

**Rspondin-1 Deficiency Enhances Beta
Cell Neogenesis in a Murine Model of Diabetes**

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Physiology
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ABSTRACT OF THESIS

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The cWnt activator, Rspondin-1 (Rspo1), has been identified as a regulator of β -cell growth and function, although its role in pathophysiological conditions such as streptozotocin (STZ)-induced diabetes is unknown. Hence, I hypothesized that Rspo1 deficiency stimulates β -cell neogenesis in STZ-diabetes. There was no difference in oral glucose handling between STZ-induced Rspo1 mice, although, Rspo1^{-/-} mice demonstrated increased insulin sensitivity compared to wild-type littermates. Moreover, β -cell mass and the total number of islets did not differ between STZ-induced Rspo1^{+/+} and Rspo1^{-/-} mice, although mice with Rspo1 deficiency had reduced β -cell apoptosis and significantly enhanced numbers of insulin-positive ductal cells suggestive of β -cell neogenesis. Furthermore, the increased β -cell regeneration observed in knockout animals appeared to be associated with a more differentiated/mature β -cell phenotype in Rspo1^{-/-} versus Rspo1^{+/+} mice. Collectively, these findings indicate a role for Rspo1 as a negative regulator of *in vivo* β -cell neogenesis and survival in the face of STZ-induced diabetes.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor Dr. Patricia Brubaker for her continuous support and guidance throughout my Master's project. I am extremely grateful that Dr. Brubaker took me into her lab despite the fact that she had not been expecting to take on a graduate student at the time. For this, I can really not express enough gratitude. Being a part of the Brubaker lab has helped me become more confident in myself as a scientist, and I will always be grateful for being given the opportunity to grow and learn from such a remarkable role model and mentor. I would also like to thank all the members of the Brubaker lab for welcoming me into the lab and providing continuous support, guidance and advice whenever it was needed. I'd like to especially thank Shivangi Trivedi, Charlotte Dong, Samantha Li and Manuel Gil Lozano for their companionship and our numerous venting sessions! I thank Angelo Izzo for all his technical support and for teaching me many of the techniques that I have used to perform my experiments. I would also like to thank both Leila Tick and Sara Johnson for taking great care of my research animals. Thank you to my committee members Dr. Allen Volchuk and Dr. Minna Woo for their feedback and guidance throughout the course of this research project.

Finally, thank you to my wonderful family and friends for their constant support and encouragement. I would not be here without their massive support and patience. I feel that the last two years in the Brubaker lab has allowed me to grow both as a student and as a person. Once again, I am very grateful for the experience I gained in the Brubaker lab and I hope to continue to build on the knowledge that I have acquired here in my future endeavors.

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LIST OF ABBREVIATIONS

ABC	Avidin/biotin complex
Amer1/WTX	APC member recruitment 1/Wilms tumour gene on the X chromosome
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli gene product
Arx	Aristaless related homeobox
AUC	Area-under-the-curve
β-TrCP	β -transducin repeat-containing protein
BCM	β -cell mass
Brn4	Brain 4
BSA	Bovine, from serum albumin
BSC	Biological safety cabinet
CA-II	Carbonic anhydrase-II
CK-19	Cytokeratin-19
cDNA	Complementary deoxyribonucleic acid
CK1	Casein kinase 1
CMF-PBS	Calcium-magnesium free phosphate buffered saline
Cre	Cyclization recombination
cWnt	Canonical Wnt
Dvl/Dsh	Disheveled
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DKK	Dickkopf
DNA	Deoxyribonucleic acid
e	Embryonic day
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Ex4	Exendin-4
FBS	Fetal bovine serum
FCCM	Fat cell-conditioned medium
FoxM1	Forkhead box protein M1
Frz	Frizzled
GLP-1	Glucagon-like peptide-1
GIP	Glucose-dependent insulinotropic polypeptide
GLUT2	Glucose transporter 2
GSIS	Glucose-stimulated insulin secretion
GSK3	Glycogen synthase kinase 3
HBSS	Hank's buffered saline solution
Hes-1	Hairy and enhancer of split 1
Hnf1β	Hepatocyte nuclear factor 1 β
Hnf6	Hepatocyte nuclear factor 6
HRP	Horseradish peroxidase
IAPP	Islet amyloid polypeptide
ip	Intraperitoneal
Ins2	Insulin2
ITS	Insulin-transferrin-selenium

ITT	Insulin tolerance test
IRS2	Insulin receptor substrate 2
Irx1/2	Iroquois homeobox protein 1/2
K_{ATP}	ATP-sensitive potassium channel
KGF	Keratinocyte growth factor
LEF	T cell factor/lymphoid enhancer factor
LGR 4/5	Leucine-rich repeat containing G-protein-coupled receptor 4/5
LIF	Leukemia inhibitory factor
LRP 5/6	Low-density lipoprotein receptor-related protein 5/6
MACS	Magnetic assisted cell sorting
Maf	v-maf musculoaponeurotic fibrosarcoma oncogene homolog
mRNA	Messenger ribonucleic acid
Myc	Myelocytomatosis oncogene
Ngn3	Neurogenin 3
Nkx2.2	Nk2 homeobox 2
Nkx6.1	Nk6 homeobox 1
NeuroD1	Neurogenic differentiation 1
OGTT	Oral glucose tolerance test
Pax	Paired box
PBS	Phosphate buffered saline
Pdx1	Pancreatic and duodenal homeobox 1
PI3-kinase	Phosphoinositide-3 kinase
PO	<i>Per os</i>
PP	Pancreatic polypeptide
Ptf1a	Pancreas specific transcription factor 1a
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
Rspo1	Roof plate spondin-1
siRNA	Small interfering RNA
Sox9	SRY-box containing gene 9
SNARE	Soluble <i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
SNP	Single nucleotide polymorphism
STZ	Streptozotocin
T1DM	Type 1 (insulin-dependent) diabetes mellitus
T2DM	Type 2 (insulin-independent) diabetes mellitus
TCF	T-cell factor
TCF7L2	Transcription factor 7-like 2
TBST	Tris buffered saline and Tween
TGFα	Transforming growth factor α
TSP1	Thrombospondin type 1
TNFα	Tumour necrosis factor alpha
ub	Ubiquitin
VEGF	Vascular endothelial growth factor
WD	Week of human development
Wnt	Wg (wingless) + Int1 (chromosomal integration site of mouse mammary tumor virus on mouse chromosome 15)
ZNFR3	Zinc and ring finger 3

Symbols and Units

α	Alpha
β	Beta
δ	Delta
γ	Gamma
k	Kilo- ($\times 10^3$)
m	Milli- ($\times 10^{-3}$)
μ	Micro- ($\times 10^{-6}$)
n	Nano- ($\times 10^{-9}$)
%	Percent
°C	Degrees Celsius
sec	Second (s)
hr	Hour (s)
min	Minute (s)
d	Day
kDa	Kilodalton
U	Units
g	Gram
l	Litre
t	Time
w/v	Weight per volume
xg	Times gravity (relative centrifugal force)
rpm	Revolutions per min
M	Molar
mol	Moles

1. INTRODUCTION

1.1 Rationale

Diabetes mellitus is characterized by hyperglycemia as a result of insulin deficiency or insulin resistance. This complex metabolic disorder can be classified into two main categories: insulin-dependent diabetes mellitus (T1DM) and insulin-independent diabetes mellitus (T2DM). T1DM is an autoimmune disease that results in the selective destruction of pancreatic β -cells, resulting in absolute insulin deficiency. It accounts for 5-10% of all diabetes and is associated with the presence of islet-cell antibodies (1). The second, more common form of diabetes, is T2DM, which comprises more than 90% of patients with the disease (1). T2DM is a complicated metabolic disorder but, put simply, it can be characterized by a progressive decline in β -cell mass and function, as well as chronic insulin resistance (2). T2DM has become a major global problem, as over 300 million people worldwide are currently diagnosed with this disease (1). Concurrent with the obesity epidemic, the diabetes epidemic is taking place in both developed and developing nations, mainly due to increased urbanization and changes in increasingly sedentary lifestyles (3). The severe economic burden on governments and individuals incurred by diabetes and its associated complications highlights the importance of developing new therapeutic interventions that promote the formation of new β -cells, either *in vitro* for transplantation, or *in vivo* through increased β -cell regeneration (4). It is thus crucial to further understand the biology of β -cell regeneration to achieve this end. This goal of this study was to better understand the regulatory effects of R-spondin 1 (Rspo1) on the β -cell under conditions of metabolic stress such as diabetes, thereby providing a potential therapeutic target for the preservation and/or restoration of pancreatic β -cell mass.

1.2 Beta cell development and regeneration

1.2.1 Development of the endocrine pancreas

The pancreas consists of three main cell types: exocrine acinar tissue which produces digestive enzymes, endocrine cells (islets of Langerhans) which produce key hormones that regulate blood glucose levels, and ductal cells which collect digestive enzymes produced from acinar cells. Exocrine cells make up the majority of the pancreas, while endocrine islet cells constitute only 1-2% of the total pancreatic mass. The pancreatic islets are comprised of four main endocrine cell types: α , β , δ , and pancreatic polypeptide (PP) cells, which produce the hormones glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively.

In mice, the first morphological signs of pancreas formation begin at embryonic day (e) 9.5 (5). At this point, the dorsal pancreatic bud begins to form, followed by the two ventral/lateral buds one day later (5). As the buds appear, the expanding pancreatic epithelium initiates the expression of the transcription factors Pdx1 (pancreatic duodenal homeobox gene-1), Sox9 (SRY-box containing gene 9), Nkx2.2 (Nk2 homeobox 2), Nkx6.1 (Nk6 homeobox 1), Ptf1a (pancreas transcription factor 1a), and Hnf1 β (hepatocyte nuclear factor 1 β), thereby forming a pool of multipotent progenitor cells for exocrine and endocrine cell formation in what is termed the first developmental transition (5-7). Expansion of the dorsal and ventral buds continues, followed by rotation of the gut, allowing for the fusion of the two buds into a single organ at e12.5-e14 (5;6). A limited number of these first transition epithelial cells differentiate into immature endocrine cells expressing glucagon (e9.5) and/or co-expressing glucagon and insulin (e10-e10.5) (6). However, the contribution of these early endocrine cells to mature islet formation is unclear (7).

The second developmental transition marks the period when the majority of endocrine cell differentiation occurs (e12.5-e16.5) (7). Several gain- and loss-of-function

models have been utilized to demonstrate the expression of various transcription factors during pancreatic development, using approaches such as immunohistochemical, protein and/or mRNA analyses. The most important transcription factor for driving pancreatic progenitor cells towards an endocrine cell fate is neurogenin 3 (Ngn3) (7). Ngn3 is expressed in pancreatic endocrine precursors (8), hence making it a useful marker of immature endocrine cells (Figure 1.1). Its expression starts on e9-e9.5, peaks on e15.5 during the major wave of endocrine cell formation, and then is greatly reduced at birth, with very low but detectable levels in the adult pancreas (6;7;9). Ngn3 expression is required for the development of all four endocrine cell lineages of the pancreas, as mice lacking Ngn3 fail to develop any pancreatic endocrine cells and die postnatally from hyperglycemia (8). Early transcription factors such as Sox9 (10) and Hnf6 (hepatocyte nuclear factor 6) (11) function as positive regulators of Ngn3 expression, while Hes-1 (hairy and enhancer of split-1) inhibits Ngn3 expression and endocrine differentiation through the Notch signaling pathway (9). The Notch signaling pathway is crucial in pancreas development, as normal Notch signaling results in the expression of Hes-1 and adoption of the exocrine cell fate; enforced activation of Notch signaling results in impaired differentiation of pancreatic progenitors; and reduced Notch signaling leads to an up-regulation of Ngn3 expression (9;12). Increased Ngn3 through the inhibition of Notch signaling subsequently allows for the activation of a number of endocrine transcription factors required for the various endocrine lineages, such as NeuroD1 (neurogenic differentiation 1) (5;8). These transcription factors are differentially and temporally regulated, but basically, PP-cells are characterized by Nkx2.2; δ -cells by Pax4 (paired box 4), Pax6 (paired box 6) and Pdx1; α -cells by Arx (aristaless related homeobox), Irx1/2 (iroquois homeobox protein 1/2), Brn4 (brain 4), Pax6, and MafB (v-maf musculoaponeurotic fibrosarcoma oncogene homolog B); and β -cells by MafA (v-maf

musculoaponeurotic fibrosarcoma oncogene homolog A), Nkx2.2, Nkx6.1, Pax4, Pax6 and Pdx1 (reviewed in (5;6)). Finally, the third developmental transition (e16.5-birth) involves formation of individual endocrine cells in organized islet-like clusters (6). However, the islets are not fully mature and functional until after birth, and undergo additional remodeling and maturation for 2-3 weeks after birth until weaning (6).

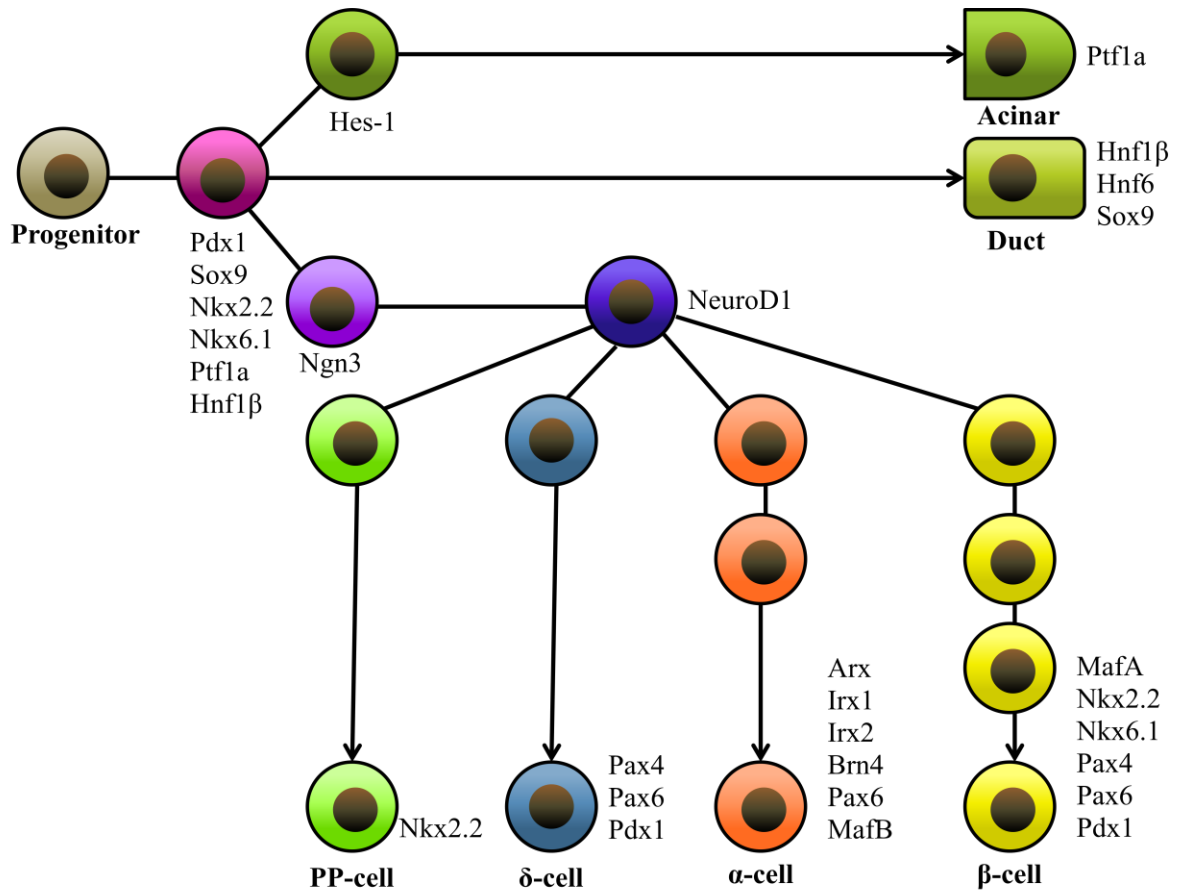


Figure 1.1 Overview of pancreas development and the transcription factors involved.

The transition of pancreatic progenitor cells to mature endocrine cells is regulated by the sequential activation and interaction of several transcription factors. Most epithelial cells of the early pancreatic buds are pre-differentiated precursors of exocrine and endocrine cells. These cells express a combination of transcription factors, including Pdx1, Sox9, Nkx2.2, Nkx6.1, Ptf1a and Hnf1 β . Activation of the Notch signaling pathway results in the expression of Hes-1 and adoption of the exocrine fate, resulting in the formation of mature acinar cells. Inactivation of Notch signaling results in an up-regulation of Ngn3 expression, thereby producing a pool of committed endocrine precursors. These Ngn3-expressing endocrine precursor cells subsequently result in the activation of a number of endocrine transcription factors required for the various endocrine lineages, such as NeuroD1. PP-cells are characterized by Nkx2.2; δ -cells by Pax4, Pax6 and Pdx1; α -cells by Arx, Irx1/2, Brn4, Pax6, and MafB; and β -cells by MafA, Nkx2.2, Nkx6.1, Pax4, Pax6 and Pdx1 (*Modified from Van Hoof et al. (5)*)

1.2.2 Pre- and post-natal beta cell growth and function

The most considerable increases in fetal β -cell mass occur during the second and third developmental transitions, as mentioned above. During late fetal gestation (e16-e20), the numbers of β -cells double approximately every day (13), with the greatest increase in β -cell mass occurring during the last 2 days of gestation in rats (14). However, in the rat fetus, β -cell division only accounts for 10-20% of total β -cell growth (14). The remaining 80-90% of growth is attributed to differentiation of pancreatic progenitor cells to β -cells, a process called neogenesis (14). In human fetal pancreas, β -cell mass expands rapidly from ~20 weeks (15). The extremely low replication rate of endocrine cells noted in human fetal pancreas suggests that, similar to rodents, massive differentiation from nonendocrine progenitor cells is likely to be the major mechanism of β -cell expansion during this period (16). In support of this theory, large numbers of proliferative islet precursors expressing insulin and the duct-specific marker, cytokeratin, have been observed during fetal islet formation (17), suggesting that the conversion of ductal cells to β -cells may contribute to the expansion of β -cell mass seen in late fetal gestation.

