages (e.g. microglia, Kupffer cells) adopt highly specific phenotypes to maintain tissue homeostasis. They can do this by acting as local defense sentinels, removing toxins, and controlling cell turnover through phagocytosis. These homeostatic processes are mediated by paracrine signals, such as nucleotides, neurotransmitters and growth factors, that are released by neighboring cells. The signals that drive these homeostatic processes in tissue macrophages have been studied in depth, such as “find-me” signals (e.g ATP), which attract macrophages to sites of inflammation.

4.1 Activation of beta cells leads to production of danger signals

The beta cell secretes high levels of ATP (25 μM) during insulin secretion (Detimary et al., 1996; Hazama, 1998). Macrophages are well-known sensors of ATP (Alonso-Torre and Trautmann, 1993). Our group has shown that beta cells communicate with islet macrophages through ATP during beta cell activation (Weitz et al., 2018). The amount of ATP released during beta cell stimulation is sufficient to activate inflammatory responses in other tissues (Melani et al., 2005). However intriguingly, there is no inflammation in the islet during normal physiology. This raises the question: if ATP levels are high, why is there no inflammation in the islet?

We hypothesize that another signal, acetylcholine, is released from local cholinergic nerves and inhibits inflammatory responses in macrophages. It is known that cholinergic stimulation of beta cells, promotes insulin secretion, but the role of acetylcholine as a regulator of inflammation in the islet is completely unknown. This
“cholinergic anti-inflammatory reflex” has been described in other tissues, but never in the islet.

4.2 Parasympathetic axons innervate islet macrophages and modulate physiological Ca\(^{2+}\) responses to danger signals (ATP)

Acetylcholine is rapidly degraded by acetylcholinesterase. Therefore, a cholinergic nerve must innervate the target cell in order for acetylcholine to act as a signal. The experimental approach consisted of using immunohistochemistry to identify cell types which are innervated by cholinergic nerves. It is known that beta cells are innervated by parasympathetic nerves (Taborsky, 2011). We found that cholinergic nerves from mouse islets innervate islet macrophages to the same level at which beta cells are innervated.

Figure 4-1. Cholinergic nerves from mouse islets innervate islet resident macrophages. (A) Pancreas section from ChAT-GFP mouse showing cholinergic nerves in green, and macrophages labeled with Iba1 in red. Islet denoted by dotted line. (B zoom) high magnification of macrophage B shown in A. (C) Quantification of percentage of nerves contacting macrophages. (D) Quantification of the percentage of macrophages contacted by cholinergic nerves. DAPI = negative control.
We next sought to identify what are the physiological effects of cholinergic stimulation of islet macrophages. In recent publications, activation of nicotinic receptors by acetylcholine has been shown to inhibit macrophages Ca\textsuperscript{2+} responses to ATP stimulation (Nemethova, Michel, Gomez-Pinilla, Boeckxstaens, & Schemann, 2013). The α2 and α7 nicotinic receptor subtypes were implied to be responsible for the reduction of Ca\textsuperscript{2+} responses. In addition to Ca\textsuperscript{2+} responses, nicotine is capable of inhibiting ATP dependent IL-1 release in alveolar macrophages through activation of nicotinic receptors (Hecker et al., 2015). Similarly, we found nicotine inhibited Ca\textsuperscript{2+} responses to ATP in tissue macrophages, Figure 4-2.

**Figure 4-2.** Cholinergic stimuli dampens macrophage functional Ca2+ responses to ATP in living pancreatic slices. (A) Basal (3 mM glucose) (B) basal + ATP (C) basal + ATP +

![Image](image_url)
Acetylcholine (ACh). (D) Raw traces of intensity levels from individual macrophage responses as seen denoted by the yellow arrows in the islet (denoted by white dotted line). (E) Quantification of results from cholinergic stimuli. Acetylcholine, and nicotine inhibit macrophage responses as compared to a normalized first ATP response. The muscarinic antagonist (oxotremorine) does not significantly block ATP responses in tissue macrophages. Our results showed that there is likely a functional significance for islet macrophage innervation by parasympathetic nerves. This functional significance should be investigated in future studies.

4.3 Summary and discussion of findings

In summary, these initial evidences support the hypothesis that there may be a cholinergic anti-inflammatory mechanism in the pancreatic islet. Our results indicate that macrophages receive cholinergic input from parasympathetic nerves. We expect our studies to identify parasympathetic nerves as a source of anti-inflammatory signals that efficiently dampen the potentially pro-inflammatory environment in the islet. In future studies, we will need to address how local endogenous mechanisms may activate the tissue resident macrophages.

In human islets, cholinergic innervation is sparse therefore, questioning that there is little translational relevance. Interestingly, there is a source of acetylcholine in the human pancreatic islet: the alpha cell (Rodriguez-Diaz et al., 2011). Therefore, human islet macrophages may receive cholinergic input during alpha cell stimulation. Although the source of acetylcholine release may be different, cholinergic regulation of macrophage function is relevant in both the human and mouse condition. Non-neuronal sources of acetylcholine have been described in other regions of the body such as the spleen, where a
subset of memory CD4+ T cells provide an endogenous source of acetylcholine to local
tissue macrophages (Pavlov and Tracey, 2012). New clinical trials have shown that the
acetylcholinesterase inhibitor galantamine has antidiabetic effects in human patients
(Consolim-Colombo et al., 2017). This is the first experiment to test if a local “cholinergic
anti-inflammatory loop” exists in the pancreatic islet.
CHAPTER 5: The pericyte of the pancreatic islet regulates capillary diameter and local blood flow

This chapter concentrates on paracrine signaling in the islet with relation to the islet pericyte. This work was done in collaboration with Dr. Joana Almaca. My contribution to this chapter is providing assistance using the living pancreatic slice technique to perform physiological recordings to identify the molecular signals detected by islet pericytes.

5.1 Pericytes cover the microvasculature in mouse and human islets

The pathogenesis of type 2 diabetes is associated with dysfunction of the pancreatic islet and of its vasculature (Ballian & Brunicardi, 2007; Richards, Raines, & Attie, 2010). The microvasculature of the islet originates from few feeding arterioles that branch into a dense network of capillaries that allows efficient insulin release into the circulation (Ballian & Brunicardi, 2007). Capillary tubes are made of a thin layer of endothelial cells covered by pericytes. Pericytes were first characterized by Ebert in the 19th century as mural cells embedded within the endothelium basement membrane (Bergers & Song, 2005). Pericytes interact closely with endothelial cells and are crucial for proper capillary function, giving structural stability, participating in angiogenesis, and controlling vascular permeability and blood flow (Armulik, Abramsson, & Betsholtz, 2005; Armulik, Genove, & Betsholtz, 2011).

While a wide body of literature exists on islet endothelial cells, the function of the islet pericyte is largely unknown (Jansson et al., 2016). Studies on islet pericytes focused on the role they play in angiogenesis, fibrosis, vascular stabilization and metastasis in beta cell tumors (Chu et al., 2013; Hayden et al., 2008; Song, Ewald, Stallcup, Werb, & Bergers, 2005; Xian et al., 2006), in graft revascularization after transplantation (Juang et al., 2015).
and on their mesenchymal stem cell potential (Crisan, Corselli, Chen, & Peault, 2012; Crisan et al., 2008). Previous studies have shown that pericyte ablation in the whole organism impairs glucose-stimulated insulin secretion and leads to beta cell dedifferentiation (Sasson et al., 2016), which could be due to altered platelet-derived growth factor signaling (Chen et al., 2011). Islet pericytes have further been shown to secrete nerve growth factor to potentiate insulin granule exocytosis (Houtz, Borden, Ceasrine, Minichiello, & Kuruvilla, 2016). These studies suggest that pericytes enhance beta cell function and insulin secretion, but it is unclear if these effects are direct or mediated by pericytes impacting vascular function. Surprisingly, the role of pericytes in regulating islet vascular function has not been addressed. Islet blood flow is thought to be regulated at the pre-capillary and capillary levels (Brunicardi et al., 1996; Y. M. Liu, Guth, Kaneko, Livingston, & Brunicardi, 1993; McCuskey & Chapman, 1969), but control at the capillary level remains controversial (Jansson et al., 2016). Because pericytes regulate capillary diameter in the retina, brain and kidney (Crawford, Wildman, Kelly, Kennedy-Lydon, & Peppiatt-Wildman, 2013; Hall et al., 2014; Peppiatt, Howarth, Mobbs, & Attwell, 2006), we hypothesized that islet pericytes regulate blood flow in the islet by adjusting capillary diameter.

To test our hypothesis, we conducted immunohistochemical and electron microscopy studies of mouse and human pancreatic sections from non-diabetic and type 2 diabetic individuals to investigate the anatomical properties of islet pericytes. We further performed physiological recordings in living pancreas slices to examine functional responses of islet pericytes and capillaries. We focused on how beta cell activity and sympathetic input affect pericyte function and capillary diameters in the islet. To study the
role of islet pericytes in controlling islet perfusion, we performed in vivo imaging of intraocular islet grafts and measured changes in pericyte activity, capillary diameter, and blood flow in response to hyperglycemia and sympathetic agonists. These approaches allowed us to establish the pericyte as an active component of the islet vasculature that mediates vascular responses to increased beta cell activity and autonomic nervous input. Our results further indicate that these pericytic functions are likely compromised in type 2 diabetes.

The expression of genes and proteins and the location of pericytes overlap with those of vascular smooth muscle cells and other mesenchymal cells (fibroblasts/myofibroblasts) in the periendothelial compartment. A proper identification of pericytes thus requires assessing their location, morphology, and expression of markers (Armulik et al., 2011). We examined the expression of pericytic and endothelial cell markers by immunohistochemistry and ultrastructural features by transmission electron microscopy in pancreatic sections from mice and humans. A subset of vascular cells in mouse and human islets were immunoreactive for two bona fide pericytic markers: chondroitin sulfate proteoglycan 4 (neuron-glial antigen 2, NG2; Figures 1A and 1B) and platelet-derived growth factor receptor-beta (PDGFR-β; Figure 1C). NG2-labeled pericytes constituted ~3% of the human or mouse islet cell population (2.56 ± 0.25 % in mouse and 2.61 ± 0.37 % in human islets). Islet pericytes were closely associated with endothelial cells, extending cytoplasmic processes along the length of the capillaries (Figures 5-1A-1C). The long cytoplasmic processes spanned several endothelial cells and occasionally bridged neighboring capillary branches (Figures 1A’ and 1C). Many pericyte cell bodies were located at capillary branching points. At the ultrastructural level, pericytes
and their processes were found embedded within the vascular basement membrane (Figures 5-1E and 5-1F).