In neonatal rodents, β -cell expansion is still evident, although at a reduced rate compared with late fetal growth (13). Interestingly, although β -cell volume continues to increase with age, α - and δ -cells do not appear to exhibit further growth after postnatal day 25 (18). As with prenatal islet growth, both β -cell replication and neogenesis contribute to newly formed β -cells in the postnatal rodent pancreas (19). Furthermore, an increased rate of β -cell apoptosis is observed at weaning, resulting in substantial remodeling of the pancreas (20). However, these changes do not result in a reduced β -cell mass, as new and more functionally mature β -cells compensate for the loss (21). In adult rodents, increases in β -cell number (hyperplasia) and β -cell size, as well as low rates of β -cell apoptosis, contribute to a

linear increase in β -cell mass with age and body weight (22). Dor et al. (23) reported that β -cell replication, and not β -cell neogenesis, is the primary mechanism of increased β -cell growth in the adult rodent. However, the presence of potential β -cell progenitor cells has been found in the adult human pancreas, including the exocrine compartment and ductal epithelium, although it is not clear whether these β -cell precursors contribute to adult β -cell growth under normal conditions, or only under specific circumstances such as obesity or pregnancy (24).

The maturation of β -cell function is an important step in the culmination of β -cell development. In mature rodent β -cells, glucose enters the cell via glucose transporter 2 (GLUT2) (25) and becomes phosphorylated by the rate-limiting enzyme glucokinase (26). Glucose metabolism in the β -cell ultimately results in a net increase in the ATP/ADP ratio, thus triggering depolarization of the plasma membrane by closure of the ATP-sensitive potassium (K_{ATP}) channels (1;27). Membrane depolarization subsequently induces the initiation of calcium influx via voltage-dependent calcium channels (27). In response to increases in intracellular calcium concentration, insulin granules fuse with the plasma membrane via soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery (27). The rapid exocytosis of insulin granules through these SNARE-dependent processes occurs within the initial 5-10 min of stimulation and is referred to as the first phase of insulin secretion (27). The subsequent sustained, but less robust, release is referred to as the second phase of insulin secretion (27), thus resulting in a biphasic pattern of insulin secretion. However, this biphasic insulin secretion does not fully mature until after birth, as a low response to glucose was reported in fetal rat islets at e19.5, likely due to defective β -cell membrane depolarization (28). Nevertheless, by postnatal day 2-3, insulin

secretion returned to levels seen in adults, likely due to increased expression of K_{ATP} channels (28;29).

1.2.3 Modulators of beta cell mass

β -cell mass is dynamic throughout adulthood and fluctuates in response to various physiological (i.e. aging and pregnancy) and pathophysiological (i.e. obesity and insulin resistance) states. The mechanisms responsible for alterations in β -cell mass involve changes in β -cell replication/proliferation, β -cell neogenesis, β -cell size and β -cell death/apoptosis.

The regenerative and proliferative capacity of most organs declines with age. In the pancreas, β -cell hypertrophy and hyperplasia contribute to increased β -cell mass in young (1-7-month old) rats, but only β -cell hypertrophy is responsible for increases in β -cell mass found in 20-month-old rats (22). Furthermore, β -cells of aged (1-year-old) mice have extremely low rates of replication (30). Hence, the rarity of β -cell replication in aged mice, as well as minimal evidence of β -cell turnover, suggests that murine aged β -cells are mostly in a quiescent G_0 state (30). This is also seen in aged human islets (50.2 ± 3.5 years), as β -cell replication decreases in an age-dependent manner, whereas rates of β -cell apoptosis and islet neogenesis are not significantly altered (31). The reduced β -cell proliferation associated with aging may in part be due to age-related decreases in the expression of cell cycle activators (i.e. forkhead box M1 (FoxM1)) with simultaneous increases in cell cycle inhibitors (reviewed in (32)). Since β -cell replication contributes to adaptations in β -cell mass, albeit at low levels, these changes may restrict the ability of the aging pancreas to adapt to changing demands for insulin secretion and thus increase the risk of developing T2DM (31).

Physiological alterations in β -cell mass also occur during pregnancy. Rodent studies have shown that β -cell mass increases 50% during pregnancy in order to compensate for the increased metabolic demands of the developing fetus (33;34). This occurs primarily through

increases in β -cell size and β -cell proliferation, induced by the pregnancy hormones prolactin and placental lactogen (34). However, in contrast to rodents, adaptive increases in pancreatic β -cell area during pregnancy in humans are not accompanied by changes in mean β -cell size, replication, or apoptosis frequency (35). Instead of an increase in islet size or β -cells per islet, increased β -cell expansion is due to a greater number of small islets (35). Furthermore, Butler et al. (35) observed an increased incidence of ductal cells positive for insulin, as well as scattered single β -cells. Therefore, in humans, increases in β -cell adaptation during pregnancy are a result of an increased number of β -cells in “new small islets” (35). After pregnancy, decreased β -cell replication and reduced β -cell size, along with concomitant increases in β -cell apoptosis, ensures the return of β -cell mass to normal levels, at least in postpartum rodents (36). Failure to compensate for the increased metabolic demands of pregnancy can lead to gestational diabetes and an increased risk of developing T2DM later in life (21).

β -cell plasticity is further demonstrated in obese individuals who do not develop diabetes. These individuals exhibit a compensatory increase in β -cell mass in order to adapt to the increased metabolic load and obesity-associated insulin resistance (37). In obese humans, increased β -cell mass occurs through increases in β -cell replication, neogenesis and β -cell hypertrophy (2;37). Similarly, obese rodents demonstrate an expansion in β -cell mass as a result of massively increased islet size and β -cell replication (38); both glucokinase and insulin receptor substrate 2 (IRS2) are required for this β -cell hyperplasia to occur (39).

Although there is an initial compensatory increase in β -cell mass, 25-30% of obese individuals eventually lose the ability to adapt to increasing insulin resistance (40). Consequently, a progressive decline in β -cell mass results in the development of T2DM (37). This β -cell loss and reduced β -cell mass is attributed to a marked increase in β -cell apoptosis,

with no significant changes in the levels of β -cell replication seen in either humans or rodents (2;41). Although rodents exhibit a decrease in the rate of β -cell neogenesis (41), no change in neogenesis was observed in islets from diabetic humans (2). Many factors could trigger the increase in β -cell apoptosis that occurs during the development of T2DM (37). For example, genetic factors, the development of endoplasmic reticulum stress, chronic hyperglycemia, chronic hyperlipidemia, oxidative stress, and certain cytokines can lead to the activation of β -cell apoptotic signaling cascades (37).

It is unclear whether the β -cell failure associated with diabetes is due only to a reduced β -cell number (as described above) or also to an impairment in β -cell function (42). Several rodent studies have shown that hyperglycemia results in reduced expression of key hormones, transcription factors, and β -cell metabolic enzymes necessary for the maintenance of normal β -cell differentiation and function (42-44). For example, islets of diabetic rats exhibit a marked decrease in the mRNA expression levels of insulin, islet amyloid polypeptide (IAPP), GLUT2 and glucokinase, as well as β -cell ion channels/pumps (43;44). A recent study by Talchai et al. (42) challenges the widely accepted belief of increased β -cell apoptosis as being the major contributor of β -cell failure. Using a variety of models, these authors demonstrated that the failure of sustainable β -cell mass and function under metabolic stress (i.e. pregnancy, aging, obesity) is due to β -cell dedifferentiation, not β -cell death (42). Levels of β -cell apoptosis were not found to be significantly altered, although the number of dedifferentiated β -cells expressing markers of progenitor cells was increased (42). However, the contribution of β -cell dedifferentiation in human conditions of metabolic stress is unknown.

1.2.4 Potential sources of new beta cells

The generation of new β -cells to restore functional β -cell mass in diabetic patients would be a promising therapeutic intervention that could lead to islet replacement therapies. The issue of β -cell regeneration in the pancreas is a controversial aspect of diabetes research due to the continual debate over the source of endogenous pancreatic progenitor cells. The potential mechanisms of β -cell regeneration include proliferation of β -cells (as discussed above), transdifferentiation from one differentiated cell type into another, and neogenesis from non- β -cell precursors.

1.2.4.1 Beta cell transdifferentiation

Recently, two reports have shown that transdifferentiation of α -cells to β -cells can be a source of insulin-positive cells in the adult mouse (45;46). Thorel et al. (46) used a transgenic model of diphtheria-toxin-induced β -cell-ablation, and showed that lineage-tracing to label glucagon-producing α -cells reveals large numbers of newly generated β -cells deriving from α -cells. Similarly, Chung et al. (45) demonstrated that neogenic β -cells arise primarily from α -cells. Hence, following pancreatic duct ligation and alloxan treatment, α -cell proliferation is induced, followed by intermediate cells co-expressing both glucagon and insulin (45). These double-hormone positive cells gradually decline in number as β -cells with a mature phenotype, defined as lack of glucagon and expression of MafA, become predominant (45). Interestingly, another study by Collombat et al. (47) showed that Pax4 misexpression in glucagon-expressing cells results in a conversion of α -cells to β -like cells. This causes a decrease in circulating glucagon, which results in the reactivation of neurogenin-3 in cells within the ductal epithelium and their subsequent differentiation into α -cells to compensate for the glucagon shortage (47). The newly formed α -cells then continue their conversion into β -like cells with Pax4 misexpression (47). Thus, transdifferentiation of

α -cells to β -cells appears to play an important role in β -cell regeneration, at least under some conditions.

Exocrine acinar cells have also been evaluated as a possible source of pancreatic progenitor cells. Baeyens et al. (48) showed that the addition of epidermal growth factor (EGF) and leukemia inhibitory factor (LIF) to adult rat exocrine cells *in vitro* results in a dramatic increase in β -cell number. These newly formed β -cells express Pdx1, GLUT2 and C-peptide, and are able to restore normoglycemia when transplanted into diabetic mice, suggesting that they have differentiated into mature β -cells (48). Consistent with this finding, Zhou et al. (49) demonstrated that fully differentiated exocrine cells can be directly reprogrammed into ‘ β -cell-like’ cells in adult animals through the ectopic expression of Ngn3, Pdx1, and MafA. Although these cells do not organize into islet structures and remain as single cells or small clusters, they express markers of mature and functional β -cells (i.e. GLUT2, glucokinase, Nkx6.1 and Nkx2.2) (49). Therefore, transdifferentiation of exocrine acinar cells to β -cells may also contribute to β -cell regeneration under the correct stimuli.

Finally, several studies have demonstrated that treatment with specific growth factors or transcription factors is sufficient to cause transdifferentiation of non-pancreatic tissue such as hepatocytes (50), bone marrow (51) and intestinal cells (52) towards a mature β -cell phenotype. Thus, the formation of new β -cells from transdifferentiation of various pancreatic and non-pancreatic tissues represents one method of generating β -cells in adult life.

1.2.4.2 Beta cell neogenesis from non-beta cell precursors

Islet neogenesis refers to the formation of insulin-producing cells from the adult stem/progenitor cells of the pancreas (53). Xu et al. (54) reported that exendin-4 (Ex4), a glucagon-like peptide 1 (GLP-1) receptor agonist, increases β -cell mass when administered to rats with 90-95% partial pancreatectomy. This effect is due to increased β -cell

proliferation and neogenesis, and results in the amelioration of glucose intolerance in these animals (54). These authors found that the GLP1-receptor is expressed in both ducts and β -cells, and later studies demonstrated the positive effects of GLP-1 in inducing the differentiation of Pdx1-positive pancreatic ductal cells into insulin-secreting cells (55). Several studies provide strong support that the newly generated insulin-positive cells after pancreatic injury are also derived from pancreatic ducts. For example, Bonner-Weir et al. (56) demonstrated that following expansion, human ductal tissue can be directed to differentiate into islet endocrine cells with Matrigel overlay and the addition of keratinocyte growth factor (KGF). These 'cultivated human islet buds' are composed of cytokeratin-19 (CK-19) positive duct cells and hormone-positive islet cells, and are able to secrete insulin in response to high glucose (20 mM) (56). Furthermore, the Bonner-Weir group (57;58) genetically marked ductal cells using carbonic-anhydrase II (CA-II) as a duct-cell-specific promoter in lineage-tracing experiments using the Cre-Lox system, and found that following ductal ligation, CA-II expressing ductal cells act as pancreatic progenitors to give rise to new β -cells. Li and colleagues (59) have also examined the mechanisms by which adult pancreatic duct cells may contribute to regeneration of the pancreas. After 90% partial pancreatectomy in adult rats, pancreatic duct cells experience a loss of differentiated phenotype with decreased expression of Hnf6 (59). This results in enhanced proliferation and increased expression of progenitor-like markers such as Pdx1, Sox9 and Ngn3 (59). As insulin-positive cells 'bud' from the ducts, expression of Sox9 and Ngn3 is reduced, whereas expression of Nkx6.1, GLUT2, and MafA gradually increases (59). Therefore, it is proposed that following injury, duct cells dedifferentiate to a progenitor-like phenotype, followed by expansion and redifferentiation into differentiated endocrine cells following the normal embryonic developmental program (59). Therefore, several studies show that the ductal

epithelium in humans and rodents contains pancreatic progenitor cells that are capable of producing insulin-positive cells.

Similar to Inada and colleagues (57), Solar et al. (60) employed the Cre-Lox system to genetically mark ductal cells using Hnf1 β as a duct-cell-specific promoter in lineage-tracing experiments. In contrast to the studies mentioned above, these authors found that the ductal epithelium does not make significant contributions to exocrine or endocrine cell regeneration after birth (60). Partial duct ligation (PDL), as well as alloxan followed by EGF and gastrin treatment, resulted in increased β -cell mass as expected (60). However, there was very little contribution of Hnf1 β -expressing cells, suggesting that the pancreatic duct epithelium does not contribute a significant source of progenitors in the β -cell growth response seen after PDL (60). These findings support the work of Dor and colleagues (23), as these authors used lineage tracing of genetically marked β -cells in mice to demonstrate that no new β -cells are formed from non-insulin expressing stem cells or progenitor cells, either postnatally or following 70% pancreatectomy (23;61). Consequently, the ductal cell neogenesis hypothesis remains controversial and further studies are required to fully understand whether and how new islets originate postnatally from ducts (61).

1.3 Canonical Wnt (Wg (wingless) + Int1 (chromosomal integration site of mouse mammary tumor virus on mouse chromosome 15)) signaling

1.3.1 Canonical Wnt signaling pathway

Wnts are a large family of highly-conserved secreted glycoproteins that are associated with the regulation of many developmental processes, including proliferation of stem/progenitor cell populations, cell fate determination, and tissue homeostasis (62-64). The most heavily studied Wnt pathway is the canonical (c), or β -catenin-dependent, Wnt

signaling pathway. In the absence of Wnt ligands, the Wnt antagonists Dickkopf (DKK) and Kremen inhibit the Wnt-induced complex of the seven-transmembrane receptor protein, frizzled (Frz) and its co-receptor, lipoprotein receptor-related protein 5 or 6 (LRP5/6) (65). As a result, cytosolic β -catenin is targeted by a degradation complex composed of axin, adenomatous polyposis coli gene product (APC), APC member recruitment 1/Wilms tumour gene on the X chromosome (Amer1/WTX), casein kinase 1 α (CK1 α), and glycogen synthase kinase 3 (GSK3) (Figure 1.2; (65-67)). CK1 and GSK3 sequentially phosphorylate conserved serine and threonine residues on the amino terminal of β -catenin, resulting in β -catenin recognition by an E3 ubiquitin (ub) ligase subunit, β -TrCP (β -transducin repeat-containing protein), and its subsequent ubiquitination and proteosomal degradation (65;68). This continual degradation of β -catenin prevents β -catenin from reaching the nucleus, ensuring that the repressor protein Groucho is free to bind to members of the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, thereby preventing the transcription of cWnt target genes (65). Activation of cWnt signaling requires interaction between Wnt ligands and the Frz and LRP5/6 co-receptors (65). Wnt binding leads to internalization of the Frz and LRP co-receptor, as well as the recruitment of the intracellular scaffolding protein dishevelled (Dvl/Dsh), which then results in the recruitment of WTX to the plasma membrane and phosphorylation of LRP6 by CK1 γ and GSK3, thereby dissociating the degradation complex (65-67). This leads to inhibition of β -catenin phosphorylation and degradation. Stabilization of β -catenin allows for β -catenin to accumulate and enter the nucleus where it forms complexes with TCF/LEF and activates the transcription of cWnt target genes such as cyclin D1 and/or c-myc (65).