We calculated that pericytes cover around 40% of capillaries in mouse and human islets (Figures 1D and 1I). In islets from type 2 diabetic donors there was a significant decrease in the number of pericytes (Figures 5-1G-1I). Pericyte coverage of human islet capillaries was inversely correlated with the duration of the disease (Figure 5-1J). Islets from an individual that had diabetes for 10 years were nearly devoid of pericytes (Figure 5-1G). In the obese mouse model of type 2 diabetes (ob/ob mouse), islet blood vessels also had significantly fewer pericytes, which likely caused capillary dilation that characterizes the islet vasculature in this mouse strain (Dai et al., 2013; Hellstrom et al., 2001).

**Figure 5-1.** Capillaries in mouse and human islets are covered with pericytes (A-C) Z-stack of confocal images of mouse (A and C) and human islets (B) showing pericytes and
endothelial cells respectively immunostained for chondroitin sulfate proteoglycan (NG2, neuron-glial antigen 2, green) and for CD31 (PECAM, red). Nuclei are shown in blue. (A’) and (B’) higher magnifications of (A) and (B). Pericytes in mouse islets also express platelet-derived growth factor receptor-beta (PDGFRβ green) (C). Scale bars, 50 µm (A and B) and 10 µm (A’, B’ and C). (D) Quantification of the ratio of pericyte number to endothelial cell number in confocal images in mouse and human islets. Dots represent confocal images pooled from > 3 pancreas per group. Average ratios ± SEM are shown in green. (E and F) Transmission electron microscopic images of a pericyte cell body (E) and cytoplasmic processes wrapping capillaries in mouse islets (E and F). An alpha cell can be seen (α). Pericyte processes are embedded within the endothelial basement membrane (F). The pericyte cytoplasm is shown in green. Scale bars, 5 µm (E) and 2 µm (F). (G and H) Z-stack of confocal images of an islet from a type 2 diabetic individual (duration of disease = 10 years), showing pericytes (NG2, green), endothelial cells (CD31, red) and beta cells (insulin, blue). (H) Higher magnifications of pericytes covering capillaries in islets from a non-diabetic individual (upper panel) and type 2 diabetic individual (shown in (G), lower panel). Scale bars, 50 µm (G) and 20 µm (H). (I) Quantification of the ratio of NG2-immunostained area to CD31-immunostained area in human islets from non-diabetic or type 2 diabetic individuals (T2D). Dots represent the ratios of individual islets pooled from > 4 pancreases per group. Ratios ± SEM are shown in green (unpaired t-test; p-value shown in the graph). (J) Correlation between pericytic coverage of islet capillaries and the duration of type 2 diabetes ($r^2 = 0.32$, $p = 0.03$).
5.2 A subset of islet pericytes expresses contractility markers

Because pericytes have contractile properties, we examined the expression of markers of cell contractility. Alpha smooth muscle actin (\( \alpha \text{SMA} \)) is a major constituent of the contractile apparatus (Joyce, Haire, & Palade, 1985). We found that \( \sim 50\% \) of islet pericytes surrounding capillaries expressed \( \alpha \text{SMA} \) (Figures 5-2A and 5-2D), in contrast to the paucity of \( \alpha \text{SMA} \) in pericytes of other capillary beds (Crisan et al., 2012). Vascular smooth muscle cells of the feeding arteriole were also immunoreactive for \( \alpha \text{SMA} \) (Figure 5-2A). Most of the \( \alpha \text{SMA} \)-positive cells in mouse islets were also immunoreactive for NG2 (65 ± 4\%) and PDGFR\( \beta \) (98 ± 1 \%). The heterogeneity of pericytes is in line with observations in other microvascular beds that pericytes are a very diverse population (Shepro & Morel, 1993). Furthermore, staining for \( \alpha \text{SMA} \) was not distributed equally within the individual pericytes (Figures 5-2B and 5-2C), suggesting localized contractile function in pericyte processes wrapping capillaries.
Figure 5-2. A subset of islet pericytes expresses alpha smooth muscle actin

(A and B) Z-stack of confocal images of a mouse islet immunostained for N2 (pericytes, green), CD31 (endothelial cells, blue), and alpha smooth muscle actin (αSMA, red). White arrows point to pericytes that express both NG2 and αSMA. αSMA expression is not equal throughout the pericytes cytoplasm. Vascular smooth muscle cells with their circular processes around the arteriole show strong αSMA staining. A high magnification of (A) showing islet capillaries is shown in (B). (C) Pericyte cell bodies (arrows) can be seen extending αSMA-labeled processes between different capillary branches. (D) Quantification of the fraction of NG2-positive pericytes that expresses αSMA in mouse (M) and human (H) islets. Dots represent individual islets pooled from > 3 mice or human donors. Scale bars, 50 μm (A) and 10 μm (B and C).

5.3 Functional characterization of islet pericytes

To characterize islet pericytes physiologically, we adapted the pancreatic slice technique (Marciniak et al., 2014) to study responses to vasoactive substances in pericytes in their native environment. In living pancreatic slices, the different tissue components are preserved allowing to study interactions between endocrine cells and the vasculature, nerves, immune cells, and surrounding exocrine acini (Figure 5-3). Pericytes are electrically excitable cells and their contractile activity is controlled by changes in cytosolic free Ca$^{2+}$ concentration (Burdyga & Borysova, 2014). We used transgenic mice that express the genetically encoded Ca$^{2+}$ indicator (GCaMP3) in an inducible Cre recombinase dependent manner. Cre recombinase expression was driven by the NG2 promoter and thus, besides NG2-expressing oligodendrocyte precursor glia in the central nervous system, only vascular smooth muscle cells and pericytes express the Ca$^{2+}$ sensor [Figures 5-3A, 5-3A’,
and 5-3B; (Zhu, Bergles, & Nishiyama, 2008)]. By confocal time-lapse imaging of living pancreatic slices of NG2-GCaMP3 mice, we tested the effects of different neural, hormonal, and local paracrine signaling molecules on cytosolic Ca\textsuperscript{2+} responses in islet pericytes. We compared these responses to those of pericytes in the acinar tissue and vascular smooth muscle cells in arteries.

The catecholamines noradrenaline and adrenaline are vasoactive substances that induce changes in cytosolic Ca\textsuperscript{2+} levels in pericytes (Borysova, Wray, Eisner, & Burdyga, 2013; Burdyga & Borysova, 2014; Peppiatt et al., 2006). Applying adrenaline (100 µM) elicited increases in cytosolic Ca\textsuperscript{2+} in capillary and feeding arteriole mural cells (Figure 5-3C). Compared to Ca\textsuperscript{2+} responses in mural cells of the feeding arteriole, those in islet pericytes were more prolonged and showed occasional Ca\textsuperscript{2+} spikes (Figure 5-3C). In vascular smooth muscle cells of arteries, adrenaline induced a smaller cytosolic Ca\textsuperscript{2+} response, which, after a transient increase, was followed by repetitive, propagating Ca\textsuperscript{2+} spikes (Figure 5-3C). Adrenaline induced a small and transient response only in a subset of acinar pericytes (~ 50%).

Differences in Ca\textsuperscript{2+} responses between islet pericytes, vascular smooth muscle cells, and acinar pericytes were also observed with other vasoactive substances (Figures 5-3D and 5-3E). While some stimuli elicited responses in all types of mural cells (adrenaline and L-NAME; Figure 5-3E, left panel), other stimuli activated selectively pericytes (Figure 5-3E, middle panel) or smooth muscle cells (Figure 5-3E, right panel). Inhibiting nitric oxide synthase with L-NAME raised cytosolic Ca\textsuperscript{2+} in all types of mural cells (Figure 5-3E, left panel), likely by reversing nitric oxide decreases in IP\textsubscript{3}-stimulated Ca\textsuperscript{2+} release from intracellular stores that result from increasing cGMP levels and activating cGMP-
dependent protein kinase I (Borysova & Burdyga, 2015; Ruth et al., 1993) present in pericytes (Tian, Fessenden, & Schacht, 1999) and smooth muscle cells (Feil et al., 2002). Islet and exocrine pericytes differed in their responses to calcitonin gene related peptide (CGRP) and acetylcholine (ACh; Figure 5-3E), reflecting the known heterogeneity of pericyte populations (Rucker, Wynder, & Thomas, 2000; Shepro & Morel, 1993).

Figure 5-3. Recording Ca$^{2+}$ responses in living pancreatic slices reveals functional differences between mural cell populations

(A and A’) Z-stack of confocal images of a pancreatic slice from an NG2-GCaMP3 transgenic mouse processed for immunohistochemistry after a physiological experiment.
Shown is an islet immunostained for GFP (GCaMP3, green) and NG2 (red). The Ca$^{2+}$ sensor GCaMP3 is expressed in pericytes (colocalization appears yellow in merged images). Cell nuclei are shown in blue. Scale bars, 20 µm (A) and 10 µm (A’). (B) Confocal image of a pancreatic slice from an NG2-GCaMP3 transgenic mouse showing mural cells expressing GCaMP3 (green) and islet endocrine cells (backscatter signal, red). GCaMP3 is expressed by different mural cells located around arteries, arterioles and within the islet parenchyma. Scale bar, 20 µm. (C) Representative traces showing changes in mean GCaMP3 fluorescence intensity induced by adrenaline (100 µM) in islet pericytes (black), acinar pericytes (purple) and mural cells on the feeding arteriole (gray) or around the artery (blue). Increases in GCaMP3 fluorescence indicate increases in cytosolic Ca$^{2+}$ levels and are expressed as ΔF/F. Dashed horizontal line indicates the zero value. Each trace corresponds to one cell. (D) Representative traces showing changes in cytosolic Ca$^{2+}$ levels in islet pericytes (black) and smooth muscle cells (VSMCs, gray) induced by ATP (100 µM). Dashed horizontal line indicates the zero value. (E) Quantification of the percentage of islet pericytes (black bars), acinar pericytes (white bars) and smooth muscle cells (gray bars) that respond to different vasoactive substances: adrenaline (Adr, 50-100 µM), 1-nitroarginine methyl ester (L-NAME, 10 µM), endothelin-1 (ET-1, 10 nM), acetylcholine (ACh, 100 µM), kainate (Kai, 10 µM), calcitonin gene-related peptide (CGRP, 10 µM) and ATP (100 µM). (nd, not determined; N = 3 – 22 cells were examined per substance).