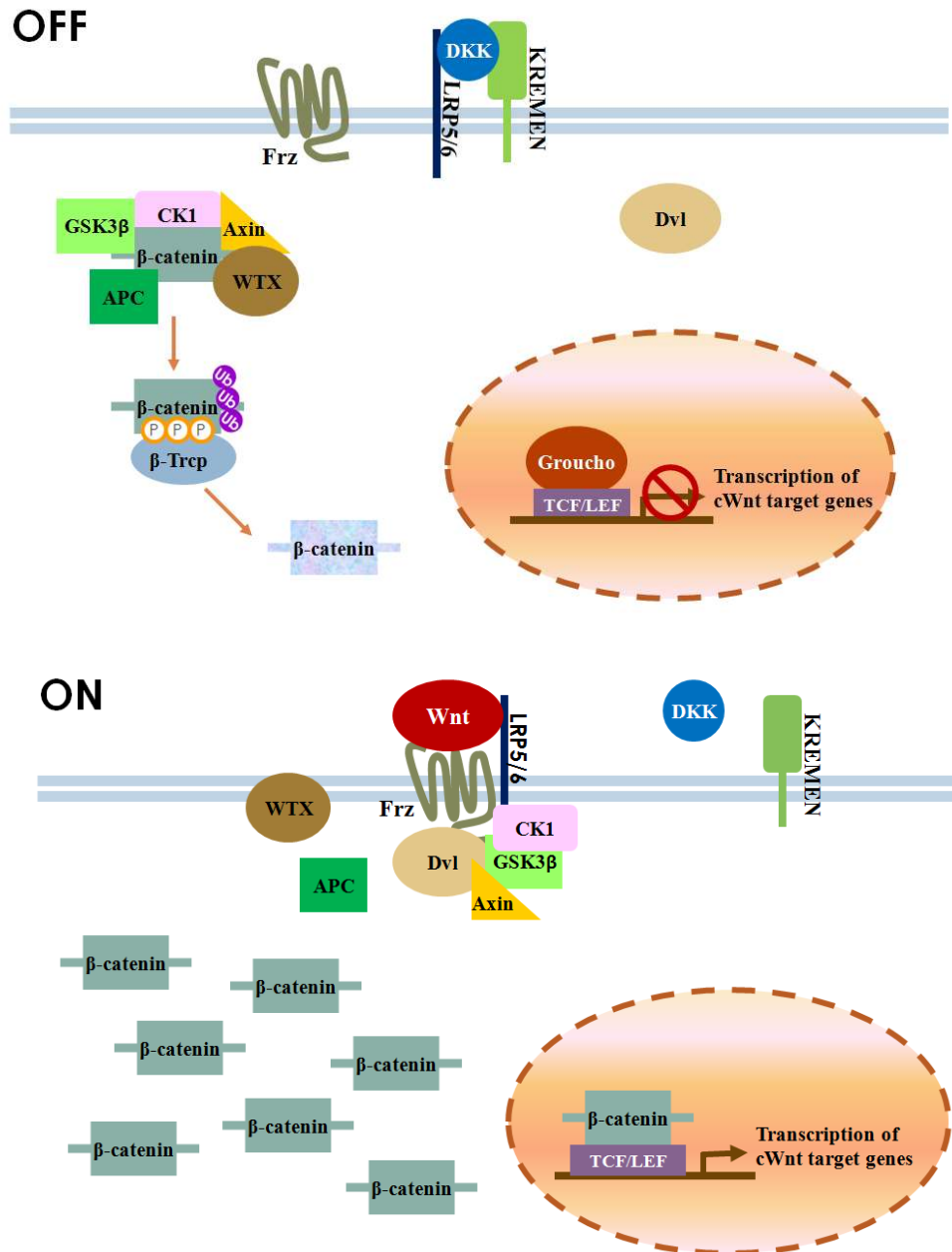


Figure 1.2 Overview of the canonical Wnt (cWnt) signaling pathway. “OFF” state: In the absence of a Wnt ligand, β -catenin is targeted by the degradation complex of Axin, APC, GSK3 β , and other co-factors. This results in the ubiquitination and subsequent degradation of β -catenin, thereby preventing its translocation into the nucleus to activate cWnt target gene expression. “ON” state: In the presence of Wnt ligands, Wnt binding to the Frizzled receptor and LRP co-receptors induces phosphorylation of LRP and recruitment of Axin, thus dismantling and inhibiting the degradation complex. This allows β -catenin to accumulate and enter the nucleus, where it interacts with the TCF/LEF family of transcription factors to activate the transcription of cWnt target genes.

1.3.2 cWnt signaling pathway in pancreatic development

Wnt proteins play crucial roles in the development of multiple tissues, including the pancreas. Indeed, components of the cWnt pathway have been found to be expressed in the developing mouse pancreas (69). For example, several Wnt ligands and various Frz receptors, in addition to other modulators of Wnt signaling such as LRP co-receptors, Wnt antagonists, GSK3, Dvl, secreted DKK proteins, and β -catenin have been found in the surrounding mesenchyme and/or epithelium of the developing mouse pancreas (69), in addition to postnatal human pancreas, human and rodent islets, and rodent cell lines (70;71). The TCF7L2 (formerly known as TCF4) gene has also been found to be expressed in human and rodent islets, as well as β -cell lines (64;70;72-74).

Although the presence of cWnt components suggests that Wnt signaling is present and likely active in these tissues, the role of cWnt signaling in pancreatic development remains controversial, as several studies report conflicting results on its role in exocrine versus endocrine development. For example, in the studies of Murtaugh et al. (75) and Wells et al. (76), deletion of the β -catenin gene in pancreatic epithelium of the developing mouse pancreas results in a severe reduction of exocrine cell mass, due to decreased proliferation among early exocrine progenitors and a subsequent near-complete loss of acinar cells. Furthermore, both studies found that β -catenin is essential for the formation of exocrine acini, but is dispensable for endocrine differentiation and function, as the conditional β -catenin knockout in the developing pancreas causes no significant alterations in endocrine cell mass or function, leaving pancreatic islets generally intact (75;76). In addition, an initial study reported that the overexpression of Wnt1 or Wnt5a in the developing mouse pancreas disrupts normal development, causing a reduction in endocrine cell number and lack of islet

formation (69). These studies indicate that cWnt signaling may not have a dominant role in the development or function of islets (64).

In contrast, studies from Dessimoz et al. (77) found defects in the development of endocrine pancreas in the absence of β -catenin. In addition, Figeac et al. (78) showed that *in vivo* knockdown of TCF7L2 results in an almost 30% reduction of total β -cell mass in the pancreases of 7-day-old neonatal Wistar rats. Therefore, these studies demonstrate that, in contrast to the previously mentioned studies, inactivation of cWnt signaling alters β -cell growth in the developing rodent pancreas (76).

Differences in timing and expression levels of Cre recombinase between different Pdx1-Cre lines may be one possible explanation for the contrasting phenotypes observed in some of these studies (64;76). Alternatively, Heiser et al. (79) reported that the expression of constitutively active β -catenin early in development prevents proper differentiation and expansion of pancreatic progenitor cells, whereas expression later in development causes increased proliferation that result in dramatically enhanced pancreatic growth. These observations strongly suggest that the timing of the activation or inactivation of Wnt signaling is essential in regulating proper pancreatic development (68).

1.3.2 cWnt signaling pathway in mature pancreatic islets

In addition to pancreatic development, the cWnt pathway is involved in β -cell growth and function in mature pancreatic islets. For example, Rulifson et al. (63) demonstrated that Wnt signaling stimulates islet β -cell proliferation. The addition of Wnt3a protein to MIN6 β -cells (63;71) and mouse islets (63) enhanced β -cell proliferation *in vitro* through the upregulation of essential β -cell cycle regulators. Furthermore, overexpression of activated β -catenin in β -cells *in vivo* results in β -cell expansion, increased insulin production and serum levels, and enhanced glucose handling (63). Moreover, overexpression of TCF7L2 in isolated

human and mouse islets protects islets from glucotoxicity and cytokine-induced apoptosis and improved insulin secretion and function (73), indicating a protective effect on β -cell survival. In contrast, activation of axin, a negative regulator of cWnt signaling, results in impaired β -cell growth and reduced β -cell mass (BCM) with a corresponding downregulation in cWnt target gene expression, leading to impaired glucose tolerance (63). Similarly, mice expressing constitutively active GSK3 β , another inhibitor of cWnt signaling, under the control of the rat insulin promoter exhibit impaired glucose tolerance as a result of decreased β -cell proliferation and mass (80). Consistent with these findings, reduced TCF7L2 gene expression by siRNA-mediated knockdown in human and rodent islets results in increased β -cell apoptosis, decreased β -cell proliferation, and reduced levels of active Akt, an important β -cell survival factor (73). Likewise, depletion of β -catenin or knockdown of TCF7L2 in rodent β -cells also results in decreased β -cell proliferation and reduced glucose-stimulated insulin secretion (GSIS) (72;81).

In addition to these findings, Fujino et al. (82) showed that LRP5 contributes to glucose-induced insulin secretion, as mice with LRP5 deficiency show impaired glucose tolerance as a result of decreased glucose-induced insulin secretion, whereas the addition of Wnt3a and Wnt5a to isolated islets from LRP5 wild-type mice stimulates glucose-induced insulin secretion. Consistent with this data, Wong et al. (71) demonstrated that the addition of Wnt3a stimulates glucose-independent insulin secretion in MIN6 β -cells. Similarly, mice lacking the Wnt antagonist DKK-2 demonstrate reduced basal blood glucose and improved glucose tolerance, as well as decreased hepatic glucose output and enhanced hepatic glycogen accumulation (83). Furthermore, Schinner et al. (84) reported that activation of Wnt signaling with adipocyte-derived Wnt molecules in fat cell-conditioned medium results in increased insulin secretion and activation of cyclin D1 and glucokinase gene transcription in

islets and INS-1 cells. Thus, cWnt signaling appears to be greatly beneficial for β -cell growth and survival, as well as endocrine cell function. In support of this notion, Grant et al. (85) found that single nucleotide polymorphisms (SNPs) within the gene TCF7L2 contribute to an increased risk of developing T2DM in Icelandic, Danish, and US cohorts. Since then, several studies have confirmed that the TCF7L2 gene is probably the strongest genetic marker for individuals with T2DM in various ethnic populations (86). However, the mechanisms through which TCF7L2 variants affect glucose metabolism are not fully understood.

It has been proposed that the incretin hormone GLP-1 may be a link between TCF7L2 function and the development of TD2M. Indeed, it has been demonstrated that TCF7L2 is involved in the regulation of proglucagon gene transcription and the production of GLP-1 (an insulin secretagogue) in the intestinal endocrine L-cells (87). Additionally, it has recently been shown that mice expressing the dominant negative form of TCF7L2 specifically in glucagon-expressing cells have reduced glucagon, and consequently, reduced GLP-1 expression, in the gut and brain, although no effect was seen on glucagon expression in the pancreas of these mice (21;88). As a result, these mice had impaired oral glucose handling in association with attenuated plasma insulin levels (88). Moreover, Ip et al. (89) found that TCF7L2 represses hepatic gluconeogenesis as a result of increased insulin- and feeding-stimulated expression of TCF7L2, as well as increased insulin activated phosphorylation of β -catenin.

In human studies, Schafer et al. (90) reported that non-diabetic subjects with TCF7L2 risk alleles have impaired insulin secretion due to a reduced response to GLP-1. Since there were no defects in GLP-1 secretion, the impaired GLP-1-induced insulin secretion is likely to be a result of a functional defect in GLP-1 signaling in β -cells rather than a reduction in GLP-1 secretion (90). Consistent with these findings, depletion of TCF7L2 with siRNA in

human islets leads to a decreased ability of GLP-1 to stimulate GSIS, thus providing further support for the hypothesis that changes in TCF7L2 result in a functional defect of GLP-1 signaling (73). Interestingly, islets from humans with T2DM and isolated human islets treated with TCF7L2 siRNA both have decreased expression of receptors for the incretin hormones, GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), resulting in impaired glucose-, GLP-1- and GIP-stimulated insulin secretion (74). This study thereby offers an alternative mechanism for the impaired β -cell function seen with changes in TCF7L2 in T2DM.

The majority of studies, such as those described above, associate reduced β -cell cWnt activity and the resulting decreased levels of TCF7L2 with impaired β -cell function. In contrast, Lyssenko et al. (91) found that TCF7L2 mRNA expression levels are increased in human islets of diabetic patients. Furthermore, the level of TCF7L2 expression in islets was found to be inversely correlated with insulin secretion (91), thereby suggesting that increased, rather than decreased, levels of TCF7L2 in islets increases the risk of T2DM by inhibiting insulin secretion. On the other hand, Shu et al. (74) found that, although mRNA levels of TCF7L2 were increased in islets from three diabetic rodent models, protein levels were decreased. This finding was further supported by a drastic downregulation of TCF7L2 protein expression in pancreatic sections from patients with T2DM (74). Thus, this study demonstrated for the first time that TCF7L2 mRNA and protein levels are oppositely regulated in diabetes, thereby providing a potential answer for the conflicting data.

1.4 R-spondin 1

1.4.1 *Function of R-spondin proteins*

The roof plate-specific spondin (R-spondin; Rspo) protein family is a group of four secreted proteins (Rspo1-4) that have been implicated as agonists of the cWnt signaling pathway. The Rspo family members are evolutionary conserved proteins that share 40-60% amino acid sequence identity and are approximately 35 kDa in size (92). All four Rspo family members contain an N-terminal signal peptide, two cysteine-rich furin-like domains, one thrombospondin type 1 (TSP1) domain, and a C-terminal region with positively charged amino acids (93). It has been suggested that the furin-like domains are required for activation of cWnt signaling, whereas the TSP1 and C-terminal domains are dispensable for function (93-95). Despite their significant structural similarities, the developmental expression patterns of the Rspo proteins have been found to be strikingly different (96).

Mouse Rspo1 was first identified by Kamata et al. (93) from the screening of genes specifically expressed in the central nervous system during development. Rspo1 expression was found in the boundary region between the roof plate and neuroepithelium of mouse embryos, suggesting that Rspo1 may contribute to dorsal neural tube development (93). This study was one of the first to demonstrate a link between Rspo1 and the cWnt signaling pathway, as the expression of Rspo1 was reduced in Wnt1/3a double knockout mice (93). Consistent with this finding, Kazanskaya et al. (94) showed that Rspo2 synergizes with Wnt3a to enhance activation of nuclear β -catenin and upregulate expression of myogenic markers, thereby indicating that Rspo2 is required for cWnt-mediated myogenesis in *Xenopus* muscle development. Furthermore, the expression of Rspo2, Wnt8, and Wnt3a were found to significantly overlap in *Xenopus* embryos (94), therefore providing support for the notion that expression of Rspo proteins overlaps with expression of Wnt proteins during

development (93;94). Since then, numerous studies have shown that Rspo proteins act as secreted activators of the cWnt signaling pathway, thus leading to the induction of cWnt target genes (92;94;95;97-102).

Wnt signaling is essential in cell fate determination during embryonic development. Therefore, Rspo's ability to positively modulate cWnt signaling and play a role in embryogenesis is of no surprise. Indeed, several studies have demonstrated the importance of Rspo1 as a regulator of sexual development and in the development of the female reproductive system (98;103-106). For example, Chassot et al. (98) showed that differentiation of female gonads requires activation of the cWnt signaling pathway by Rspo1, as overexpression of β -catenin rescues the masculinization of the reproductive system seen in female Rspo1 knockout mice. Furthermore, Rspo1 is required for normal mammalian mammary gland and ovary development (103;106). In humans, mutations in the Rspo1 gene have been identified in XX individuals with female to male sex reversal, palmoplantar hyperkeratosis and predisposition to squamous cell carcinoma of the skin (105). In addition, both Rspo1 and Rspo2 have been found to play roles in Wnt-mediated osteoblast differentiation and bone development (107;108). Rspo2 signaling through the cWnt pathway is also required for skeletal myogenesis and limb development (99), as well as normal development of lungs (109). Likewise, Rspo3 is required for mouse placental development (110), and both Rspo1 and Rspo3 triggered Wnt activation of vascular endothelial growth factor (VEGF) further mediates the effects of these two Rspo proteins on angioblast and vascular development (111;112). Finally, Rspo4 plays a crucial role in nail morphogenesis, as individuals with autosomal recessive mutations in the furin-like cysteine-rich domain of this protein exhibit an absence or severe hypoplasia of all fingernails and toenails (anonychia and hyponychia, respectively) (113).

In addition to its role during vertebrate development, Rspo1 has been described as a potent mitogen for gastrointestinal epithelial cells in normal mice (100;114). For example, injection of human Rspo1 into normal mice leads to the significant proliferation of intestinal crypt epithelial cells in the small intestine and colon through the activation of β -catenin (114). Rspo1's mitogenic effects have also proven to be beneficial in an animal model of inflammatory bowel disease by stimulating crypt cell growth, accelerating mucosal regeneration, and restoring intestinal architecture (114;115). Moreover, Rspo1 significantly reduces the overproduction of proinflammatory cytokines and preserves mucosal barrier function in inflammatory bowel disease, hence demonstrating that Rspo1 may have therapeutic potential in this disease. Interestingly, although Kim et al. (114) found that treatment with human Rspo1 of the murine colon carcinoma cell line CT26 had no effect on tumour volume, a recent report has demonstrated the presence of recurrent gene fusions involving Rspo2 and Rspo3 in 10% of colon tumours (116), although the reasons for the conflicting results are not well known. Lastly, Rspo1 treatment in a tumour necrosis factor alpha (TNF α)-transgenic mouse model of arthritis protects bone and cartilage from inflammation-related damage by preserving the structural integrity of joints (108). Therefore, these data show that Rspo1 can have therapeutic effects in two different models of inflammation-associated disease.

1.4.2 R-pondin proteins and the cWnt signaling pathway

Although Rspo proteins have been linked to the cWnt signaling pathway, the exact mechanism by which these proteins regulate the cWnt pathway are not clearly defined. Initial studies report conflicting results about the receptors for Rspo proteins (95;97;100;102). For example, it was first demonstrated that Rspo proteins function as ligands for the Wnt receptors Frz8 and LRP6, thereby allowing for LRP6 phosphorylation

and activation of endogenous β -catenin levels, ultimately leading to induced expression of cWnt target genes (95;102). In contrast, two other papers report that, instead of directly activating LRP6, Rspo1 interferes with the Wnt antagonists DKK-1 and Kremen (97;100). Rspo1 antagonizes DKK-1 through an interaction with Kremen, thus preventing internalization of LRP6 and allowing increased levels of LRP6 to remain on the cell surface for Wnt activation (97;100). Interestingly, this group also reported that although all four Rspo proteins activate the cWnt pathway to some extent, Rspo2 and Rspo3 are more potent than Rspo1, and Rspo4 is relatively inactive (97).

It has recently been reported that, in contrast to the previous studies, Rspo proteins actually function as ligands of the leucine-rich repeat containing G-protein-coupled receptors 4 and 5 (LGR4 and LGR5, respectively) (117-119). These studies have shown that Rspo binding to the LGR4 and LGR5 receptors by their furin-like cysteine-rich domains potentiates cWnt signaling, and consequently increases β -catenin activity, through enhanced LRP6 phosphorylation and Frz-LRP complexes (117-119). A recent paper by Hao et al. (120) took a more in-depth look into the mechanism by which Rspo proteins and LGR4/5 receptors potentiate Wnt signaling. These authors identified the transmembrane E3 ubiquitin ligase zinc and ring finger 3 (ZNRFF3) as a negative regulator of the Wnt pathway, as ZNRFF3 inhibits Wnt signaling through ubiquitination-mediated degradation of Frz and LRP6 (120). Moreover, Hao et al. (120) demonstrated that Rspo inhibits the actions of ZNRFF3. Rspo binding to the extracellular domain of ZNRFF3 induces the association of ZNRFF3 and LGR4, resulting in membrane clearance of ZNRFF3 (Figure 1.3; (120)). This allows for accumulation of Frz and LRP receptors on the plasma membrane and therefore enhances Wnt signaling (120). Thus, multiple receptor complexes have been proposed for Rspo-induced potentiation of Wnt signaling: 1) Rspo-LGR4/5-ZNRFF3; 2) Rspo-induced inhibition of DKK1-Kremen-

LRP5/6; 3) Rspo-Frz-LRP5/6; and 4) Frz-LRP6-LGR4/5 supercomplexes in the presence of both Wnt and Rspo ligands (117;118;120;121).

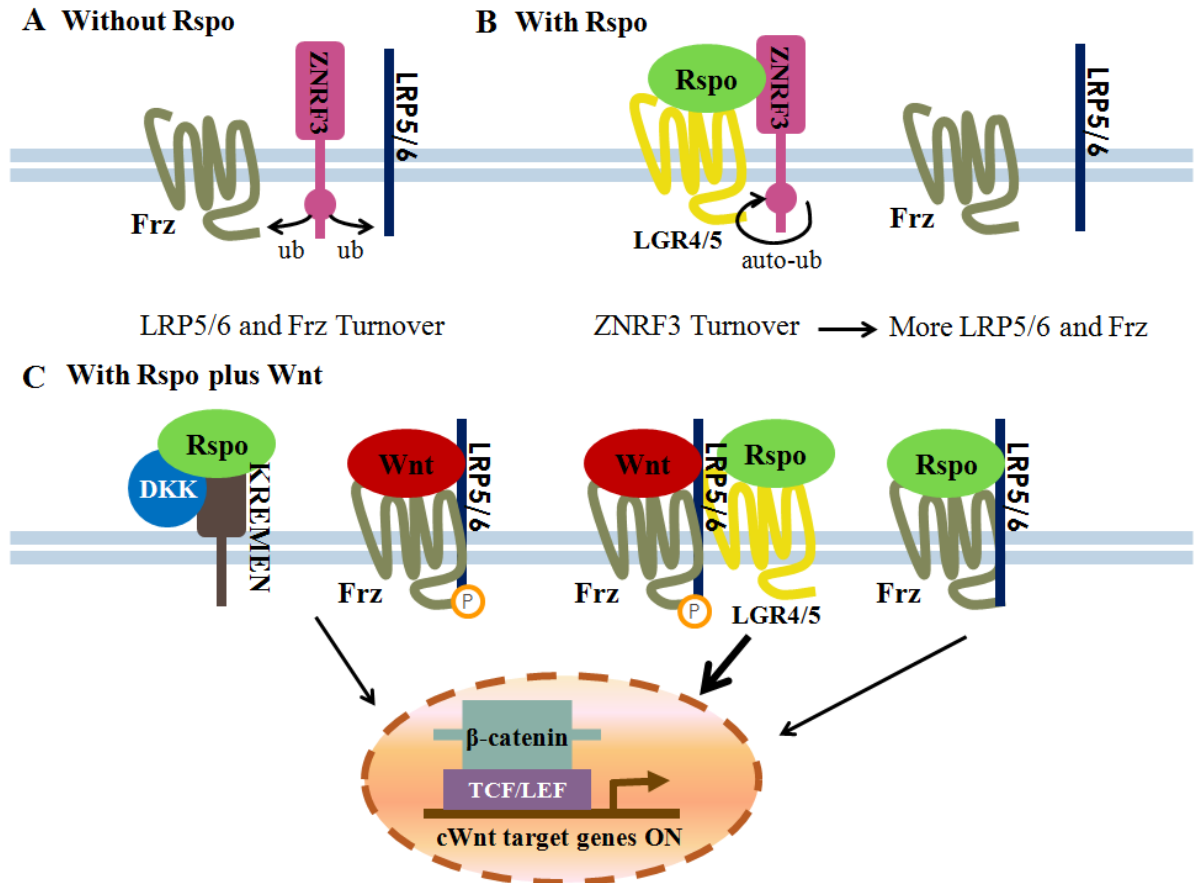


Figure 1.3 Rspo-mediated activation of cWnt signaling. **A** ZNRF3 inhibits Wnt signaling through ubiquitination-mediated degradation of the Frizzled and LRP5/6 receptors. **B** Rspo binding to the extracellular domain of ZNRF3 induces the association of ZNRF3 and LGR4/5, resulting in the autoubiquitination and membrane clearance of ZNRF3. This allows Frizzled and LRP5/6 receptors to accumulate on the plasma membrane. **C** The stabilization of Frizzled and LRP5/6 receptors allows for Wnt ligands to bind to the Frz-LRP5/6 receptor complex and activate the cWnt signaling pathway. Thus, Rspo-mediated activation of several receptor complexes for the potentiation of cWnt signaling have been reported: 1) Rspo-LGR4/5-ZNRF3; 2) Rspo-induced inhibition of DKK1-Kremen-LRP5/6; 3) Rspo-Frz-LRP5/6; and 4) Frz-LRP6-LGR4/5 supercomplexes in the presence of both Wnt and Rspo ligands. (Modified from MacDonald & He (121))

1.4.3 R-spondin proteins in mature beta cells

Rspo1 has been reported to be expressed in mature human pancreas (114) as well as in murine pancreas and islets and in the murine MIN6 and β TC β -cell lines *in vitro* (71). Previous *in vitro* studies from our laboratory have shown that Rspo1 induces cWnt signaling in MIN6 β -cells, leading to elevated levels of nuclear β -catenin and increased transcription of the cWnt target gene c-myc (71). In addition, treatment with Rspo1 increases MIN6 and murine β -cell proliferation, prevents cytokine-induced apoptosis, and stimulates insulin secretion in a glucose-independent manner (71). Moreover, Rspo1 mRNA expression levels are upregulated by Ex4, a GLP-1 receptor agonist, in a time-, dose- and PI3-kinase-dependent manner, although this effect is only seen under high glucose conditions (71). Therefore, these findings are consistent with previous reports of a role for Rspo1 in cWnt signaling and demonstrate, for the first time, the effects of Rspo1 as a β -cell growth factor and insulin secretagogue *in vitro* (71).

As expected, and in further support of a link between Rspo1 and cWnt signaling, mice with global Rspo1 deficiency have reduced levels of islet nuclear β -catenin-positive cells, as well as decreased mRNA expression of cWnt target genes, c-myc and cyclin D1 (122). However, unexpectedly, under basal conditions, Rspo1^{-/-} mice display better oral glucose tolerance (122). Furthermore, in contrast with the *in vitro* studies, mice lacking Rspo1 have increased β -cell proliferation, as well as an increased number of insulin-positive ductal cells in the pancreas suggestive of increased β -cell neogenesis (122). This results in an increased β -cell mass in these mice, as well as increased mRNA expression levels of insulin2, Pdx1, glucokinase, and GLUT2 (122). Together, these data demonstrate that Rspo1 is a regulator of whole-body glucose homeostasis and a negative regulator of β -cell neogenesis, at least under basal, physiological conditions (122).

1.5 Hypothesis and aims

The cWnt activator, Rspo1, has recently been identified as a novel β -cell growth factor and insulin secretagogue *in vitro*, and a regulator of β -cell function, growth and neogenesis *in vivo*. However, there are no reports to-date examining β -cell responses to Rspo1 under pathophysiological conditions of metabolic stress, such as diabetes. Streptozotocin (STZ) is one widely used model to induce experimental diabetes similar to T1DM (via one single high-dose injection) and T2DM (via multiple low-dose injections) in rodents (123-126). STZ acts as a toxic glucose analogue that enters β -cells via GLUT2 transporters, thus causing β -cell death through the alkylation of DNA and subsequent repair-induced depletion of cellular NAD^+ and ATP stores (126). As a result, STZ-induced diabetes results in gradual hyperglycemia and pancreatic insulinitis in certain inbred mouse strains (124). Furthermore, STZ-induced diabetes is considered a model of β -cell growth and neogenesis in several rodent studies (127-131). Hence, the hypothesis for the current study states that Rspo1 deficiency stimulates β -cell neogenesis in STZ-induced diabetes. My aims were therefore to i) examine the role of Rspo1 in STZ-induced metabolic challenge as a model of apoptosis and neogenesis and ii) examine the developmental patterns of insulin-positive ductal cells suggestive of β -cell neogenesis after insult with STZ.

2. METHODS

2.1 Animals

Rspo1^{-/-} mice were generated by inserting the LacZ gene into exon3 of Rspo1, followed by a neomycin resistance cassette (98). Mice were genotyped as previously described (Figure 2.1) (98). Briefly, the Rspo1 alleles were determined using primers P2 (5'-ATCCAGGGTCCCTCTTGATC-3') and P12 (5'-TTGAGGCAACCGTTGACTTC-3') for wild-type (Rspo1^{+/+}) mice with an expected band size of 605 base pairs, and primers P2 and LacZ50 (5'-AATATCGCGGCTCATTCGAGG-3') for knockout (Rspo1^{-/-}) mice with an expected band size of 416 base pairs (98). Real-time polymerase chain reaction (RT-PCR) was performed using the following conditions: i) 1 cycle at 94°C for 3 min; ii) 1 cycle at 80°C for 2 min; iii) 35 cycle at 94°C for 45 sec, 58°C for 30 sec, and 72°C for 45 sec; iv) 1 cycle at 72°C for 7 min; and v) 0°C forever. Although the Rspo1^{-/-} mice were generated in France on a C57Bl/6 and SV129 mixed strain (98), the Toronto Rspo1^{-/-} mice were back-crossed onto a mixed CD1 background (C57Bl/6/SV129/CD1) (122). A comparison of the phenotype of the two strains of mice showed no differences (122); therefore all studies were performed using mice from the Toronto colony only. Mice were genotyped as previously described (98) and were given *ad libitum* access to water and standard rodent chow with a 12-hr light/dark cycle. Age- and sex-matched wild-type and Rspo1^{-/-} littermates from heterogeneous crossings were used in all experiments, and all animal protocols were approved by the University of Toronto Animal Care Committee.

To determine the effects of Rspo1 deficiency in a model of STZ-induced diabetes, a modified protocol was used as previously described (132). Briefly, after a 5 hr fast, STZ (40 mg/kg body weight; Sigma-Aldrich, Oakville, ON, Canada) was freshly prepared in 0.1 mM

sodium citrate pH 5.5 and approximately 200 μ l was injected intraperitoneally (ip), once daily for five consecutive days. The specific dose of STZ was chosen based on (124;132).

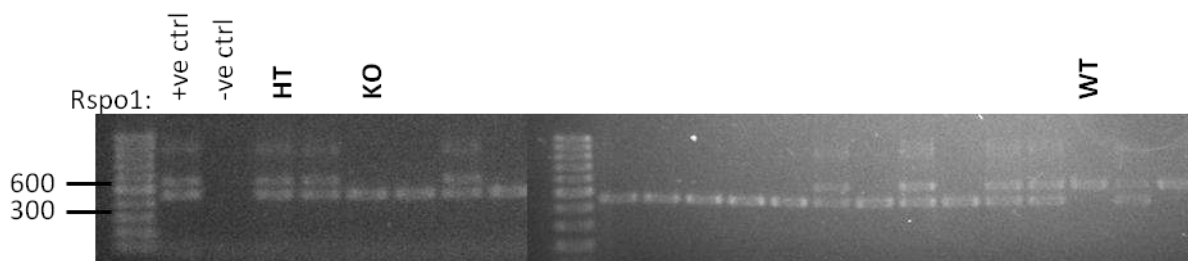


Figure 2.1 Characterization of *Rspo1* mice genotypes.

2.2 Metabolic tests

Body weight and morning blood glucose was measured periodically throughout the experimental period (Figure 2.2) using the One Touch Basic glucose meter (a kind gift from Lifescan, Canada, Burnaby, BC, Canada). Mice were fasted overnight for oral glucose tolerance tests (OGTT) or 6 hr for insulin tolerance tests (ITT). Basal blood samples were collected from the tail vein at $t = 0$ min for measurement of glucose using the One Touch Basic glucose meter. Mice were then gavaged with glucose (1.5 mg/g; *po*) for the OGTT or injected (ip) with human biosynthetic insulin (0.3 U/kg; Humalog, Eli Lilly, Toronto, ON, Canada) for the ITT. Additional blood samples were collected from the tail vein at $t=10, 20, 30, 60, 90$ and 120 min for glucose measurements. For the OGTT, a blood sample was also collected from the tail vein at $t=10$ min for measurement of plasma insulin concentrations using an Insulin enzyme-linked immunosorbent assay (ELISA) for small sample volumes (Crystal Chem, Chicago, IL, USA). The trapezoidal rule for area-under-the-curve (AUC) calculations was used to measure glucose excursions from both the OGTTs and ITTs.

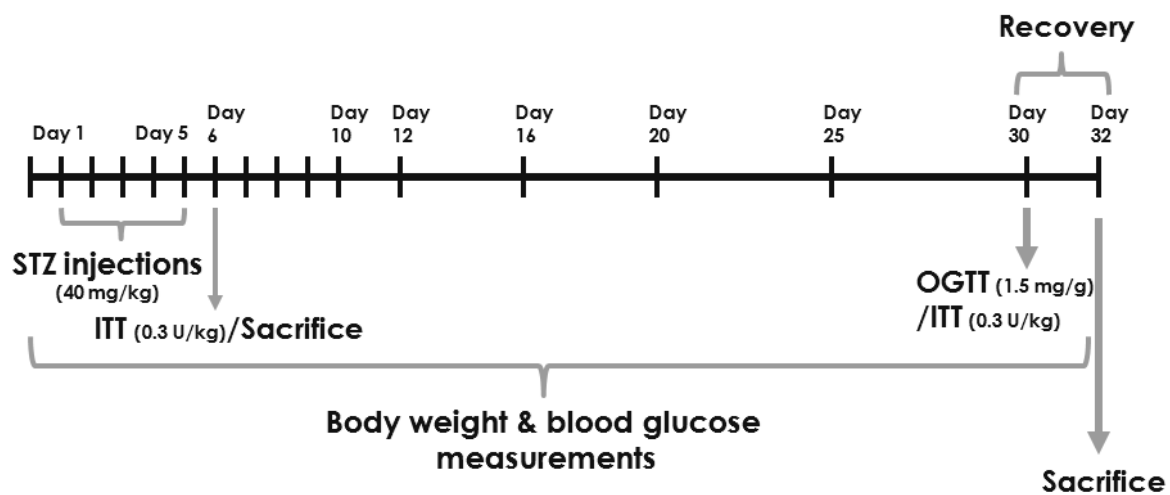


Figure 2.2 Experimental protocol for 6-d and 32-d STZ-induced diabetes studies.

2.3 Immunohistochemistry/Immunofluorescence

Mouse pancreatic tissues were divided into three sections and the head of the pancreas was weighed, fixed in formalin (Sigma-Aldrich), paraffin-embedded and sectioned at the Pathology Research Program Laboratory (Toronto General Hospital, Toronto, ON, Canada). The remaining splenic and body of the pancreas were flash-frozen for mRNA (messenger ribonucleic acid) analysis.