5.4 Pericyte activation is associated with changes in vascular diameter

Pericytes’ potential for contractility has been demonstrated ex vivo in brain and retinal slices (Peppiatt et al., 2006). Because vasoactive substances elicited Ca$^{2+}$ responses in islet pericytes (Figure 5-3), we sought to determine whether pericyte activation changes blood
vessel diameter in the islet. To correlate changes in cytosolic Ca\(^{2+}\) levels in pericytes with changes in vessel diameter, we labeled blood vessels in NG2-GCaMP3 mice with an intravascular injection of fluorescent \textit{Lycopersicon esculentum} lectin (Figures 5-4A and 5-4B).

Applying the vasoconstrictor endothelin 1 elicited a strong and prolonged contraction of islet capillaries (average reduction in capillary diameter of 29 ± 4 %, N = 5 vessels / 3 slice preparations; Figures 5-4C-5-4F), which was preceded by robust, long-lasting increases in cytosolic Ca\(^{2+}\) levels in pericytes (Figure 5-4D). We exposed slices to three different stimuli and found that each time the same subset of islet capillaries responded (Figures 5-4E, 5-4G-5-4I). On average, only 20% of the microvasculature responded to a vasoactive stimulus (19.9 ± 2.4%, N = 6 islets / 4 slice preparations; Figures 5-4H and 5-4I), which correlates with the proportion of capillaries covered by pericytes expressing \(\alpha\)SMA (Figures 5-1 and 5-2).

5.5 Beta cell activation inhibits islet pericytes and induces vascular dilation

Functional hyperemia is an increase in tissue blood perfusion during periods of heightened cellular metabolism. Whether or not this process involves pericytes of the microcirculation remains controversial. In pancreatic islets there is a positive correlation between endocrine cell activity and blood flow (Hellerstrom, Westman, Zachrisson, & Hellman, 1960; Jansson et al., 2016). Hyperglycemia and its associated beta cell activation increase islet capillary blood pressure (Carlsson, Jansson, Ostenson, & Kallskog, 1997), dilate islet arterioles (Lai, Jansson, Patzak, & Persson, 2007), accelerate blood flow and increase blood volume (Nyman, Ford, Powers, & Piston, 2010; Short, Head, & Piston, 2014). We hypothesized that activating beta cells with high glucose concentrations decreases islet pericyte activity,
which relaxes pericytes and dilates capillaries. We found that exposing pancreatic slices to an increase in glucose concentration from 3 mM to 16 mM decreased cytosolic Ca\textsuperscript{2+} levels in pericytes and simultaneously dilated islet capillaries (Figures 5-5A-5-5C, 5-5F-5-5H2). Diameters increased by 36 ± 6% (N = 10 vessels from 4 slice preparations, Figure 5G) in a subset of islet capillaries (Figures 5-4G and 5-4H). High glucose did not decrease cytosolic Ca\textsuperscript{2+} levels in vascular smooth muscle cells or in acinar pericytes.

Figure 5-4. Simultaneous recording of pericycle activation and capillary constriction
(A) Z-stack of confocal images of a pancreatic slice from an NG2-GCaMP3 transgenic mouse after intravascular injection of fluorescent *Lycopersicon esculentum* lectin to label the blood vessels (white). (B and B’) In the slice shown in (A), GCaMP3 fluorescence (Ca\(^{2+}\) levels) in pericytes (green) and capillaries (labeled with lectin, blue) in islets (red, backscatter signal) can be visualized simultaneously. Zoomed image of (B) is shown in (B’). (C) Sequential confocal images of an islet capillary before (left panel), during (middle panels; * indicates drug application) and after exposure (right panel) of a slice to endothelin 1 (ET-1, 10 nM). White arrows point at a constricting capillary region. (D) Representative traces showing simultaneous changes in cytosolic Ca\(^{2+}\) levels in islet pericytes (green traces) and capillary diameter (black trace) induced by endothelin 1 (10 nM). Dashed line on Ca\(^{2+}\) traces shows the zero value. (E) Temporal projection of a line scan perpendicular to the vessel axis shows the temporal pattern of changes in vessel diameter. Capillary borders can be seen in white. Endothelin 1 (ET-1) induced a strong constriction of the islet capillary. (F) Quantification of changes in diameter induced by endothelin 1 based on temporal projections as shown in (E). Values were scaled to the initial diameter (before drug application). Changes in diameter are shown for four different capillaries of the same islet. Not all islet capillaries constricted upon stimulation. (G) Temporal projection of line scans of two different islet capillaries stimulated with noradrenaline (NA, 10 µM, left) and high glucose (16G, 16 mM, right). The capillary shown in the top panel is the same as the one shown in (E) that had responded to endothelin 1. The same vessel constricted upon noradrenaline and dilated upon high glucose (responsive capillary). The one shown in the bottom panel does not respond to the stimuli applied (unresponsive capillary). (H and I) Quantification of the percentage of the islet microvasculature that responded to vasoactive
stimuli such as noradrenaline (NA), high glucose (16G) or ET-1, plotted for each stimulus (H), or for different islets independently of the stimulus used (I). Scale bars, 50 µm (A, applies to B) and 5 µm (C).

Among the signaling molecules released from beta cells that could mediate the effects on pericyte activity, we focused on ATP because ATP and its breakdown products ADP and adenosine have strong vascular effects (Burnstock & Ralevic, 2014). Of these, adenosine is a potent vasodilator (Berne, Knabb, Ely, & Rubio, 1983; Collis, 1989; O'Farrell et al., 2017). In islet pericytes, both adenosine and NECA (a non-specific agonist of adenosine receptors) decreased cytosolic Ca\(^{2+}\) levels (Figures 5-5D and 5-5I). The effect of exogenous adenosine mimicked that of high glucose; both inhibited cytosolic Ca\(^{2+}\) transients and baseline Ca\(^{2+}\) levels in islet pericytes (Figures 5-5C and 5-5D). Adenosine also inhibited cytosolic Ca\(^{2+}\) in smooth muscle cells in arterioles but not acinar pericytes.

To test if endogenous adenosine mediated the inhibitory effects of high glucose concentration on pericytes, we stimulated beta cells with 16 mM glucose concentration and applied theophylline, a non-specific antagonist of adenosine receptors reported to prevent islet blood flow increases after in vivo glucose administration (Carlsson et al., 2002). Theophylline abolished high glucose-induced capillary dilation (Figures 5-5A, 5-5B and 5-5F) and reversed the inhibition of cytosolic Ca\(^{2+}\) in islet pericytes (Figures 5-5A, 5-5B, and 5-5H), as did a selective adenosine A1 receptor antagonist [PSB36; Figure 5-5E]. These results indicate that, upon high glucose stimulation, adenosine accumulates in the islet and activates A1 receptors in islet pericytes, relaxing these cells and dilating capillaries.
Figure 5-5. High glucose stimulation of beta cells inhibits pericytes and dilates capillaries through adenosine and A1 receptors
(A) Temporal projections of line scans show changes in vessel diameter (upper and middle panels) of a feeding arteriole (upper panel) and an islet capillary (middle panel) and of cytosolic Ca\textsuperscript{2+} levels in a nearby capillary pericyte (green, lower panel) induced by increasing extracellular glucose concentration from 3 mM (3G) to 16 mM (16G) in a living pancreatic slice. High glucose increased capillary, but not arteriole, diameter and simultaneously decreased cytosolic Ca\textsuperscript{2+} in the pericyte. Theophylline (20 µM, in 16G), a non-specific antagonist of adenosine receptors, reversed the effects of high glucose. (B) Traces of responses as in (A) show the average change in vessel diameter (upper panel, N = 3 capillaries) and cytosolic Ca\textsuperscript{2+} levels in pericytes (lower panel). Dashed line on Ca\textsuperscript{2+} traces shows the zero value. Each trace corresponds to one pericyte. (C-E) Traces showing changes in cytosolic Ca\textsuperscript{2+} levels in islet pericytes induced by (C) high glucose (16 mM, 16G), (D) adenosine (50 µM) in 3 mM glucose and (E) A1 adenosine receptor antagonist (PSB36, 100 nM) in 16 mM glucose. Changes in baseline cytosolic Ca\textsuperscript{2+} levels (lower panel) are shown at a higher gain. Y-axis bars correspond to 20% change (ΔF/F). Each trace corresponds to one pericyte. Horizontal dashed lines show the zero value, and vertical dashed lines when stimuli were applied. (F) Quantification of the changes in capillary diameter of a responsive capillary induced by high glucose (16G) and the reversal by theophylline (Theo; in 16G) 2 min and 5 min after application of the antagonist. (G) Quantification of the changes in capillary diameter induced by 16 mM glucose. Each pair of symbols is one capillary (N = 10 capillaries pooled from 4 slice preparations, paired t-test). (H) Quantification of changes in cytosolic Ca\textsuperscript{2+} levels in pericytes induced by high glucose (16G) and high glucose plus theophylline (Theo, 20 µM). The area under the curve (AUC) was quantified in a 4-5 min recording in each condition. N = 4-14 pericytes from 3
slice preparations, one-way ANOVA corrected for multiple comparisons. (I) Quantification of the changes in cytosolic $\text{Ca}^{2+}$ levels in pericytes induced by adenosine (50 $\mu$M). Area under the curve (AUC) was quantified in 3 min recordings before, during and after adenosine. Data are scaled to AUC values before adenosine application (dashed horizontal line). $N = 7$ pericytes from 3 slices preparations, paired t-test.

5.6 In vivo imaging reveals functional hyperemia in pancreatic islets

Our results suggest that stimulated beta cells inhibit pericytes and thus dilate capillaries in the islet. To determine if high glucose-induced capillary dilation affects islet blood flow, we transplanted islets into the anterior chamber of the eye for in vivo imaging (Speier et al., 2008). Intraocular islet grafts are fully vascularized one month after transplantation in a pattern that closely resembles that of islets in the pancreas. Importantly, pericytic coverage of capillaries in islet grafts (Figures 5-6A and 5-6B) was similar to that found in the pancreas (Figure 5-1).

For in vivo imaging, blood vessels in islet grafts were visualized with an intravenous injection of a fluorescent dextran (Figure 5-6C). Capillary blood flow was intermittent and varied from capillary to capillary, as previously described (Y. M. Liu et al., 1993). We found that raising glycemia increased blood perfusion of islet grafts (Figures 5-6D and 5-6E). Blood started flowing through islet regions that were not perfused at lower glycemic levels (Figure 5-6D). Simultaneously, we observed a significant dilation of islet capillaries (Figures 5-6E-5-6G, Movies S4-S6). Not all blood vessels in the islet graft dilated, in line with our ex vivo findings (Figure 5-6G). The vascular dilation was similar to that elicited by high glucose concentrations in pancreas slices (Figure 5-5). We also quantified blood flow by counting red blood cells in the vascular lumen and found that
blood flow increased in blood vessels (Figure 5-6H), similarly to what has been reported for islets in the pancreas (Nyman et al., 2010; Short et al., 2014). These results indicate that raising glycemia increases islet blood flow through local dilation of capillaries in the islet microcirculation.

Figure 5-6. Raising glycemia dilates islet capillaries in vivo and increases blood flow
(A and B) Z-stack of confocal images of an islet graft 6 months after transplantation into the eye immunostained for NG2 (pericytes, green), CD31 (endothelial cells, red), and insulin (beta cells, gray). Cell nuclei are shown in blue. Pericytes cover capillaries in transplanted islets as they do in the pancreas. (B) Zoomed image of (A). (C-E) Z-stack of confocal images of the graft vasculature visualized with an i.v. injection of FITC-dextran. (D and E) Islet capillaries were imaged before and after an i.p. injection of 20% glucose (2 g/Kg body weight). Glycemia was measured at different time points. A rise in glycemia caused regions of the islet to become perfused (arrow in D) or dilated islet capillaries (arrows in E). Asterisks point at red blood cells. (F) Quantification of changes in capillary diameter in the islet before (glycemia = 147 mg/dL), 5-10 min (glycemia = 279 mg/dL) or 20-30 min (glycemia = 336 mg/dL) after injection of glucose. N = 30 capillaries from 3 islet grafts, one-way ANOVA corrected for multiple comparisons. (G) Capillaries were grouped according to their responses to a rise in glycemia: vessels that showed a progressive dilation (dilating capillaries) or vessels that did not change or exhibited a transient non-significant dilation (non-dilating). N = 13-17 capillaries from 3 islet grafts, one-way ANOVA corrected for multiple comparisons. (H) Quantification of the number of red blood cells, identified as black shadows in the vessel lumen, in different islet capillaries before (glycemia = 147 mg/dL) and after injection of glucose (glycemia 336 mg/dL). N = 4 capillaries, paired t-test. Scale bars, 50 µm (A and C), 20 µm (B, D, and E).

5.7 Control of pericyte activity by adrenergic input ex vivo and in vivo

In mouse and human islets, arterioles and capillaries are innervated by sympathetic axons (Rodriguez-Diaz, Abdulreda, et al., 2011; Tang, Peng, & Chien, 2014). We conducted immunohistochemical studies on mouse pancreatic sections and found that
tyrosine hydroxylase-positive sympathetic axons were in close contact with αSMA-positive pericytes [Figure 5-7A; see also (Rodriguez-Diaz, Abdulreda, et al., 2011)]. In slices, islet pericytes responded to application of the sympathetic neurotransmitters adrenaline and noradrenaline with strong increases in cytosolic Ca\textsuperscript{2+} (Figures 5-7B and 5-7C, see also Figure 5-3). To determine if islet pericytes respond to endogenous, locally released noradrenaline, we applied tyramine, a sympathomimetic that stimulates the release of neurotransmitters from nerve terminals (Gilliam, Palmer, & Taborsky, 2007; Graefe et al., 1999). Tyramine (50 µM) elicited an increase in cytosolic Ca\textsuperscript{2+} in islet pericytes (Figures 5-7B and 5-7C), indicating that islet pericytes responded to sympathetic nervous input. We investigated the effects of sympathetic neurotransmitters on islet blood vessel diameter and found that noradrenaline (10 µM) induced a strong contraction of a subset of islet capillaries (29 ± 6% reduction in capillary diameter, N = 5 vessels from 3 slice preparations; Figure 5-7F). Adrenaline (50-100 µM) also constricted islet capillaries (average reduction of capillary diameter of 12 ± 2%, N = 4 vessels from 3 slice preparations) but had a stronger effect on feeding arterioles (21 ± 4% reduction of arteriole diameter, N = 4 arterioles from 4 slice preparations; Figures 5-7D-7F).

To determine if sympathetic activation of pericytes affects islet blood perfusion, we transplanted islets from NG2-GCaMP3 mice into the eyes of NG2-GCaMP3 mice for \textit{in vivo} imaging. Three months after transplantation, islets were well engrafted and pericytes expressing GCaMP3 could be visualized. Similar to what we observed \textit{ex vivo}, spontaneous Ca\textsuperscript{2+} spikes could be seen in some islet pericytes before stimulation. Eye drops containing the α1-adrenergic receptor agonist phenylephrine applied topically increased cytosolic Ca\textsuperscript{2+} levels in islet pericytes (Figures 5-7G-5-7J), induced contraction of islet capillaries
(Figures 5-7J-5-7M) and decreased capillary blood flow in most vessels (Figure 5-7N). These results indicate that sympathetic agents activate islet pericytes \textit{in vivo}, leading to capillary constriction and reducing blood flow.

\textbf{Figure 5-7.} Sympathetic activation of islet pericytes leads to capillary constriction \textit{ex vivo} and \textit{in vivo}
(A) Z-stack of confocal images of a mouse islet immunostained for the sympathetic nerve marker tyrosine hydroxylase (TH, green), for αSMA (pericytes, red) and for CD31 (endothelial cells, blue). TH-labeled axons and varicosities can be seen in close contact with endothelial cells and pericytes in islet blood vessels. (B) Representative traces showing changes in cytosolic Ca^{2+} levels in islet pericytes induced by tyramine (50 µM) and noradrenaline (NA, 50 µM). Values are expressed as ΔF/F. Each trace corresponds to one pericyte. Dashed horizontal line indicates the zero value. (C) Quantification of changes in cytosolic Ca^{2+} levels in pericytes induced by tyramine (Tyr, 50 µM) and noradrenaline (NA, 50 µM). AUC was quantified in a 2 min recording in each condition and scaled to control values (in 3 mM glucose). N = 9-15 cells per group from 3 slice preparations; one-way ANOVA corrected for multiple comparisons. (D) Temporal projections of line scans through a feeding arteriole (upper panel) and a capillary (lower panel) show a reduction in vessel diameter induced by adrenaline (50 µM) in a living pancreatic slice. (E) Traces showing the average change in vessel diameter induced by adrenaline (black = capillaries, N = 3; gray = arterioles, N = 3). (F) Quantification of changes in vessel diameter for individual capillaries induced by adrenaline (50 µM) and noradrenaline (NA, 10 µM), scaled to the initial diameter (before catecholamine application, in 3G). (G and H) In vivo imaging of islets from NG2-GCaMP3 mice transplanted into the eye before and 5 min after administration of phenylephrine as eye drops. Backscattered light is shown in blue. (H) Increases in cytosolic Ca^{2+} levels in pericytes are evident in the zoomed images (upper panel, before; lower panel, 5 min after phenylephrine). (I) Trace (green) showing changes in cytosolic Ca^{2+} levels in islet pericytes in vivo before (left) and 5 min after application of phenylephrine (PE). Vertical dashed lines correspond to the 5 min period that the eye was
exposed to eye drops before rinsing with imaging buffer. Values are expressed as ΔF/F. A sustained response to phenylephrine was observed in islet pericytes in vivo, which was similar to the response to adrenaline of islet pericytes in slices (gray trace shown behind the in vivo trace). Average traces ± SEM are shown (N = 3 pericytes in vivo and in slices).

(J-L) In vivo imaging of NG2-GCaMP3 islet grafts in the eye before (upper panels) and 5 min after (lower panels) phenylephrine. Phenylephrine increased cytosolic Ca\(^{2+}\) levels in a pericyte (green) wrapping a constricting islet blood vessel (J). Arrows in (J), (K) and (L) point to constricting islet capillaries. Blood vessels were visualized with an i.v. of TRITC-dextran (red or grey) and islet tissue by backscatter (blue in J).

(M) Quantification of changes in blood vessel diameter in the islet before and after phenylephrine (N = 16 capillaries from 5 islet grafts in 3 mice; paired t-test).

(N) Quantification of the number of red blood cells, identified as black shadows in the vessel lumen, in different islet blood vessels before and after phenylephrine (N = 5 capillaries). Scale bars, 10 µm (A), 50 µm (G), 20 µm (H), and 10 µm (J, K, and L).