For determination of β -cell mass (BCM), pancreatic sections were de-waxed with xylene, rehydrated through a series of graded (100%, 95%, and 70%) ethanol baths, and washed in phosphate buffered saline (PBS). Slides were then treated for 30 min at room temperature in 3% hydrogen peroxide made fresh in PBS prior to use. Epitope antigen retrieval was performed by heating slides in 10 mM sodium citrate buffer pH 6.0 on high power for 20 min and then for 5 min at power 6. Slides were allowed to cool down to room temperature before blocking for 30 min at room temperature with normal serum block (Covance Signet, Princeton, NJ, USA) in a humid chamber. Sections were then incubated overnight at 4°C with a guinea pig anti-insulin antibody (1:200 dilution; Dako Diagnostics, Mississauga, ON, Canada). Slides were washed in PBS, incubated for 1 hr at room temperature with biotinylated anti-guinea pig antibody (1:500 dilution; Vector Laboratories, Burlington, ON, Canada) and subsequently treated for 1 hr at room temperature with either avidin/biotin complex (Vectastain Elite ABC Kit; Vector Laboratories) or streptavidin-labeled horseradish peroxidase (HRP) (Covance Signet). Subsequently, slides were stained with diaminobenzidine (DAB; Sigma-Aldrich) for 5 min, washed in distilled water, and counterstained with hematoxylin (Sigma-Aldrich). Finally, slides were dehydrated through a series of graded ethanol baths and mounted with Permount mounting medium (Fisher Scientific, Ottawa, ON, Canada). All dilutions were made in PBS. Pancreatic slides were

then scanned at the Advanced Optical Microscopy Facility (Princess Margaret Hospital, Toronto, ON, Canada) and Aperio ImageScope software (Aperio Technologies, Vista, CA, USA) was used to measure β -cell and total pancreatic area per section. Total BCM for each pancreas was determined as the product of the total cross-sectional β -cell area over the total pancreatic area and the weight of the pancreas.

For determination of β -cell proliferation, co-staining of insulin and the proliferative marker, Ki67, was performed as described above. Briefly, after blocking with normal serum block, slides were incubated for 1 hr at room temperature using a rat anti-mouse Ki67 antibody (1:150 dilution; Dako Diagnostics). Slides were then washed in PBS, incubated for 1 hr at room temperature using a biotinylated mouse anti-rat secondary antibody (1:200 dilution; Vector Laboratories), and labeled with streptavidin HRP for 30 min in a humid chamber. Pancreatic slides were washed and immunoreactivity was visualized with DAB as described above. Subsequently, slides were washed in PBS, re-treated with normal serum block for 30 min, and incubated overnight at 4°C with a guinea pig-anti insulin antibody (Dako Diagnostics). Slides were then washed in PBS and incubated for 1 hr at room temperature with biotinylated anti-guinea pig antibody (1:500 dilution; Vector Laboratories, Burlington, ON, Canada). After labeling the slides for 30 min at room temperature with avidin/biotin complex (Vectastain Elite ABC Kit; Vector Laboratories), slides were subsequently treated with Warp Red chromagen (Biocare Medical, Concord, CA, USA) for 2 min at room temperature. Slides were washed, dehydrated, and mounted as described above, and all dilutions were made in PBS. Staining of negative controls in all procedures was performed in the same manner as positive sections, with the exception of the primary antibody being omitted. Representative images are shown in Figure 3.10. The number of

proliferating β -cells was calculated as the total number of $\text{Ki67}^+:\text{insulin}^+$ cells over the total number of insulin^+ cells.

For determination of β -cell apoptosis, co-staining of insulin and the apoptotic marker, cleaved caspase 3, was performed using a rabbit anti-cleaved caspase 3 antibody (1:200 dilution; Cell Signaling Technology, Danvers, MA, USA) as described above. Slides were incubated for 1 hr at room temperature using a biotinylated goat anti-rabbit secondary antibody (1:200 dilution; Vector Laboratories) and further visualized as previously described. Slides were then incubated overnight at 4°C with a guinea pig-anti insulin antibody (Dako Diagnostics), followed by a wash and 1 hr incubation at room temperature with a biotinylated anti-guinea pig antibody (1:500 dilution; Vector Laboratories, Burlington, ON, Canada), as described above. Representative images are shown in Figure 3.10. The number of apoptotic β -cells was calculated as the total number of cleaved caspase 3 $^+:\text{insulin}^+$ cells over the total number of insulin^+ cells.

For determination of β -cell neogenesis, sections were co-stained for insulin and Ki67 or cleaved caspase 3, as described above. Representative images are shown in Figure 3.11. β -cell neogenesis was determined as the total number of insulin^+ ductal cells per section.

To analyze the developmental patterns of newly formed β -cells, immunofluorescence co-staining was performed with a variety of antibodies/antisera. Pancreatic sections were de-waxed and rehydrated as described above, and antigen retrieval was performed by heating slides in 10 mM sodium citrate buffer pH 6.0 as previously mentioned. Slides were allowed to cool down to room temperature before sections were blocked with 10% normal donkey serum (made in Tris buffered saline and Tween; TBST) and incubated overnight at 4°C with a guinea pig anti-insulin antibody (1:200 dilution; Dako Diagnostics) and one of the following primary antibodies/antisera (also shown in Table 2.1): rabbit anti-carbonic

anhydrase II (CA-II; 1:1000 dilution; Abcam, Cambridge, MA, USA), rabbit anti-cytokeratin-19 (CK-19; 1:100 dilution; Abcam), rabbit anti-glucagon (1:200 dilution; Dako Diagnostics), rabbit anti-GLUT2 (1:1000 dilution; Millipore, Billerica, CA, USA), rabbit anti-amylin/IAPP (1:100 dilution; Abcam), rabbit anti-MafA (1:2000 dilution; Bethyl Laboratories, Montgomery, TX, USA), and mouse anti-Nkx6.1 (1:1000 dilution; Beta Cell Biology Consortium, Nashville, TN, USA). The sections were then washed in TBST for 30 min and incubated for 1 hr at room temperature with Alexa Fluor 488-conjugated donkey anti-guinea pig IgG (H+L) and Cy3-conjugated donkey anti-rabbit or anti-mouse IgG (H+L), all of which were performed at a 1:400 dilution in TBST and obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Multiple dilutions in TBST were attempted in the various antisera for optimization of staining. Nuclear counterstaining was performed using 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and immunofluorescent images were acquired using a Zeiss AxioPlan confocal microscope (Carl Zeiss Canada, Toronto, ON, Canada). Staining of negative controls in all procedures was performed in the same manner as positive sections, with the exception of the primary antibody being omitted, as described above. Representative images of control and positive staining are shown in Figure 3.12.

Table 2.1. Primary antibodies used for IHC staining

ANTIGEN	SPECIES	SOURCE	DILUTION
Ki67	Rat	Dako Diagnostics (M7249)	1:150
Cleaved Caspase 3	Rabbit	Cell Signaling Technology (9661; Asp175)	1:200
Insulin	Guinea Pig	DAKO (A0564)	1:250
Carbonic Anhydrase II (CA-II)	Rabbit	Abcam (ab6621)	1:1000
Cytokeratin-19 (CK-19)	Rabbit	Abcam (ab15463)	1:100
Glucagon	Rabbit	Dako Diagnostics (A0565)	1:200
GLUT-2	Rabbit	Millipore (07-1402)	1:800-1000
IAPP (Amylin)	Rabbit	Abcam (ab53952)	1:100
MafA	Rabbit	Bethyl Laboratories (IHC-00352)	1:2000
Nkx6.1	Mouse	Beta Cell Biology Consortium (F55A10)	1:1000

2.4 Ribonucleic acid (RNA) isolation and semi-quantitative real-time polymerase chain reaction (qRT-PCR)

The splenic end of the pancreas was used for RNA extraction. Samples were sonicated in β -Mercaptoethanol (Sigma-Aldrich) and Buffer RLT (RNeasy kit; Qiagen, Toronto, ON, Canada) and total RNA isolated using the RNeasy kit according to the manufacturer's instructions (Qiagen). RNA was quantified by spectrophotometry in a Synergy Mx Monochromator-Based Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) using Gen5 1.10 Reader Control and Data Analysis Software (BioTek). A ratio of absorbance at 260 nm and 280 nm around 2.0 (1.7-2.1) was considered as valid purification of RNA for further experiments. RNA was then converted to complementary deoxyribonucleic acid (cDNA) by reverse transcription using Superscript II Reverse Transcriptase, according to the manufacturer's instructions (Invitrogen Life Technologies Inc, Burlington, ON, Canada). First-strand cDNAs were treated with ribonuclease H to remove RNA, and semi-quantitative RT-PCR (qRT-PCR) was performed using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a Chromo4 Continuous Fluorescence Detection unit with Opticon Monitor Analysis Software V 3.1 (Bio-Rad Laboratories, Mississauga, ON, Canada). Taqman Gene Expression Assays were used (ribosomal protein 18S no. Hs99999901_s1, insulin2 no. Mm00731595_gH and glucagon no. Mm00801712_ml; Applied Biosystems) and all reactions were performed in duplicate. Ribosomal protein 18s RNA was used as the endogenous control for all quantitative analyses of mRNA expression. Relative quantification of insulin2 (ins2) and glucagon mRNA expression was calculated using the $\Delta\Delta$ cycle threshold [$\Delta\Delta C(t)$] method (133).

2.5 Primary ductal cell culture

Although the *in vitro* primary ductal cell culture methods were unsuccessful, they have been included in this thesis for the sake of completeness. Pancreatic duct components were isolated from 8-9 week old *Rspo1*^{+/+} and *Rspo1*^{-/-} mice using the following two protocols.

The first protocol was adapted from Kitamura et al. (134). Mice were anesthetized using isoflourane (Abbott Laboratories, Chicago, IL, USA) and 1-2 ml of M199 medium (Gibco Life Technologies, Burlington, ON, Canada) containing 1 mg/ml collagenase P (Roche Applied Science, Mississauga, ON, Canada) was injected into the common bile duct. The swollen pancreas was then removed and incubated in the same solution for 17 min at 37°C with gentle shaking every 3-4 min to disrupt the pancreas until the suspension turned homogenous. After incubation, cellular aggregates were diluted in 30 ml of ice-cold M199 medium containing 10% fetal bovine serum (FBS) to stop the digestion reaction, and the digested pancreata was dispersed by gently pipetting up and down with a glass pipette, using 30 ml of the same medium. The tissue suspension was applied onto a pre-wet 500 µm polyester mesh (Corning Incorporated, Corning, NY, USA) and the collected tissue was spun down to collect the pellet. The pellet was then resuspended in RPMI 1640 medium (Gibco Life Technologies) supplemented with 10% FBS, 5.5 mM glucose, and Antibiotic-Antimycotic (10000 U/mL of penicillin, 10000 µg/mL of streptomycin, 25 µg/mL amphotericin B; Gibco Life Technologies) and cultured at 37°C in 5% CO₂. After 3 days in culture, medium was changed with fresh RPMI supplemented medium. After 7 days in culture, medium was replaced with serum-free RPMI supplemented with 8 mM glucose and 2 g/L BSA (Sigma-Aldrich). Nicotinamide (10 mM; Sigma-Aldrich), 1 g/L ITS (1.0 mg/ml recombinant human insulin, 0.55 mg/ml human transferrin, 0.5 µg/ml sodium selenite; Sigma-Aldrich), and 10 ng/ml keratinocyte growth factor (KGF; Sigma-Aldrich), were added

immediately prior to use, with medium changed every 2-3 days. At this point, small clusters of 'cobblestone'-shaped cells indicative of ductal cells would begin to appear, but these all died off by week 3 of culturing.

The second protocol for ductal cell isolation was adapted from Dr. Susan Bonner-Weir's group (personal communication; (135)). Once again, mice were anesthetized and the pancreas was removed as described above. After digestion for 17 min at 37°C, the digested pancreas was placed on a Ficoll gradient as previously described (136). Briefly, Ficoll 400 (Sigma-Aldrich) was diluted to 6 ml of 25% (w/v), and 4 ml each of 23%, 20% and 11% (w/v) with sterile 1xHank's buffered saline solution (HBSS). Ficoll were layered in a sterile 50 ml Falcon tube in order of decreasing density with the digested pancreas laid carefully on top. This was then centrifuged at 1350xg for 10 min at 4°C with acceleration set to slow and the deceleration turned off. The rest of the experiment was done on ice in a biological safety cabinet (BSC) when possible and centrifuged at 4°C. The pellet was collected and, after adding 40 ml of RPMI medium, this islet-depleted tissue was divided into four 50 ml tubes, centrifuged at 57xg for 5 min, washed with calcium and magnesium free-phosphate buffered saline (CMF-PBS), and centrifuged again at 57xg for 5 min. For dispersion of the cells, a freshly prepared Trypsin solution containing 1.5 ml of 0.25% Trypsin EDTA in 20 ml PBS-CMF was added to each tube and vortexed for 15 sec. The dispersed cells were then placed in a 37°C Precision Scientific Reciprocal Shaking Bath (Model 25; Thermo Scientific, Rockford, IL, USA) at 25xg for 4 min. After shaking, 10 ml of ice-cold RPMI medium was added to all tubes to halt the enzymatic reaction with gentle pipetting on ice. Next, the sample was run through a pre-wet 40 µm cell strainer (Falcon BD Biosciences, Mississauga, ON, Canada), placed in a 50 ml tube, and centrifuged at 57xg for 5 min. The pellet was resuspended in 2-3 ml of de-gassed PBS-CMF buffer solution supplemented with 750 mg/l

EDTA(Bioshop Canada, Burlington, ON, Canada) and 5 g/l BSA (Sigma-Aldrich), and vortexed. At this point, cell number was determined using a hemocytometer and trypan blue (Sigma-Aldrich), as a cell count of at least 10^5 cells was required for further steps as per requirements of the Magnetic Assisted Cell Sorting (MACS) system (Miltenyi Biotech, Auburn, CA, USA). The remaining isolation of ductal cells was performed as described by the MACS system using the MS column for positive selection of small sample sizes (http://www.miltenyibiotec.com/download/datasheets_en/42/MiltenyiBiotec_DataSheet_Anti-Rat-IgG-MicroBeads_130-048-502.pdf; a kind gift from Miltenyi Biotech). After magnetic separation, the positive fraction was centrifuged at $228 \times g$ for 5 min and resuspended in 5 ml of RPMI medium supplemented with 11 mM glucose, 10% FBS and Antibiotic-Antimycotic (10000 units/mL of penicillin, 10000 $\mu\text{g/mL}$ of streptomycin, 25 $\mu\text{g/mL}$ amphotericin B; Gibco Life Technologies). Cells were recounted as described above and $>10^4$ cells were plated on 10 cm petri dishes with the same RPMI medium. After 3 days in suspension, aggregated tissue was collected and separated by centrifugation (5 min) three times. Cells were then collected, washed, and plated onto collagen-coated plates (rat tail collagen type 1; Sigma-Aldrich) in DMEM/F12 medium (Gibco Life Technologies) supplemented with 17 mM glucose, 10% FBS, 20 ng/ml epidermal growth factor (EGF; Cedarlane, Burlington, ON, Canada) and Antibiotic-Antimycotic (10000 U/mL of penicillin, 10000 $\mu\text{g/mL}$ of streptomycin, 25 $\mu\text{g/mL}$ amphotericin B; Gibco Life Technologies). After 24 hrs in this culture medium, the media was changed to serum-free DMEM/F12 medium supplemented with 17 mM glucose, with 1 g/l ITS, 2 g/l BSA, 10 nmol/l nicotinamide, 5 nmol/l exendin-4 (Bachem, Torrance, CA, USA) and 20 ng/ml EGF added fresh immediately prior to use. After 5-7 days in culture, cells were then layered with Matrigel (VWR International, Mississauga, ON, Canada)

according to the manufacturer's instructions for thin gel overlay (http://www.bdbiosciences.com/external_files/dl/doc/manuals/live/web_enabled/354234_Guidelines.pdf), with the exception of an overnight gelling time at 37°C. The cells were then coated with Matrigel and allowed to gel overnight before additional serum-free DMEM/F12 supplemented media was added. At this point, the presence of 'cobblestone'-shaped cells was noticeable, but cell numbers were very low even after 2-3 weeks in culture with growth factors.

2.6 Statistical Analysis

All results are presented as mean \pm SEM. Statistical analysis was performing using Statistical Analysis System software (SAS v 9.1.3, SAS Institute, Cary, NC, USA) using one- or two-way analysis of variance (ANOVA), followed by a Student's t test or one-way ANOVA, as well as post hoc analysis, as appropriate. Statistical significance was defined as $p < 0.05$.

3. RESULTS

3.1 Phenotypes of $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice are similar throughout the STZ-studies.

To examine the effects of $Rspo1$ deficiency in a model of diabetes, mice were injected (ip) with multiple low-doses of STZ (40 mg/kg body weight) for five consecutive days. Although mice treated with STZ had similar levels of non-fasting blood glucose in the first days of STZ administration (Figure 3.1A), they developed progressive hyperglycemia by the end of the 32-d STZ study (Figure 3.1B), with blood glucose levels reaching similar values as previously reported (132). However, no marked differences in morning-fed blood glucose levels were noted between wild-type and knockout mice throughout the STZ studies. Wild-type and knockout mice demonstrated similar trends in body weight throughout both the short- (6-d) and long- (32-d) term STZ studies (Figure 3.2). Although there was a slight decrease in body weights of $Rspo1^{+/+}$ mice between day 4-20 in the long-term STZ studies, by the end of the experimental protocol, body weights were similar for both groups of mice (Figure 3.2B).

Pancreatic weights of $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice in non-STZ-treated control (Figure 3.3A) and 6-d STZ (Figure 3.3B) studies were not different between the two groups of mice. Interestingly, pancreatic weights of $Rspo1^{-/-}$ mice were significantly reduced compared to their wild-type littermates by the end of the 32-d STZ studies ($p < 0.05$; Figure 3.3C).