5.8 Summary and discussion of findings

Our study establishes that pericytes play an essential role in islet microvascular function. Pericytes extensively cover capillaries in mouse and human islets and respond to vasoactive substances released by the neighboring endothelium, sympathetic nerves, and beta cells. By changing cytosolic Ca\(^{2+}\) levels, these vasoactive substances alter pericytic contractile activity, thus regulating islet capillary diameter. Pericyte coverage of islet capillaries is strongly reduced in diabetic humans and mice, suggesting that under diabetic conditions islets lose pericyte control of vascular diameter and with it their ability to determine their own blood supply.
Although they constitute only 3% of the human or mouse islet cell population, pericytes contact several endothelial cells through long cytoplasmic processes, covering approximately 40% of the islet vasculature. Pericyte density or coverage correlates with endothelial barrier properties (Shepro & Morel, 1993). Our data suggest that the islet endothelial barrier has an intermediate leakiness (Richards et al., 2010): not as tight as the microvasculature of the retina or brain (pericyte : endothelial cell ratio of 1:1) but tighter than that of the lungs (ratio of 1:10). Our results challenge the idea that islet pericytes are few scattered vascular smooth muscle-like cells (Chu et al., 2013; Jansson et al., 2016; Lukinius, Jansson, & Korsgren, 1995). Instead, islet pericytes form a well-structured network around capillaries that may communicate with mural cells of the feeding arteriole and collecting veins. Islet pericytes express alpha smooth muscle actin (αSMA) and tropomyosin (Joyce et al., 1985), both essential proteins for contractile function. These findings indicate islet pericytes play an active role in the regulation of blood vessel diameter in the islet, as they do in other microvascular beds (Attwell, Mishra, Hall, O'Farrell, & Dalkara, 2016; Hall et al., 2014; Peppiatt et al., 2006).

Dilation of pre-capillary arterioles is considered to be the main mechanism that increases islet blood flow during heightened cellular metabolism (Guyton & Hall, 2006; Jansson et al., 2016). Yet we found that stimulating beta cells diminishes pericyte activity and leads to capillary dilation. That this happens ex vivo in pancreas slices indicates that islet capillaries can dilate actively and independently of the increase in blood pressure that could result from an opening of the feeding arteriole. Moreover, reversing glucose-induced pericyte relaxation reversed capillary dilation, demonstrating that local capillary dilation is a regulated process linked to a reduction in pericyte activity. Vasoactive substances known
to dilate feeding arterioles also decreased pericyte activity and dilated islet capillaries [e.g. adenosine analogs; Figure 5-5D; (Lai, Jansson, et al., 2007; Olsson, Jansson, Andersson, & Carlsson, 2000)]. Based on our results, we propose a mechanism for functional hyperemia where beta cell-derived signals inhibit islet pericytes, causing intra-islet capillaries to dilate.

What makes islet pericytes relax when beta cells are active? In beta cells, cytoplasmic ATP is transported into insulin granules by the vesicular nucleotide transporter (VNUT) and secreted together with insulin upon stimulation with increases in glucose concentration (Detimary et al., 1996; Geisler et al., 2013; Hazama et al., 1998). Once secreted, different ectonucleotidases rapidly degrade extracellular ATP to ADP, AMP, and adenosine, all of which play pivotal roles in the control of vascular tone (Burnstock & Ralevic, 2014; Yegutkin, 2008). Adenosine accumulates in metabolically active tissues, where it has potent vasodilator effects (Berne et al., 1983; Collis, 1989; O'Farrell et al., 2017; Tabrizchi & Bedi, 2001). Mouse pancreatic islets display broad nucleotidase activity in endocrine cells (e.g. NTPDase3) or in capillaries inside the islets [e.g. ecto-5'-nucleotidase; (Lavoie et al., 2010)]. It is therefore likely that ATP released from beta cells is eventually degraded to adenosine in the islet.

Endogenous adenosine may relax islet pericytes by activating potassium channels and inducing hyperpolarization (Hamilton, Attwell, & Hall, 2010). Adenosine has been shown to open K_{ATP} channels on retinal pericytes (Li & Puro, 2001) by binding to A1 receptors and activating Gαi, which antagonizes the inhibitory effect that ATP has on channel conductance (Terzic, Tung, Inanobe, Katada, & Kurachi, 1994). Our results suggest that endogenous adenosine acts on Gαi-coupled A1 receptors (Figure 5-5E). As
shown for retinal pericytes, activating A1 receptors could open $K_{\text{ATP}}$ channels and hyperpolarize islet pericytes, thus reducing cytoplasmic $\text{Ca}^{2+}$ levels. Pericytes would relax as a result, allowing for the capillary distension that increases blood flow locally.

Our findings further indicate that sympathetic nerves act on islet pericytes to reduce islet blood flow. Sympathetic nervous input increases cytosolic $\text{Ca}^{2+}$ in islet pericytes and decreases capillary diameter. This mechanism helps explain the reduction in islet blood flow that is associated with increased sympathetic tonus (Atef, Ktorza, Picon, & Penicaud, 1992; Jansson, Eizirik, & Sandler, 1989; Pettersson, Henriksnas, & Jansson, 2009). Pericyte activation and relaxation thus provide mechanisms for localized, bidirectional regulation of blood flow in the islet. While we focused on the effects of beta cell activation and sympathetic input, pericytes may respond to additional local cues. Previous studies have shown that the endothelium-derived vasoactive factors nitric oxide and endothelin-1 are major regulators of islet blood flow by acting on the feeding arteriole (Jansson, 1994; Lai, Persson, et al., 2007; Moldovan et al., 1996; Olsson et al., 2000; Svensson, Ostenson, Sandler, Efendic, & Jansson, 1994). We show that these endothelium-derived vasoactive substances activate islet pericytes as well. We haven’t yet studied their effects on islet blood flow. Nevertheless, the emerging picture is that the responses of capillary pericytes are coordinated with those of feeding arterioles, providing an additional level of blood flow control.

Islet pericytes could function as an electrical syncytium, as proposed for mural cells in the retina (Borysova et al., 2013; Zhang, Wu, Xu, & Puro, 2011). Indeed, islet vascular cells express the gap junction proteins connexin 43 (endothelial cells) and connexin 45 [smooth muscle cells; (Theis et al., 2004)]. Smooth muscle cells and pericytes could also
be electrically coupled to endothelial cells (Figueroa & Duling, 2009). Gap junctions between these cells would spread the effects of local signaling to other islet regions, including the feeding arteriole, and ensure efficient regulation of blood flow, as shown in other tissues (Iadecola, Yang, Ebner, & Chen, 1997; Peppiatt et al., 2006; Segal & Duling, 1986). Interestingly, synchronized activity of beta cells also affects intraislet blood flow. Altering beta cell electrical coupling, by knocking out connexin 36, the gap junction protein that connects islet beta cells (Serre-Beinier et al., 2000), not only disrupts pulsatile insulin secretion (Head et al., 2012; Ravier et al., 2005), but also glucose-dependent increases in islet blood flow (Short et al., 2014). By producing a unified, temporally restricted secretory burst, coordinated beta cell activity may ensure robust and concerted vascular responses that efficiently increase blood flow throughout the islet.

Our findings showing that pericytes play an active role in regulating islet blood flow also have pathophysiological implications. Diabetes is a microvascular disease characterized by pericytic dropout and dysfunction and altered vascular responses (Dodge & D'Amore, 1992; Silva et al., 2017). Our study now shows that diabetic microvascular complications also affect the islet. The striking loss of pericytic coverage in capillaries in islets from obese mice and individuals with type 2 diabetes likely impairs the adaptation of islet blood flow to increased islet metabolism and sympathetic input. This may compromise hormone release into the circulation, thereby exacerbating glucose intolerance. Islet pericyte dysfunction could thus contribute to the natural history of type 2 diabetes.
CHAPTER 6: Paracrine interactions within the pancreatic islet determine the glycemic set point

This chapter is work in collaboration with Dr. Rayner Rodriguez-Diaz. My contribution to this project was to longitudinally follow glycaemia as well as perform technical analysis with measuring hormone secretion from pancreatic islets. In addition to molecular assays, support was provided to Dr. Rodriguez-Diaz for glucose tolerance tests when administering ophthalmic eye solutions.

6.1 The pancreatic islet imposes its glycemic set point on the organism

The normal glycemic (normoglycemia) level, or, the quantity of mg of glucose per deciliter of blood of each animal species is unique. The variability of glycemia levels is astounding. Normoglycemia of one species may be lethal for another species (Davalli et al., 1995; Graham, Bellin, Papas, Hering, & Schuurman, 2011). Hyperglycemia in humans (high blood sugar levels, above 200mg/dl in humans) can lead to symptoms including fatigue, polydipsia, cardiac arrhythmia, and seizures. On the other hand, hypoglycemia (low blood sugar levels below 50 mg/dl) can lead to anxiety, nausea, vomiting and headache. In type 1 diabetics, the pancreatic islets of Langerhans are destroyed and blood sugar levels are unregulated. In this case, untreated severe hypoglycemia can be a lethal. For these reasons, the glucostat needs to be tightly regulated. The biological reasons for the organism specific glycemic set point are unclear.

Glucose homeostasis requires a complex crosstalk between organs which serve as metabolic regulators of glucose including, the liver, the brain (hypothalamus) and the pancreatic islet (Matschinsky et al., 2006; Schuit, Huypens, Heimberg, & Pipeleers, 2001). Because of the complex crosstalk between these organs, it has been difficult to discern,
which if one specific organ is responsible for the glucose set point of the organism. Studies from xenotransplantation models have shown that the pancreatic islet transfers the glycemic set point to the transplanted recipient (Carroll et al., 1992; Georgiou & Mandel, 1987). These studies suggest that the pancreatic islet likely contributes to setting the species normoglycemia level. However, we do not know the mechanisms in which the organism specific pancreatic islet assigns its necessary set point. This paper aims to identify the paracrine interactions within the islet that are necessary to fine tune the human pancreatic islet to maintain glucose homeostasis.

We used a xenotransplantation approach to determine whether the pancreatic islet serves as the bona fide glucostat in the body. Our strategy consisted of isolating the homeostatic contribution of the islet by transplanting islets from different species into the anterior chamber of the eye or under the kidney capsule of immunodeficient nude mice rendered diabetic with streptozotocin. As donors for the islets we used three species that differ widely in their normoglycemia, namely humans, cynomolgus monkeys, and C57Bl6 mice (Figure 6-1A). When islets from these species were transplanted into diabetic nude mice they restored normoglycemia to values that were indistinguishable from those of the respective donors [Figures 6-1B and 6-1C; human (86 ± 5.2 mg/dL) versus mice with human islets (80.2 ± 8.5 mg/dL); C57Bl6 (153 ± 14 mg/dL) versus mice with C57Bl6 islets (144.8 ± 10 mg/dL), monkeys (52 ± 7 mg/dL) versus mice with monkey islets (55 ± 9.6 mg/dL); mean ± SD]. Human and monkey islets imposed lower glycemic levels, whereas islets from C57Bl6 mice engrafted under the kidney capsule forced higher glycemic levels upon the recipient nude mice. These results suggest that the islet alone can set the target glycemic values of the species.
Figure 6-1. Pancreatic islet grafts transfer the glycemic set point of the islet donor species to recipient mice

(A) Non-fasting glycemic levels of humans \((n = 5)\), C57Bl6J mice \((n = 20)\), and cynomolgus monkeys \((n = 11)\) were significantly different. (B) Nude mice rendered diabetic with streptozotocin (STZ) transplanted with islets from humans \((n = 47\) recipient mice), monkeys \((n = 22)\), and C57Bl6J mice \((n = 8)\) became normoglycemic with glycemic levels of the islet donor species. Curves are shown as average \(\pm\) SD. (C) Quantification of results shown in B. All glycemic values were significantly different from each other. Data are shown as box and whisker plots and compared with one-way ANOVA followed by Tukey’s multiple comparison tests. Asterisks denote significance \((P < 0.05)\).

6.2 Islets from species with lower glycemic set point dominate glycemia

How does transplanting islets affect the normoglycemia already established by islet grafts from a different species? We transplanted human islets under the kidney capsule of diabetic nude mice and, once normoglycemia was restored to human levels, we transplanted mouse islets into the eye (Figure 6-2A). Despite adding islet mass, this procedure did not change glycemia. When human islet grafts were removed, glycemic levels increased to reach the mouse donor’s normoglycemia (Figures 6-2A and 6-B). These
results showed that both human and mouse islets engrafted functionally. More importantly, they indicate that human islets were dominant. This can be explained by human islets having a glucose-dependency curve of insulin secretion that is shifted to lower glucose concentrations, with a glucose concentration threshold ~54-72 mg/dL for human islets versus ~90 mg/dL for mouse islets (Henquin, Dufrane, & Nenquin, 2006). Hence, the most likely interpretation of our results is that insulin secretion from human islet grafts was stimulated at lower glucose levels, thus preventing glycemia from reaching levels that would activate beta cells in the mouse islet grafts. In this scenario, islets with the lower set point impose glycemia.
(A and B) Non-fasting glyceremia of diabetic nude mice transplanted with human islets under the kidney capsule (red arrow) and then with islets from C57Bl6J mice into the eye (black arrow; n = 11 recipient mice). Human islets grafts were later removed by nephrectomy, which changed glycemic values to mouse levels (quantified in B). Data are shown as average ± SD (A) or box and whisker plots (B) and compared with one-way ANOVA followed by Tukey’s multiple comparison tests. Asterisks denote significance (P < 0.05).

(C-E) Non-fasting glyceremia shows that non-diabetic nude mice transplanted with human islets (black symbols, n = 5) acquired the human glycemic set point (C). Endogenous release of mouse insulin was inhibited in the presence of human islet grafts (D), but plasma glucagon levels were not affected (E). (F) Human insulin plasma levels in transplanted mice without endogenous islets (STZ-treated, STZ+) and with endogenous islets (STZ-; 15 measurements in 5 mice). Asterisks denote significance (P < 0.05, Student’s t-tests). (G-I) Intraperitoneal glucose tolerance test (4g/kg glucose) followed by an insulin tolerance test (0.75 U/kg insulin) performed in diabetic nude mice transplanted with human islets (G; n = 6 mice) show adequate insulin and glucagon responses to the glucose challenge (H) and the induced hypoglycemia (I), respectively. Hormone plasma levels were measured at the time points indicated in G (arrows). Asterisks denote significance (P < 0.05, Student’s t-tests).

It is possible that the diabetic mouse model we used compromised glucose counterregulation, the protective response against hypoglycemia (Farhy et al., 2008; Shi et al., 1996). This could limit the recipient mouse ability to counteract the lower glycemia imposed by human islets. To address this issue, we transplanted human islets into intact, non-diabetic nude mice and found that this procedure still moved glycemic levels to the
human set point (Figure 6-2C). In these mice, endogenous (mouse) beta cell insulin secretion was inhibited by ~85% (Figure 6-2D), while glucagon plasma levels were similar to those of control non-transplanted mice (Figure 6-2E). Plasma human insulin levels were the same whether or not transplanted mice had endogenous islets (Figure 6-2F), confirming that human islet grafts prevented glycermia from reaching levels that activate mouse beta cells. Alpha cells were not activated probably because the threshold for the glucagon counterregulatory response in mice is between 63 and 72 mg/dL (Malmgren & Ahrén, 2015), which is below the human glycemic set point (~80 mg/dL). Glucagon responses, however, could be elicited by hypoglycemia (< 50 mg/dl), and insulin responses by hyperglycemia (>120 mg/dl), demonstrating that hormone secretion from human islet grafts was not passive but appropriately regulated by changes in glycermia (Figures 6-2G-6-2I). We therefore conclude that hormone secretion from human islet grafts was responsible for maintaining normoglycemia in the recipient mouse.

### 6.3 Islet mass is not a determinant of the glycemic set point

Functional engraftment of human and monkey islets required transplanting a larger islet mass. To determine the effects of islet graft mass on glyceremia we transplanted different numbers of mouse and human islet equivalents into recipient mice (Figure 6-3). A titration of mouse islet graft mass in the eye showed that mice receiving smaller amounts of islets took longer to recover from diabetes (Figure 6-3B). However, mice in all groups returned to the typical glyceremic level of the donor mouse (Figure 6-3C) despite having different islet graft volumes and graft insulin contents (Figures 6-3D, E, and J). Doubling the number of human islet grafts under the kidney capsule (Figure 6-3F) resulted in different graft insulin contents (Figure 6-3I), but did not affect the characteristic human
glycemic set point in the recipient mouse (Figures 6-3H and 6-J. Insulin plasma levels were also independent of islet graft mass (Figures 6-3E and 6-3I). These results rule out that islet mass affected the glycemic set point.

Figure 6-3. Glycemic levels are independent of transplanted islet mass but depend on donor species
(A) Z-stacks of confocal images of the eyes of nude mice transplanted with 500, 300, 150, or 75 islet equivalents (islet backscatter shown in green and blood vessels in red. Asterisks indicates pupils; images acquired at day 70 after transplantation). (B and C) Non-fasting glycemic values show that transplanting different numbers of islets from C75Bl6J mice into diabetic nude mice reversed diabetes and produced similar levels of glycemia (quantified in B, n = 3 mice per group). Note, however, that recipient mice with a smaller mass of transplanted islets needed longer to return to normoglycemia. (D) Islet graft volumes of mice shown in A-C estimated by measuring islet backscatter (green) at days 35 (solid bars) and 70 (patterned bars) after transplantation. Mice receiving 500 islets had significantly more islet mass than those receiving 75 islets (P < 0.05, ANOVA followed by multiple comparison test). Over time, there was a small increase in islet volume in mice transplanted with fewer islets. (E) Mouse graft insulin contents were different for the four groups of mice at day 70 (gray scale columns; 500 significantly different from 75; P < 0.05, ANOVA followed by multiple comparison test), but plasma insulin concentrations (red symbols) were similar. (F) Photograph of nude mouse kidneys transplanted with 1000 or 2000 human islets. Arrows point at islet grafts, which were used for quantifications of mass in I and J. (G and H) Transplantation of 1000 and 2000, but not 500, human islets reversed diabetes in recipient nude mice (n = 3 mice per group). Mice with successful islet engraftment showed human normoglycemic values that were independent of the number of transplanted islets (quantified in H). (I) Human graft insulin contents were different for the two groups of mice at day 30 (P < 0.05, Student’s t-tests), but plasma insulin concentrations (red symbols) were similar. (J) In mice transplanted with different human (red symbols) and mouse (grey symbols) islet masses, graft insulin content did not correlate
with target glycemia (slopes of regression lines not significantly different from 0), indicating that, once above the marginal mass required to achieve glucose homeostasis, islet mass does not impact the glycemic set point.

### 6.4 Artificial manipulation of nervous input changes the glycemic set point established by mouse but not by human islets transplanted into the mouse eye

Our results support the hypothesis that the islet serves as the overall glucostat in the organism. If so, manipulating the physiology of islet grafts should change the glycemic set point. We previously showed that intraocular mouse islet grafts are re-innervated according to their innervation pattern in the pancreas and that the autonomic nervous input to intraocular grafts can be manipulated via the pupillary light reflex (Rodriguez-Díaz et al., 2012). We found that artificially manipulating the nervous input to mouse islet grafts with light changed insulin secretion and normoglycemia in recipient mice (Rodriguez-Díaz et al., 2012), indicating that the glycemic set point can be adjusted by modulating islet function.

We transplanted human islets into the eye and found that the innervation patterns of intraocular islet grafts mimicked those of islets in the pancreas (Rodriguez-Díaz, Abdulreda, et al., 2011), that is, human islet grafts were innervated almost exclusively by sympathetic axons mostly targeting blood vessels (Figures 6-4A and 6-4B). By contrast, intraocular mouse islet grafts were densely innervated by parasympathetic axons (Figure 6-4C), as they are in the pancreas (Rodriguez-Díaz et al., 2011a). Activating parasympathetic input by increasing the ambient illumination did not affect glycemia or glucose tolerance in mice with intraocular human islet grafts Figure 6-4D or in mice with mouse islet grafts under the kidney capsule (Figure 6-4D), but decreased glycemia in mice
with mouse islet grafts in the eye (Figure 6-4D, see also Rodriguez-Diaz et al., 2012). These results indicate that nervous input to intraocular grafts can be manipulated with light. This manipulation, however, does not affect human islet grafts, which is in line with anatomical findings showing that the parasympathetic innervation of the human islet is sparse (Rodriguez-Diaz, Abdulreda, et al., 2011). Our findings thus suggest that the human glycemic set point does not depend on nervous input to the islet.