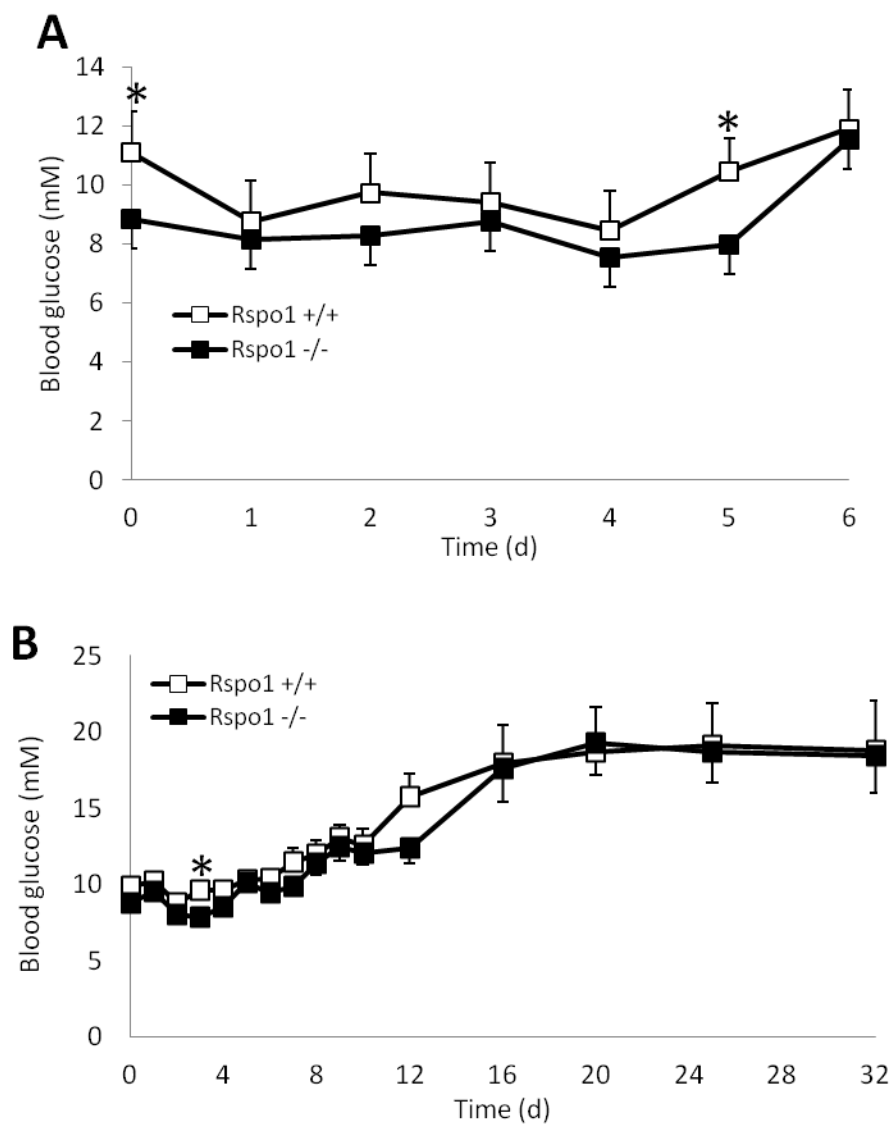


Figure 3.1 Fed glycemia of $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice does not differ throughout the STZ-induced studies. Non-fasting blood glucose levels of $Rspo1^{+/+}$ (white squares) and $Rspo1^{-/-}$ (black squares) mice over the course of 6-d (**A**; $n = 9-11$) and 32-d (**B**; $n = 11-16$) STZ studies. * $p < 0.05$ by Student's t-test

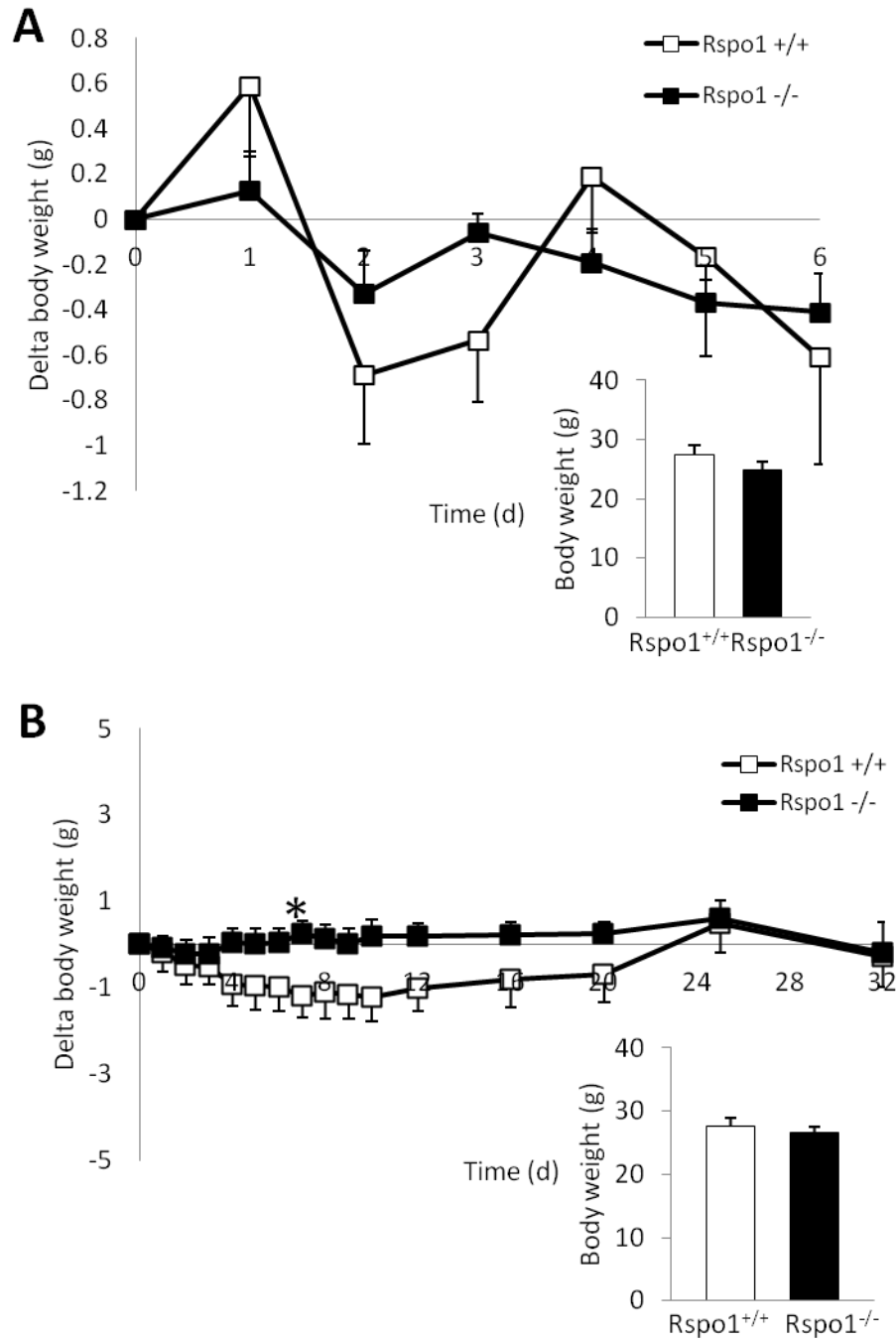


Figure 3.2 $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice have similar body weights throughout the STZ-induced studies. Delta body weights of $Rspo1^{+/+}$ (white squares) and $Rspo1^{-/-}$ (black squares) mice over the course of 6-d (**A**; $n = 9-11$) and 32-d (**B**; $n = 11-16$) STZ studies. * $p < 0.05$ by Student's t-test.

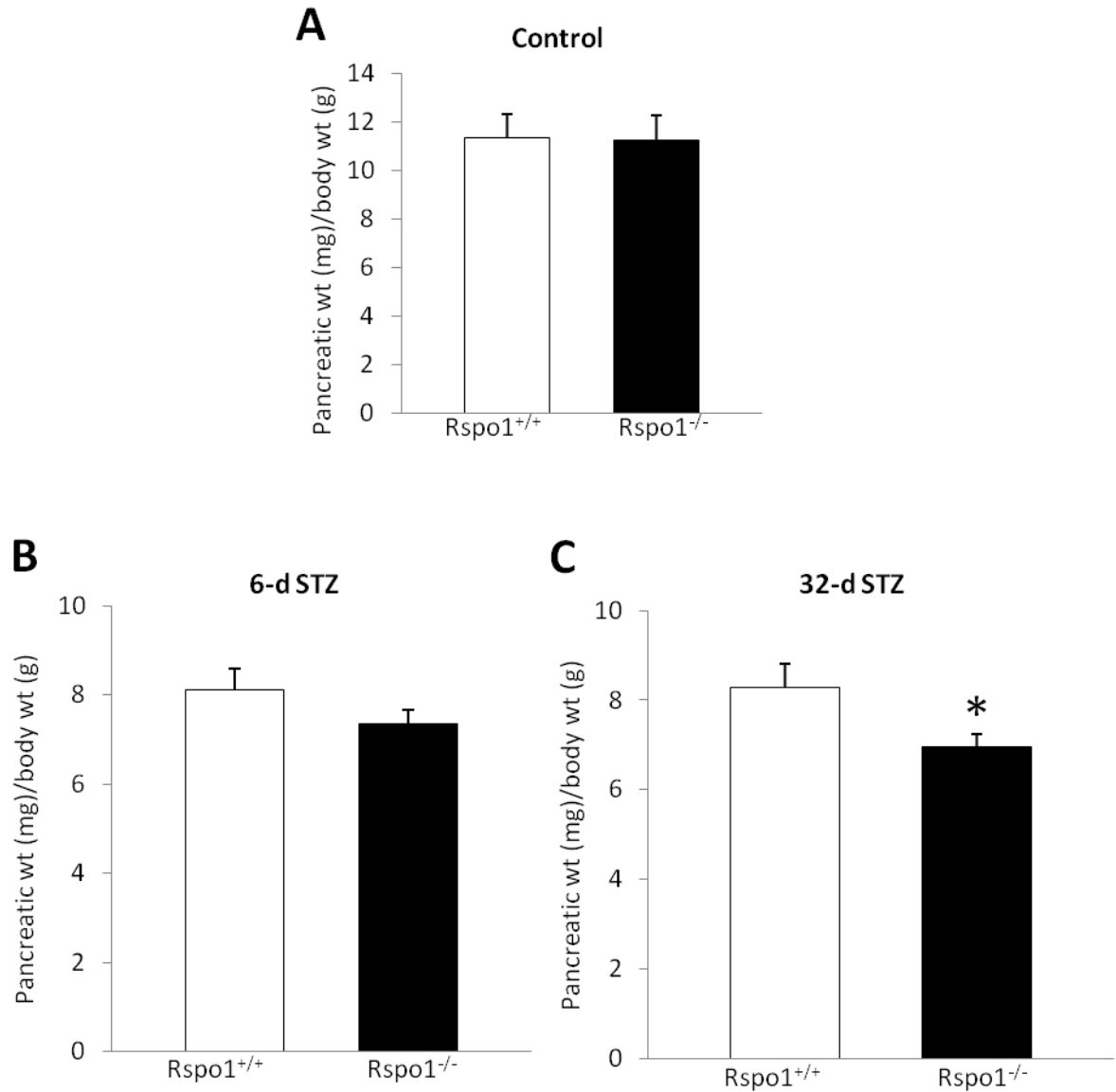


Figure 3.3 Pancreatic weights are similar between **Rspo1^{+/+}** and **Rspo1^{-/-}** mice until the end of the STZ-induced studies. Pancreatic weights of **Rspo1^{+/+}** (white bars) and **Rspo1^{-/-}** (black bars) mice normalized to body weights in control (**A**; $n = 4-5$), 6-d (**B**; $n = 9-11$), and 32-d (**C**; $n = 11-16$) STZ studies. * $p < 0.05$ by Student's t-test.

3.2 Rspo1^{+/+} and Rspo1^{-/-} mice have similar glycemetic control but improved insulin sensitivity by the end of the STZ-studies.

To examine the effect of Rspo1 deficiency in endocrine pancreas function following induction of STZ diabetes, I performed metabolic tests for whole-body glucose homeostasis. I first examined this effect in non-STZ-treated control wild-type and knockout mice. Similar to previous reports (122), fasting blood glucose levels were in the normal range for both Rspo1^{+/+} and Rspo1^{-/-} mice, with no difference noted between the two groups (Figure 3.4A inset). However, in contrast to previous work (122), there was no difference in glucose handling between Rspo1^{+/+} and Rspo1^{-/-} mice after an oral glucose challenge (Figure 3.4A). Consistent with this finding, there was also no difference in the AUC of the glucose excursions at 0 to 120 min after an oral glucose load (Figure 3.4B).

These studies were repeated in 32-d STZ-induced mice when hyperglycemia was present (Figure 3.1B). There was no difference in fasting glycemia between Rspo1^{+/+} and Rspo1^{-/-} mice by the end of the long-term studies, although Rspo1^{+/+} STZ-induced mice demonstrated impaired glucose handling in response to an OGTT ($p < 0.0001$ by two-way ANOVA between Figure 3.4B and Figure 3.5B), as expected. However, there was no difference in glycemetic responses to oral glucose administration between Rspo1^{+/+} and Rspo1^{-/-} mice after insult with STZ (Figure 3.5A-B).

Although oral glucose handling was determined to be similar between the two groups of mice, intraperitoneal ITTs were performed to examine possible differences in insulin sensitivity between Rspo1^{+/+} and Rspo1^{-/-} mice. Blood glucose levels after a 6 hr fast were similar between Rspo1^{+/+} and Rspo1^{-/-} mice in both 6-d (Figure 3.6A) and 32-d (Figure 3.6B) STZ studies although, as expected, mice exhibited impaired fasting glycemia by the end of the STZ studies ($p < 0.05$). The glycemetic response to an ITT (Figure 3.6C) was not different between Rspo1^{+/+} and Rspo1^{-/-} mice by day 6 of the STZ study. However, by the end of the

long-term STZ study, $Rspo1^{-/-}$ mice had significantly better glycemic responses to an ITT ($p < 0.05-0.0001$; Figure 3.6D), suggesting increased insulin sensitivity in these animals.

Plasma levels of insulin after a 5 hr fast were not statistically different between $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice in non-STZ-treated control mice, as well as in mice from both the 6-d and 32-d STZ studies (Figure 3.7A-C). Furthermore, no change was observed in plasma insulin levels at $t = 10$ min following an oral glucose load in both wild-type and knockout animals in both the non-STZ-treated control and 32-d STZ-induced studies (Figure 3.7D-E).

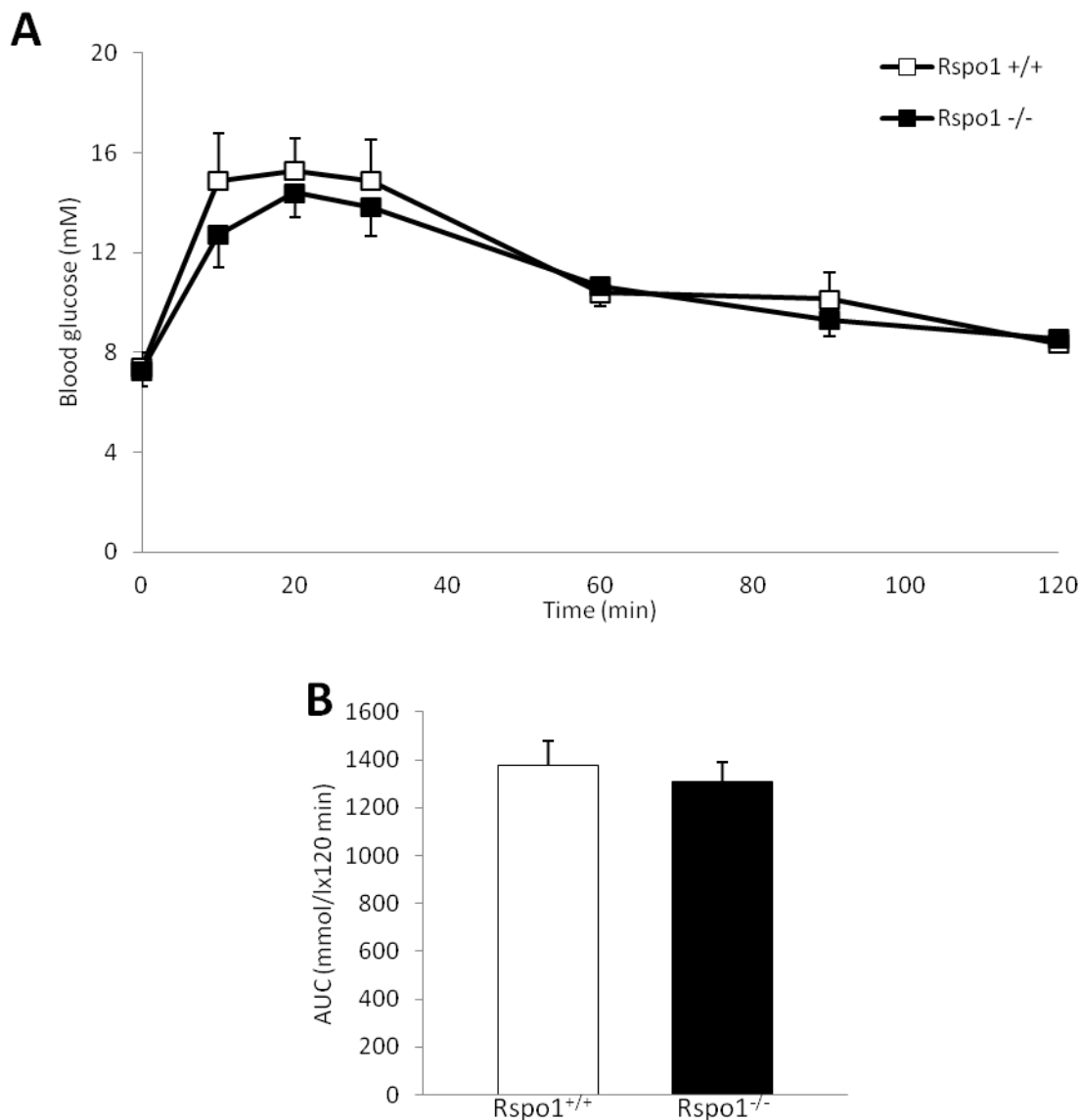


Figure 3.4 Control Rspo1^{+/+} and Rspo1^{-/-} mice have similar glycemic control in response to an oral glucose load. **A** Glycemic profiles in control Rspo1^{+/+} (white squares) and Rspo1^{-/-} (black squares) mice in response to an OGTT after an overnight fast (n = 6). **B** Area-under-the-curve (AUC) of the glucose excursions at 0 to 120 min after administration of an oral glucose load (n = 6) in control mice.