Figure 6-4. Modulating nervous input to islet grafts affects glycemia in nude mice transplanted with mouse islets, but not in mice transplanted with human islets

(A and B) Maximal projections of Z-stacks of confocal images of intraocular human islet grafts 90 days after transplantation showing immunostaining for the parasympathetic and sympathetic axon markers vAChT and TH, respectively. Notice that parasympathetic
axons of the iris do not turn into the graft and that vAChT is present in endocrine cells. By contrast, some sympathetic axons reach into the islet parenchyma along blood vessels (labeled for αSMA). These staining patterns resemble those of islets in the native human pancreas (Rodriguez-Diaz et al., 2011a, 2011b). A’ and B’ are higher magnification images of regions denoted by boxes in A and B. (C) In contrast to human islet grafts, C57Bl6J mouse islet grafts showed a high density of parasympathetic axons in the islet parenchyma (see also Rodriguez-Diaz et al., 2012). Scale bars, 50 µm (A-C) and 10 µm (A’ and B’). (D) Non-fasting glycemic values show that modulating nervous input to human islet grafts via the pupillary light reflex with ambient illumination did not change glycemic levels. By contrast, increased nervous input reduced glycemic levels in mice with intraocular mouse islet grafts, but not in mice with mouse islets transplanted under the kidney capsule (P < 0.05, Student’s t-tests). Values obtained > 2 months after transplanting 1000 human islets into both eyes (n ≥ 12 mice per group) or 300 C7Bl6J mouse islet into the right eye or the kidney of diabetic nude mice (n = 4-5 mice per group).

6.5 Beta cell secretion has to be amplified by adjacent alpha cells to establish the human glycemic set point

What are the intrinsic properties of islets from different species that dictate different levels of glycemia? A salient feature of human islets is that they contain a larger proportion of glucagon-secreting alpha cells than mouse islets (M Brissova et al., 2005; Cabrera et al., 2006). Human alpha cells secrete glucagon and acetylcholine, which are strong potentiators of glucose-induced insulin secretion in vitro (Huypens, Ling, Pipeleers, & Schuit, 2000; Rodriguez-Diaz, Dando, et al., 2011). We therefore hypothesized that alpha cell input, by increasing the efficacy of beta cell responses to glucose, affects glycemic levels. To test
this hypothesis, our strategy was to transplant human islets into diabetic nude mice and, after restoring normoglycemia, inhibit human glucagon receptors with a specific antagonist (L-168,049) that does not affect mouse glucagon receptors (Cascieri et al., 1999; de Laszlo et al., 1999). To block cholinergic signaling we topically applied the muscarinic antagonist tropicamide to mouse eyes with human islet grafts, as previously described (Rodriguez-Diaz et al., 2012). Injection of L-168,049 (50 mg/kg, ip) did not induce changes in the glycemia of control nude mice, but increased glycemia in transplanted mice by ~50 mg/dL (73% change; Figures 6-5A and 6-5B). This treatment decreased plasma human insulin levels by 44% (Figure 6-5C).

We further tested islet graft function by using intraperitoneal glucose tolerance tests and found that inhibiting the human glucagon receptor made recipient mice glucose intolerant (Figures 6-5D and 6-5E). By contrast, tropicamide did not produce changes in glucose tolerance (Figure 6-5F). We previously showed that the glucagon-like peptide-1 (GLP-1) analog liraglutide, an incretin mimetic, accelerated engraftment and diabetes reversal but did not change the glycemic set point (Abdulreda, Rodriguez-Diaz, Caicedo, & Berggren, 2016). These results indicate that local glucagon input, but not cholinergic or incretin input, was needed to produce an adequate beta cell response to the glucose challenge.

To confirm that glucagon input influences insulin secretion from human beta cells, we performed in vitro perifusion studies of hormone secretion. The glucagon receptor antagonists L-168,49 (50 nM) and des-His1-[Glu9]-glucagon (1-29) amide (1 μM) decreased insulin secretion stimulated by step increases in glucose concentration by ~25% (Figures 6-5G and 6-5H). Inhibition of glucose stimulated insulin secretion from human
islets measured \textit{in vitro} with static incubation has been reported for des-His1-[Glu9]-glucagon (1-29) amide (Huypens et al., 2000). These antagonists did not affect insulin secretion from mouse islets (Figure 6-5I), indicating that the impact of glucagon on insulin secretion is minimal in this mouse strain under these conditions.

These results suggest that the relative number of alpha cells, and hence the putative levels of intra-islet glucagon, could affect the glycemic set point in mice other than the alpha-cell-poor C57Bl6 mouse.

\textbf{Figure 6-5.} Glycemic values set by insulin secretion from human islet grafts require glucagon input from neighboring alpha cells
(A-C) Injection of the human-specific glucagon receptor antagonist L169,049 (50 mg/kg, ip, day 0) increased glycemia in nude mice with human islets transplanted into the eye (glycemic levels quantified and compared to levels before treatment in B; \( n = 6 \) mice). During treatment with L169,049, human insulin plasma levels were significantly reduced in recipient mice (C). (D and E) Local application of L169,049 to the eye (4 mM) made transplanted mice glucose intolerant in intaperitoneal glucose tolerance tests (2 g/kg glucose, quantification as area under the curve of glucose excursion shown in E, \( n = 8 \) mice). (F) Local application of the muscarinic antagonist tropicamide (0.5%, 17 mM) did not affect glucose tolerance (4 g/kg glucose) in mice transplanted with human islets (\( n = 3 \) mice). Data are shown as average ± SEM and compared with Student’s t-test. Asterisks denote significance (\( P < 0.05 \)). (G-I) Perifusion assays to measure insulin secretion showing that the glucagon receptor antagonists L-168,49 (50 nM) and des-His1-[Glu9]-Glucagon (1-29) amide (1 \( \mu \)M) diminished glucose-stimulated (3, 5, and 7 mM) insulin secretion from human islets (G and H), but not from mouse islets (I). Antagonists were added at 3 mM glucose concentration (arrow) and maintained throughout the experiment. H shows a quantification (area under curve during stimulation with 5 and 7 mM glucose concentration) of experiments shown in G. Asterisks denote statistical significance (\( n = 4 \) islet human donors; \( P < 0.05 \); ANOVA followed by multiple comparisons).

6.6 Summary and discussion of findings

Our results establish the pancreatic islet as the dominant player in determining the glycemic set point in the organism. Despite being exposed to non-physiological glycemia for months under the control of the “mismatched” islets, the recipient mice did not or could not deploy mechanisms to compensate for the chronically altered glycemic levels imposed
by the engrafted islets. The main conclusion from our studies is that the transplanted islets sense glucose levels and adjust insulin secretion until the organism reaches the species’ glycemic set point. Our results further demonstrate that paracrine glucagon signaling in the islet is critical for the beta cell to secrete the appropriate insulin amounts that sustain the human glycemic set point. This is in line with studies showing that glucagon signaling through glucagon and GLP-1 receptors contributes substantially to the beta cell’s secretory responsiveness and competence by increasing cAMP levels (Bertuzzi et al., 1995; Huypens et al., 2000; Pipeleers, in't Veld, Maes, & Van De Winkel, 1982; SAMOLS, MARRI, & MARKS, 1965).

Our findings are in sharp contrast to studies showing that the impact of alpha cells on beta cell function is negligible in rodents (King et al., 2007; Moens et al., 2002; Shiota et al., 2013; Thorel et al., 2011). The smaller proportion and spatial segregation of alpha cells in mouse and rat islets likely explains why rodent studies could not have predicted that the glycemic set point depends on islet glucagon signaling. Moreover, mouse alpha cells have an activation threshold (~70 mg/dL; (Malmgren & Ahrén, 2015]) that is much lower than the mouse glycemic set point (~140 mg/dL) and thus cannot contribute to set glycemic target values. By contrast, in the human islet under normoglycemic conditions (~90 mg/dl), alpha cell activation overlaps with beta cell activation. Indeed, when isolated human islets are exposed to step increases or decreases in glucose concentration, it is clear that both insulin and glucagon secretion are stimulated at 90 mg/dl (Figure 6-6A). This is contrary to the general notion that glucagon and insulin secretion are mutually exclusive. Although seemingly counterintuitive because these hormones have antagonistic effects on
plasma glucose levels, these findings make sense if we consider glucagon a local paracrine signal that amplifies insulin secretion to stabilize glucose levels.

The fluid dynamics in the native pancreas may hinder glucagon to reach local concentrations that activate or prime beta cells. Indeed, an in vitro study using the perfused rat pancreas model showed that glucose-induced insulin secretion occurs independently of an amplifying signal from neighboring alpha cells (Moens et al., 2002). While determining local glucagon concentration in the islet in vivo is beyond what current methods can detect, there is nevertheless a strong case to be made that local glucagon amplifies beta cell activity in the living organism. We found that local glucagon affects insulin secretion using an in vivo model that reproduces blood flow, capillarity, and ultrastructural features of the islet vasculature in the pancreas (Speier et al., 2008a; Almaça et al, 2014). In the human islet, the percentage of alpha cells is higher, alpha cells and beta cells are aligned randomly along blood vessels, and most beta cells face alpha cells (> 70%, Cabrera et al., 2006), making it likely for beta cells to be directly exposed to glucagon secretion. In mice, glycemic levels are lower when the percentages of alpha cells are higher (Figure S6), suggesting that if glucagon input is increased it may lead to similar effects. The strong insulinotropic effects of glucagon (Samols et al., 1965; Huypens et al, 2000; present study), the increased insulin secretion from beta cells overexpressing glucagon receptors (Gelling et al., 2009), and the association of glucagon receptor mutations with reduced insulin secretion and type 2 diabetes (Hager et al, Nature Genetics 1995; Hansen et al., Diabetes, 1996), all lend further support to the notion that intra-islet glucagon influences insulin secretion.

Glucagon is a major hyperglycemic hormone in the organism that counters decreases in plasma glucose levels. Indeed, glucagon secretion provides the first line of
defense in glucose counterregulation (Cryer, Davis, & Shamoon, 2003). Glucagon, however, has also been known for decades as a strong amplifier of insulin secretion (SAMOLS et al., 1965), whose effects are opposite to those of glucagon. One answer to this conundrum is to consider glucagon secretion as a mechanism that participates in two different regulatory circuits. In our view, glucagon secretion during normoglycemia reaches concentrations in the islet that amplify insulin secretion from neighboring beta cells (Figure 6-6B). As we show here, this local secretion is needed to maintain the human glycemic set point. Glucagon secretion under these circumstances is probably not strong enough to reach plasma levels that produce systemic responses. By contrast, when glycemia drops, glucagon secretion becomes strong enough to produce systemic, hyperglycemic effects, but cannot stimulate beta cells because glucose levels are no longer permissive for insulin secretion.

How can the role of glucagon secretion during normoglycemia be described in terms of homeostatic control? A regulatory system that maintains glucose homeostasis must include sensors, disturbance detectors, an integrator, and effectors. It is clear that both alpha and beta cells are specialized glucose detectors endowed with mechanisms to sense glucose. Any change (i.e. disturbance) in glucose concentration, the regulated variable, produces changes in alpha and beta cell physiology that can be considered disturbance signals (e.g. cell membrane depolarization or hyperpolarization, changes in intracellular Ca\(^{2+}\) concentration). These error signals ultimately converge on insulin granule exocytosis, which is the eventual integrator (controller) that uses the disturbance signals to send out the control signal insulin to the effector organs (liver, muscles, and fat). By increasing cAMP concentration in beta cells, glucagon secretion produces a disturbance signal that is
one of the input signals for insulin exocytosis. When activated during glucose counterregulation, by contrast, alpha cells become integrators (controllers) themselves, and glucagon acts as a control signal that directly instructs effector organs to produce and release glucose.

The glycemic set point likely arises from the dynamic interactions between alpha and beta cells. Mathematical models of glucose homeostasis predict that interactions between alpha and beta cells are beneficial because they provide more stable, efficient and accurate control of glycemia (Jo, Choi, & Koh, 2009; Koeslag, Saunders, & Terblanche, 2003). In these models, the interactions between alpha and beta cells need to be asymmetric to build a negative feedback loop for both cells. Indeed, glucagon and acetylcholine, both secreted from alpha cells in human islets, stimulate insulin secretion, whereas all known secretory products of beta cells inhibit glucagon secretion (Caicedo, 2013). This arrangement favors stability by attenuating exacerbated responses and works best with the prevailing small fluctuations in plasma glucose levels. As we show here, interrupting this feedback loop by inhibiting glucagon receptors on beta cells acutely destabilizes the glycemic set point, thus confirming the predictions made by the mathematical models.

Islet regulation of glucose homeostasis can be supplemented with feedforward mechanisms that temporarily override the glucostat. These anticipatory control mechanisms ensure that the islet is prepared for upcoming disturbances. Thus, incretin hormones produce anticipatory responses in the islet to food in the gut, and the autonomic nervous system produces anticipatory responses to food ingestion, to intense muscular activity, as well as to several other cues. Circulating signals derived from other organs may also affect islet function [e.g. bile acids from the liver, (Seyer et al., 2013)]. However, our
transplantation results indicate that these additional regulators do not supersede the islet’s intrinsic ability to establish the glycemic set point. It can be argued that human and monkey islet grafts may not respond to mouse cues, but our results show that islets from C57Bl6 mice impose higher glycemic levels on recipient nude mice without being affected by circulating factors or other compensatory mechanisms.

Based on our results, we conclude that the human glucostat depends on the functional cooperation between alpha and beta cells, not solely on the beta cell. This has implications for therapies aimed at reconstituting the beta cell population to treat diabetes because the glycemic levels set by beta cells without glucagon input would likely be pre-diabetic. In addition, new approaches to inhibit the contribution of glucagon to hyperglycemia need to be reexamined because inhibiting glucagon receptors systemically may also eliminate this crucial local input to the beta cell.

![Figure 6-6](image)

**Figure 6-6. Alpha cells and beta cells of the human islet cooperate to maintain the glycemic set point.**

(A) Glucose concentration-response relationship for insulin and glucagon secretion from human islets. Values were obtained in dynamic perifusions and represent the secretory
levels during step increases in glucose concentration \((n = 4\) human pancreas preparations). Notice that glucagon and insulin secretion overlap substantially around 5 mM glucose concentration (equivalent to 90 mg/dl). Glucagon secretion at 5 mM was significantly different from that at 11 mM glucose concentration (one-way ANOVA followed by Tukey’s multiple comparison tests). (B) Cartoon depicting our view of the glucose homeostat. Fluctuations in glucose concentration \((\Delta [\text{glucose}])\) around the glycemic set point are sensed by alpha and beta cells that continuously influence each other to fine-tune insulin secretion. Insulin serves as the control signal that regulates glucose uptake in effector organs (e.g. liver, muscles, and adipose tissue) to maintain normoglycemia. Without paracrine glucagon input to beta cells, the glucose homeostat fails to achieve target glycemic levels.
CHAPTER 7: Reseal-able, Optically accessible, PDMS-free fluidic platform for *ex vivo* interrogation of pancreatic islets.

The estimated cost of diagnosed diabetes in 2012 was $245 billion dollars. This immense cost drives a need for the development of tools to evaluate pancreatic islets. This chapter of the thesis is related to a collaboration with a bioengineer, to develop novel tools to study physiology in pancreatic islets. A microchip was developed for purposes of improving current limitations in culturing and perifusion of pancreatic islets. My role in this project was to develop the biological tools necessary for displaying the capabilities of a novel “lab on a chip” device, which allows for interrogation of islet physiology.

7.1 Current microfluidics tools used in diabetes research

Current techniques to evaluate the pancreatic islet function include measuring; hormone release, Ca$^{2+}$ imaging, oxygen consumption rates, as well as numerous other diagnostic assays. However, these current assays exist as separate tools, which are not easy to co-integrate. Commercially available perfusion systems such as the PERI4-02TM from Biorep Inc., Miami FL, involve large fluidic system volumes and require incubation of islets in bead chambers, which is burdensome to access the islet tissue. This paper aims to develop a new platform that gives researchers a new integrative tool, which allows for multidimensional analysis of pancreatic islets in an easy to use, robust lab chip. This work was in collaboration with a research group of engineers, who developed the design and fabrication of the optically accessible, PDMS-free fluidic platform. My contribution to this paper was to take advantage of the biological tool available to display the functionality of the developed lab chip.
7.2 Development of biological tools to exploit optically clear microfluidics systems

We were able to take advantage of a perfusion capable, optically clear chip, using islets isolated from transgenic mice which express the light-gated ion channel channelrhodopsin. Channelrhodopsin proteins enable researchers to control the electrical excitability of cells using light. When channelrhodopsin is activated, excitable cells become depolarized and stimulate Ca\(^{2+}\) flux into the cell. We generated F1 mice (Soma-cre x Chr2-Rosa), which expressed channelrhodopsin in the somatostatin secreting delta cell of the pancreatic islet. The islets from F1 Soma-Chr2+ mice were isolated and loaded onto the chip and mounted onto the fluorescent microscope. We performed a perifusion on the lab chip using a peristaltic pump, outfitted for the chip attached to the microscope stage. Low glucose (3 mM) was perfused with and without light stimulation and the results were analyzed using somatostatin a sensitive ELISA.

![Image](image.png)

**Figure 7-1. Optogenetics study on delta cells.** (A) Comparison of somatostatin released in each condition, calculated as cumulative signal (area under the curve), response to light is compared to the response to KCl; both anova and t-test have a p < 0.05. (B) Somatostatin
response to depolarization induced by KCl stimulus, average (±σ) of 3 wells (no light). (C) Somatostatin time response to light stimulation in two wells out of three, third well contains the control (no light). (D) Islets expressing Red Fluorescent Protein (RFP) show the delta cell structure.

We found that the optically clear chip indeed allowed light to pass through the chip and stimulate somatostatin release from Soma-Chr2+ islets. In the wells that did not receive light treatment, no increase in somatostatin protein was detected in the ELISA assay. After retrieval of pancreatic islets, they were viable and did not show any difference in the glucose response profile from physiological recordings from the current commercially available perifusion system available (Biorep).

**Figure 7-2.** Comparison of multiple microfluidic platforms for investigation of islet hormone secretion. (A) Comparison of dynamic GSIS performed in the three channels of the present device (FP-3W, solid black lines) and two standard vial chambers of the
commercial PERI4-02 machine (dotted red lines) with mouse islets obtained from the same isolation. FP-3W was run at flow rate of 50µL/min (per channel) with sampling every two minutes, while PERI4-02 vial chamber was run at 100µL/min with sampling every minute. (B) Dynamics GSIS in mouse islets performed with FP-3W installed on a PERI4-02 platform at University of Florida. FP-3W was run at 100µL/min (per channel) with sampling every minute. (C) Dynamic GSIS in B6 mice islets performed at day 1 (black line), 3 (blue) and 8 (red) after isolation; FP-3W was installed on a PERI4-02 and flow run at 100µL/min (per channel) with sampling every minute. Secreted insulin on day 8 was measured with ultrasensitive ELISA (see insert). (D) Dynamic GSIS in human islets performed with FP-3W installed on a PERI4-02 platform; FP-3W was run at 50µL/min (per channel) with sampling every two minutes.

7.3 Summary and discussion of findings

These results indicate that using this novel lab chip, we may better incorporate assays to assess islet function using this optically clear microfluidic device. Experiments using Ca\(^{2+}\) imaging techniques with fluorescent reporters such as fluo-4 can be used in combination with secretion studies to better assess islet function and predict islet health. Moreover, reduced time preparation and smaller sample size allows for increased efficiency and sensitivity of biological assays. While sophisticated devices might serve specific needs better, if they are too complicated to use, islet biologists and pharmaceutical researchers might not implement them in their routine. We hope that our microsystem could ultimately be a powerful and practical addition to the armamentarium of diabetes researchers.
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