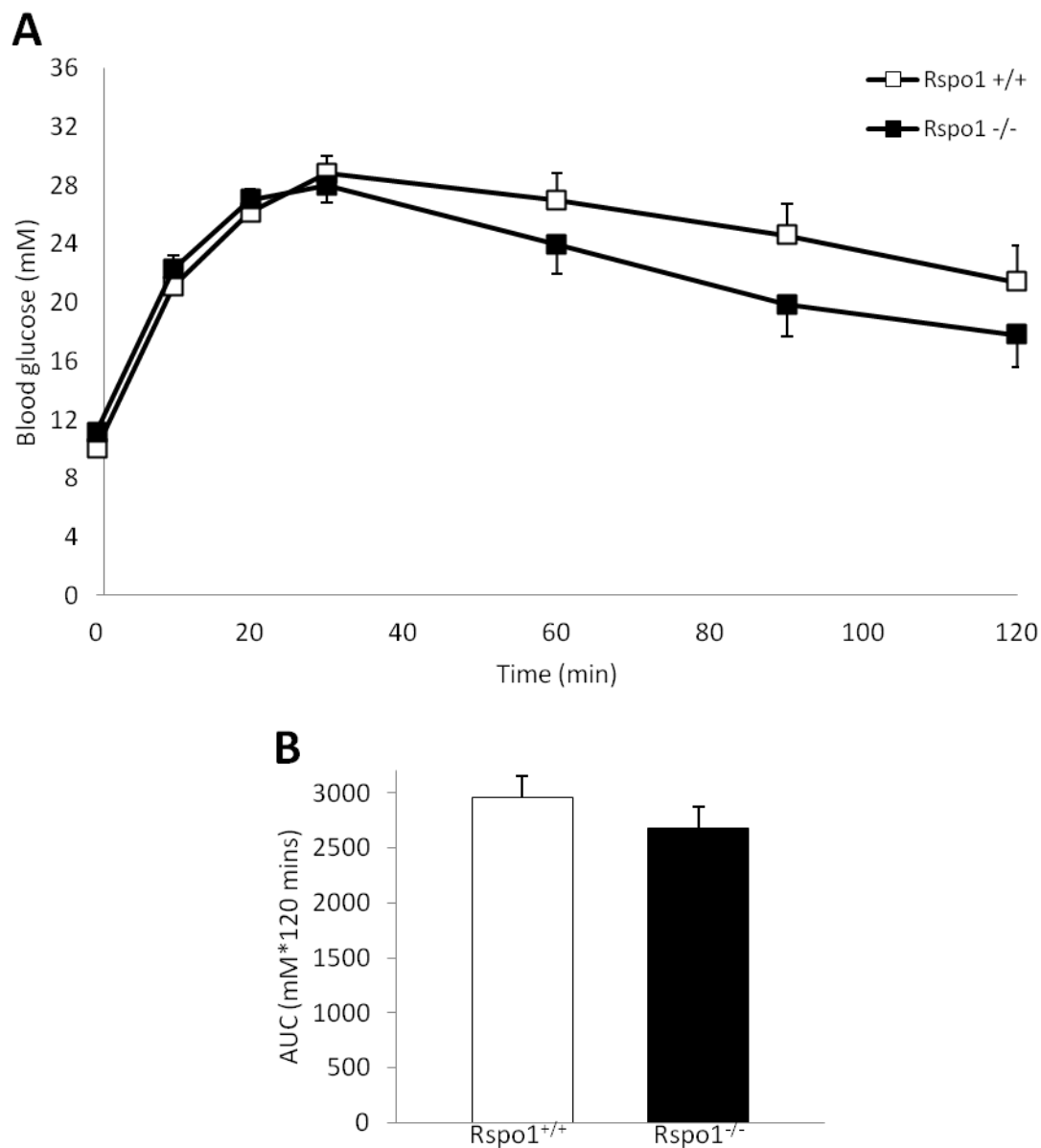


Figure 3.5 32-d STZ-induced diabetic Rspo1^{+/+} and Rspo1^{-/-} mice have similar glucose handling in response to an oral glucose load. **A** Glycemic profiles in 32-d STZ-induced Rspo1^{+/+} (white squares) and Rspo1^{-/-} (black squares) mice in response to an OGTT after an overnight fast (n = 5-16). **B** Area-under-the-curve (AUC) of the glucose excursions at 0 to 120 min after administration of an oral glucose load (n = 6) in 32-d STZ-induced mice.

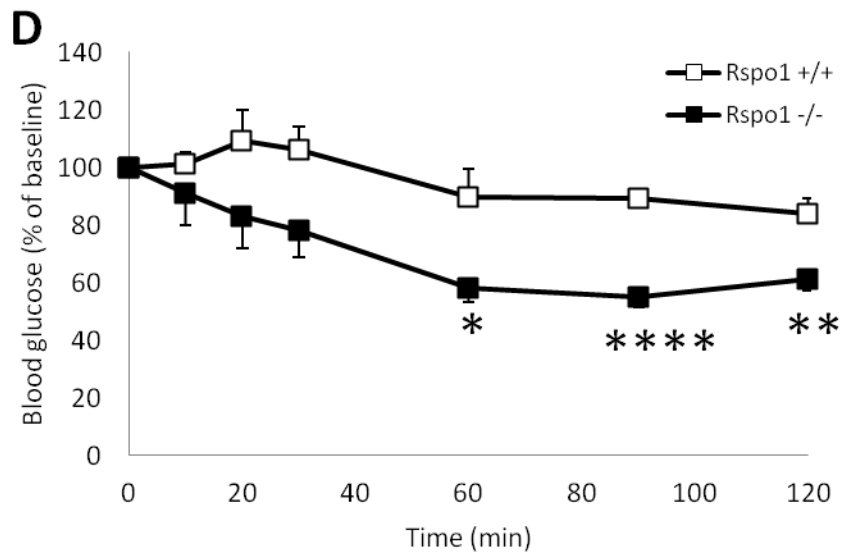
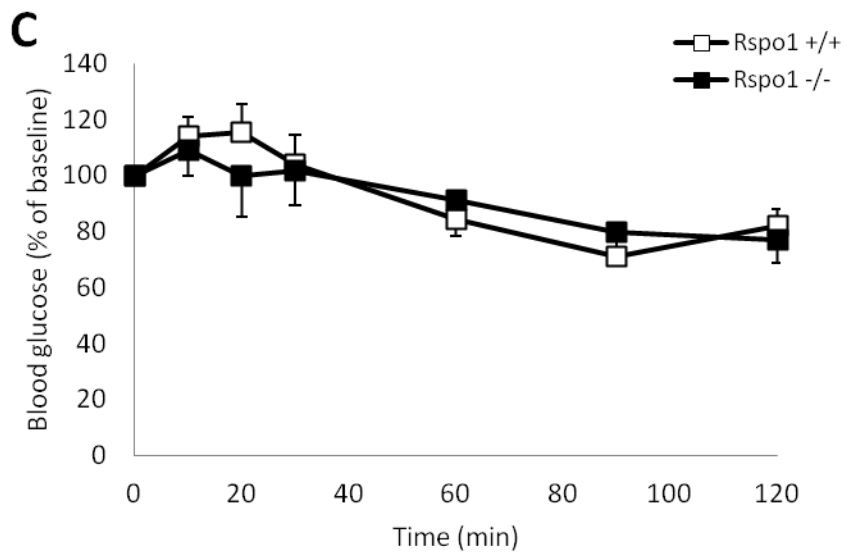
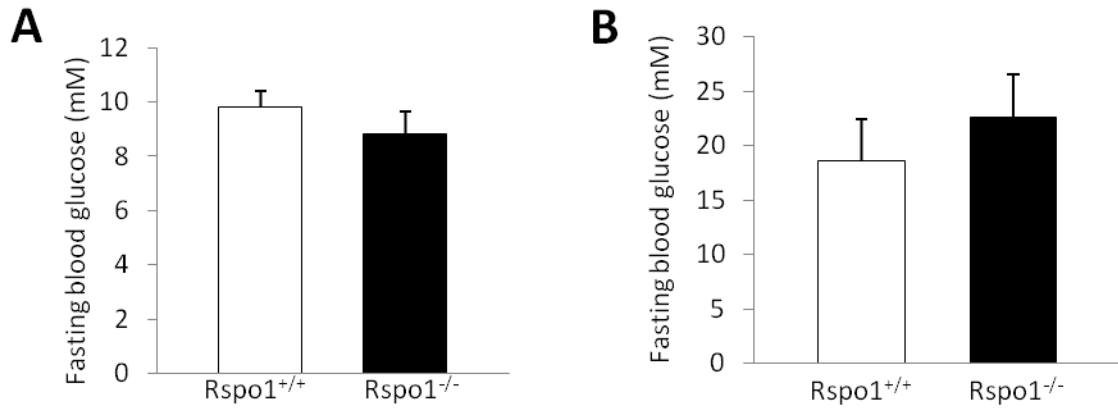


Figure 3.6 $Rspo1^{-/-}$ mice have increased insulin sensitivity by the end of the 32-d STZ studies. Blood glucose levels after a 6 hr fast in 6-d (**A**; n = 5-7) and 32-d (**B**; n = 5-16) STZ studies. **C** Glycemic responses in $Rspo1^{+/+}$ (white squares) and $Rspo1^{-/-}$ (black squares) mice during an intraperitoneal insulin tolerance test (ITT) at the end of the 6-d STZ study (n = 5-7). **D** Glycemic responses in $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice to an intraperitoneal ITT at the end of the 32-d STZ study (n = 5-16). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by Student's t-test.

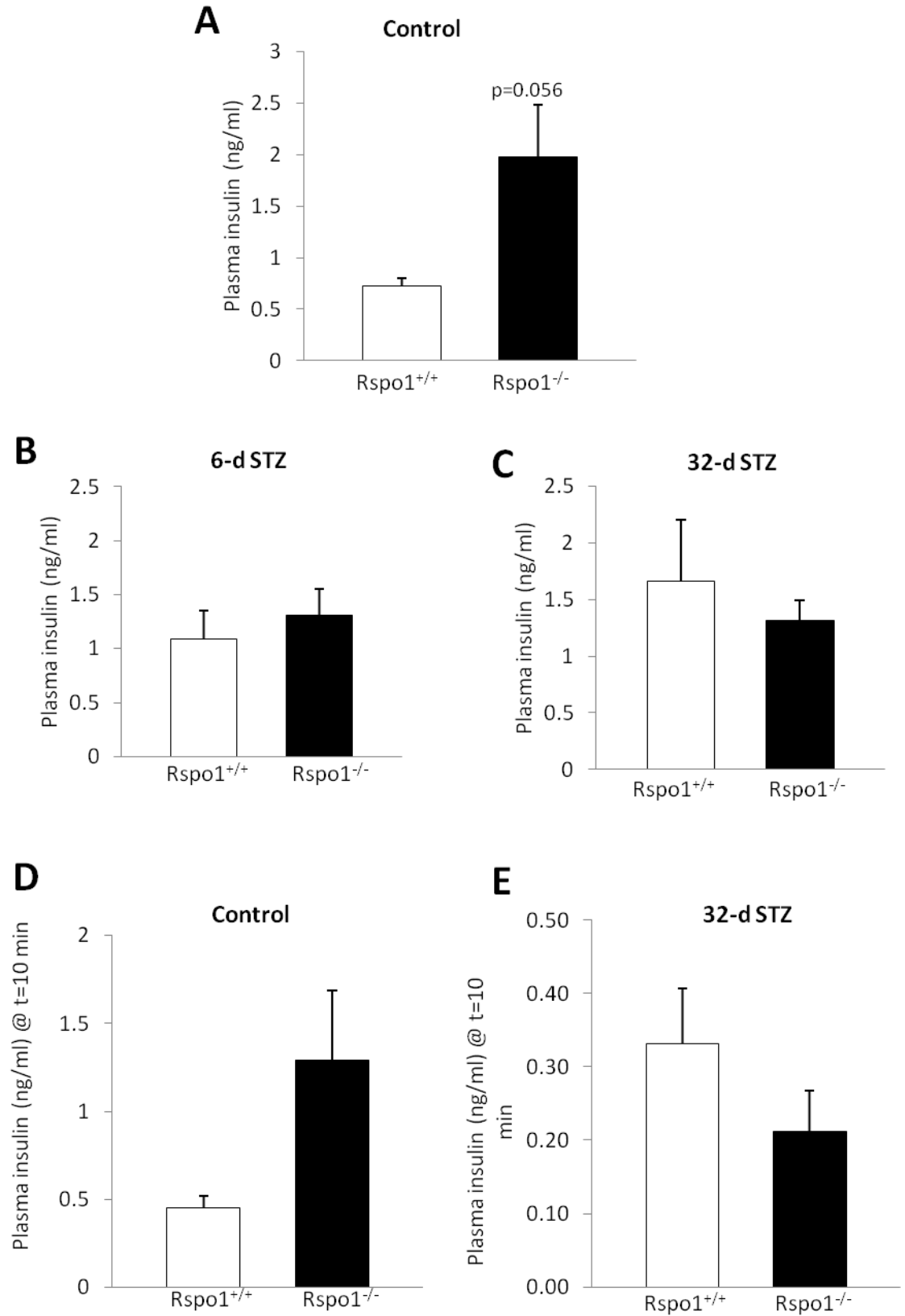


Figure 3.7 Plasma insulin concentrations do not differ between Rspo1^{+/+} and Rspo1^{-/-} mice. Levels of plasma insulin in Rspo1^{+/+} (white bars) and Rspo1^{-/-} (black bars) mice in control (A; n = 6), 6-d STZ (B; n = 9-11), and 32-d STZ (C; n = 11-16) studies after a 5 hr fast, as determined by radioimmunoassay. Plasma insulin levels in control (D; n = 3-6) and 32-d STZ (E; n = 9-13) studies at t =10 min after administration of an OGTT, as determined by an insulin ELISA.

3.3 Rspo1^{-/-} mice have reduced β -cell apoptosis and increased β -cell neogenesis in response to STZ-induced diabetes.

Rspo1 deficiency has previously been shown to increase β -cell mass under basal conditions (122). Thus, in order to determine whether mice lacking Rspo1 would continue to demonstrate this effect under STZ-stressed conditions, pancreatic sections were stained for insulin to examine total β -cell mass. Consistent with previous work (122), Rspo1^{-/-} mice from the non-STZ-treated control group had significantly increased β -cell mass, by 1.9-fold relative to wild-type controls ($p < 0.05$; Figure 3.8). Insult with STZ failed to decrease β -cell mass in Rspo1^{+/+} mice as had been expected, although STZ administration significantly reduced β -cell mass in Rspo1^{-/-} mice compared to basal levels. However, there was no difference in β -cell mass between Rspo1^{+/+} and Rspo1^{-/-} mice throughout the STZ experimental periods (Figure 3.8A). Thus, interestingly, Rspo1^{-/-} mice had a greater decrease in β -cell mass after administration of STZ than their wild-type counterparts ($p < 0.0001$), suggesting that these mice may have increased sensitivity to the early effects of the STZ. Similar trends were observed in β -cell area (Figure 3.8B-D), and no differences in the total number of islets or in islet distribution by size (arbitrarily set as 1, 2-99, or 100 and greater β -cells) were apparent between Rspo1^{+/+} and Rspo1^{-/-} mice throughout the STZ experimental protocols (Figure 3.9A-B).

Since Rspo1 deficiency has been found to increase β -cell proliferation and neogenesis in control animals (122), we investigated whether this effect would be continued after the

administration of STZ. Co-immunostaining for insulin and the proliferative marker, Ki67, in pancreatic sections of *Rspo1*^{+/+} and *Rspo1*^{-/-} mice failed to demonstrate any significant differences in cell proliferation after insult with STZ in both the 6-d (Figure 3.10A) and 32-d (Figure 3.10B) STZ studies. However, examination of β -cell apoptosis, detected by insulin and cleaved-caspase 3 co-staining, revealed that apoptosis was significantly reduced ($p < 0.05$) in *Rspo1*^{-/-} mice relative to wild-type mice in the 6-d STZ animals (Figure 3.10C), with a similar trend ($p = 0.051$) observed in 32-d STZ animals (Figure 3.10D). Consistent with previous studies (122), *Rspo1*^{-/-} mice also had significantly increased levels of insulin-positive ductal cells (by 3.1-fold, $p < 0.05$; Figure 3.11A), suggestive of β -cell neogenesis. Most importantly, *Rspo1*^{-/-} mice continued to display markedly higher levels of insulin-positive ductal cells as compared to wild-type mice in both the 6-d (by 3-fold, $p < 0.05$; Figure 3.11B) and 32-d (by 2.2-fold, $p < 0.05$; Figure 3.11C) STZ studies, whereas wild-type mice exhibited no change in the levels of insulin-positive ductal cells between control and STZ experimental studies. Increased mRNA expression of *Ins2* was also seen in the *Rspo1*^{-/-} mice relative to wild-type animals at the end of the 6-d STZ study ($p < 0.05$; Figure 3.11D). However, this difference in *Ins2* expression was lost by the end of the 32-d STZ study (Figure 3.11E). Collectively, these data indicate that *Rspo1*^{-/-} mice have increased β -cell neogenesis and enhanced protection against β -cell apoptosis compared to wild-type littermates in the face of STZ-induced diabetes.

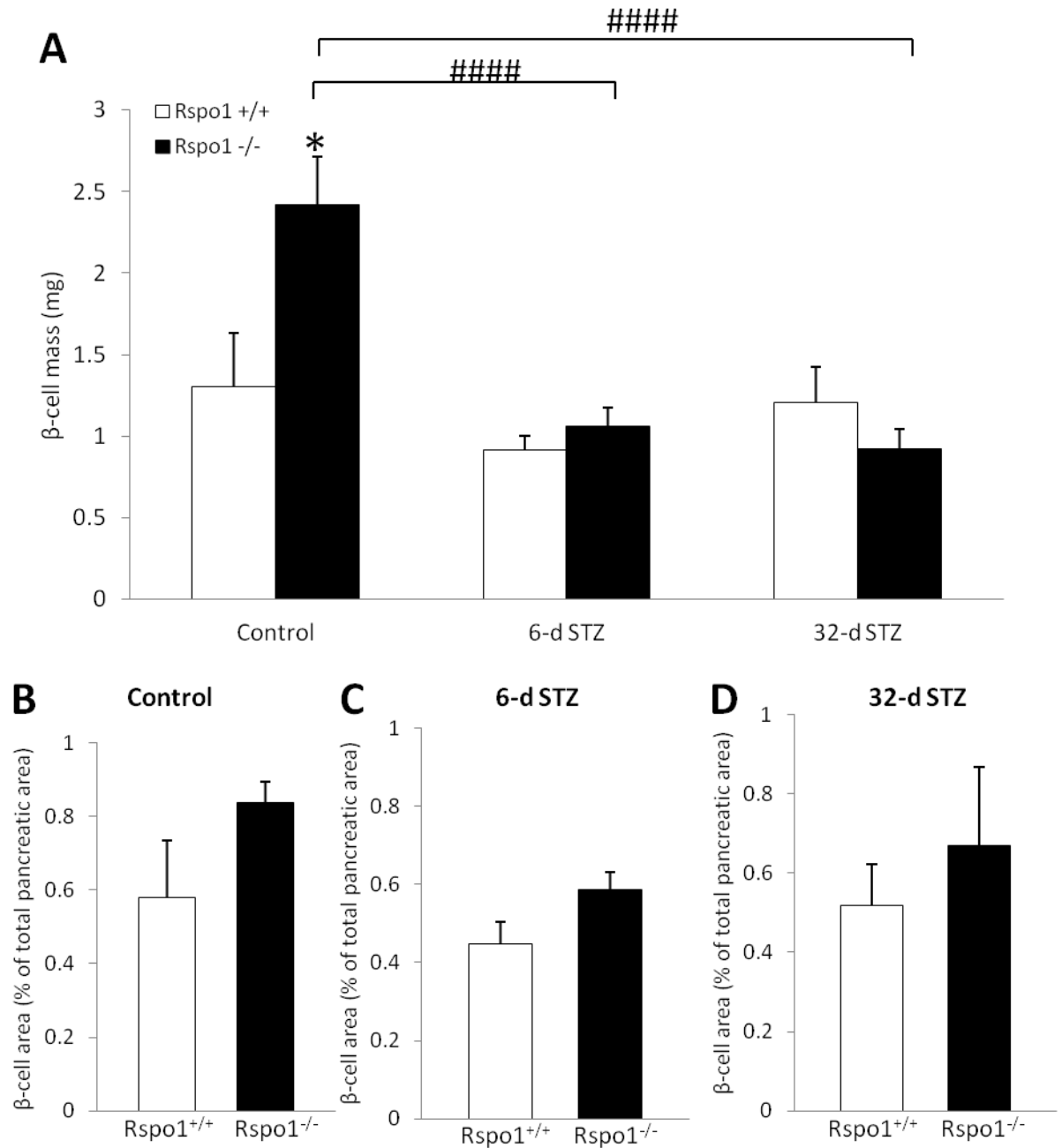


Figure 3.8 β -cell mass differs between $Rspo1^{+/+}$ and $Rspo1^{-/-}$ control mice only. β -cell mass (BCM; A) and β -cell area (B-D) was determined in control (n = 4-5), 6-d STZ (n = 9-11), and 32-d STZ (n = 11-16) studies in insulin-stained pancreatic sections from $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice. * $p < 0.05$ vs. control wild-type mice, #### $p < 0.0001$ as indicated by two-way ANOVA, followed by one-way ANOVA and n-1 custom hypotheses post-hoc tests.

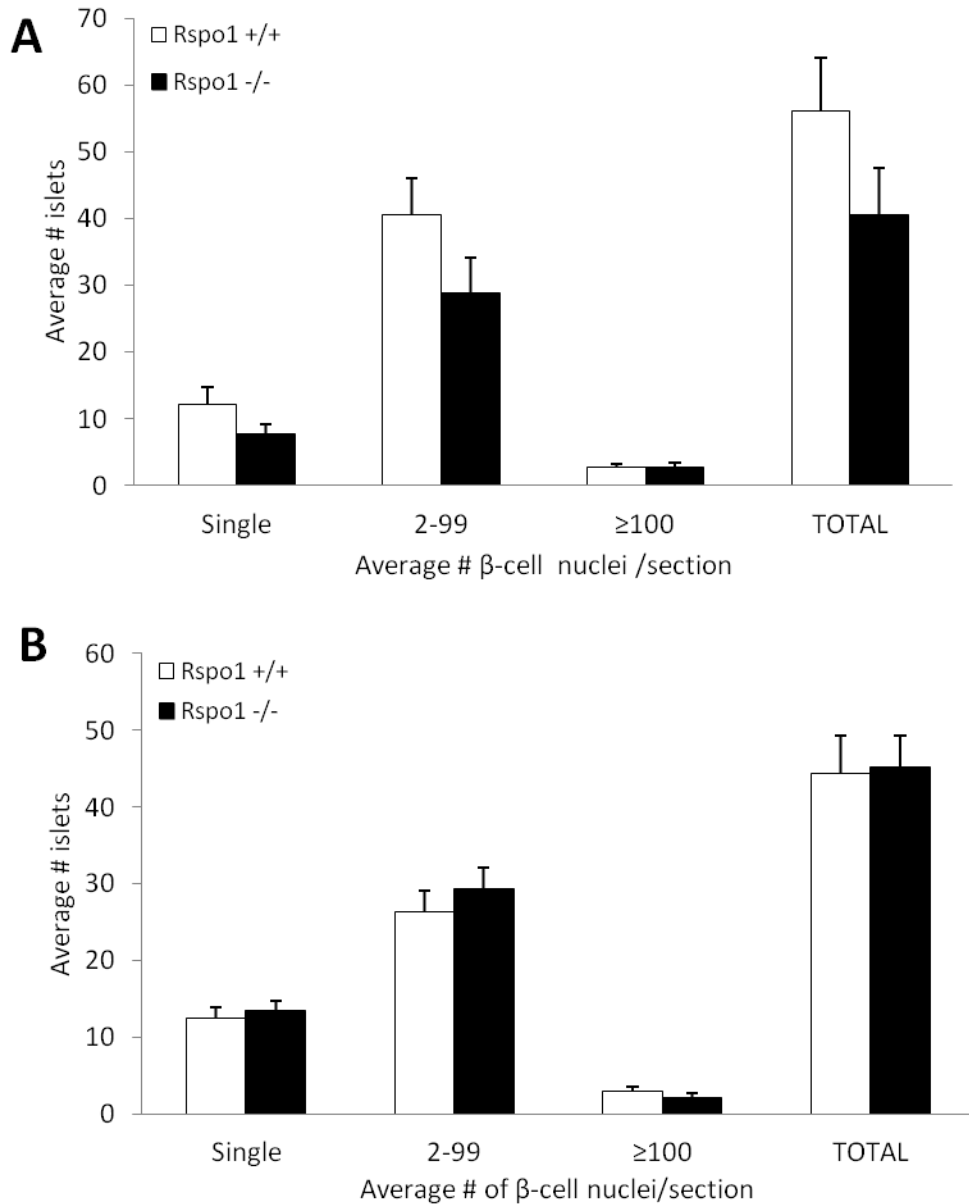


Figure 3.9 $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice have similar islet profiles. Size distribution and total number of islets per section in 6-d (**A**; $n = 9-11$) and 32-d (**B**; $n = 11-16$) STZ studies were determined in insulin-stained pancreatic sections from $Rspo1^{+/+}$ (white bars) and $Rspo1^{-/-}$ (black bars) mice. Islets were arbitrarily distributed based on size as single insulin-positive β -cells, between 2 to 99 insulin-positive β -cells, or 100 or more insulin-positive β -cells.

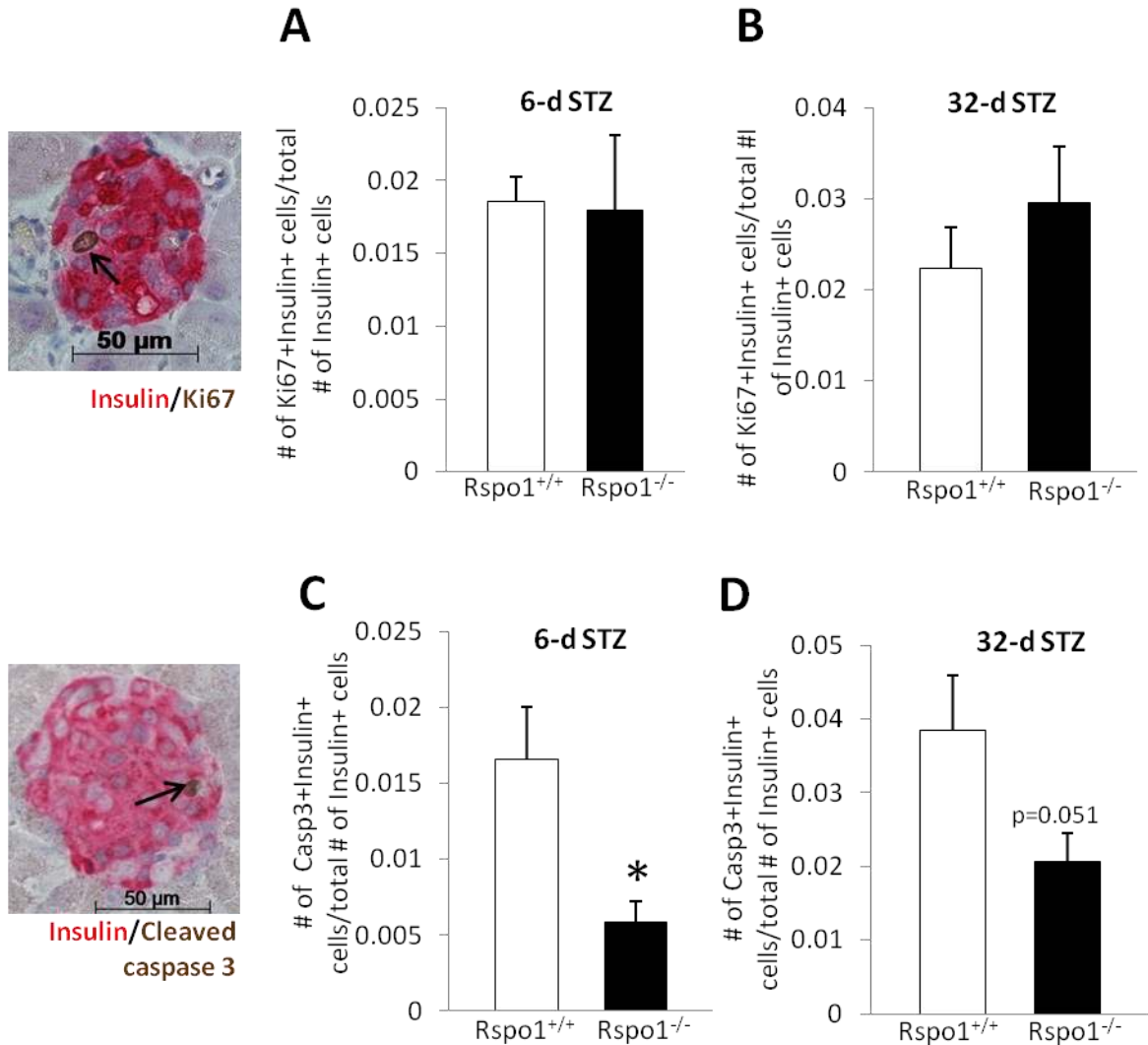


Figure 3.10 $Rspo1^{-/-}$ mice have reduced B-cell apoptosis. Proliferating β -cells were determined by co-staining for insulin and Ki67 (# of Ki67⁺:insulin⁺ cells/total # of insulin⁺ cells) in 6-d (**A**; n = 9-11) and 32-d (**B**; n = 11-16) STZ studies. Apoptotic β -cells were determined by co-staining for insulin and cleaved caspase-3 (# of cleaved caspase 3⁺:insulin⁺ cells/total # of insulin⁺ cells) in 6-d (**C**; n = 9-11) and 32-d (**D**; n = 11-16) STZ studies. Representative pictures are shown; arrows indicate Ki67- and caspase3-positive cells. Scale bars represent 50 μ m. *p<0.05 by Student's t-test.

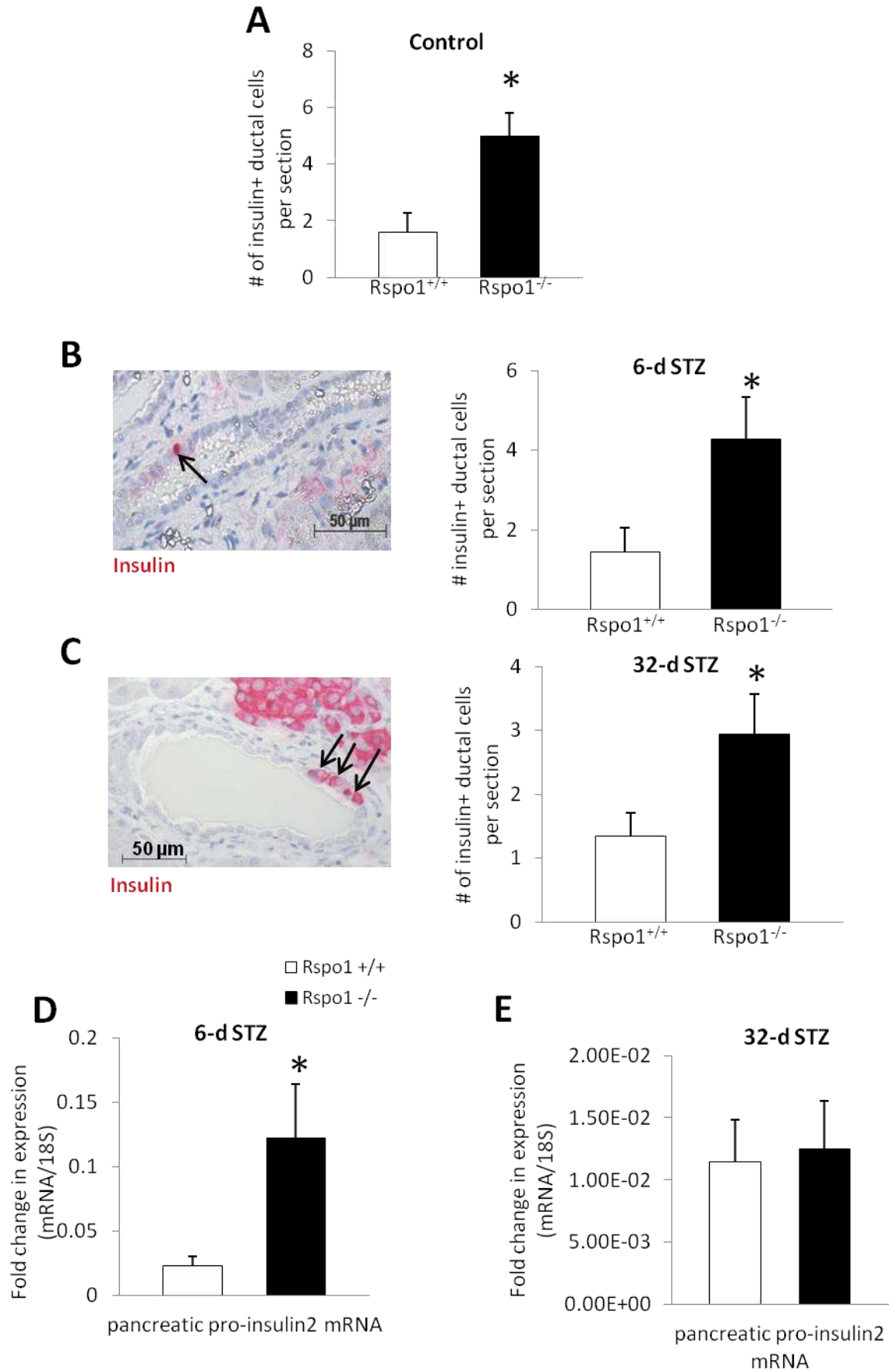


Figure 3.11 *Rspo1*^{-/-} mice have increased β -cell neogenesis. β -cell neogenesis was determined as the number of insulin-positive cells in the pancreatic ducts of control (**A**; n = 4-5), 6-d STZ (**B**; n = 9-11), and 32-d STZ (**C**; n = 11-16) studies. Representative pictures are shown; arrows indicate insulin-positive cells. qRT-PCR analyses of insulin2 mRNA in pancreatic lysates of 6-d (**D**; n = 9-11) and 32-d (**E**; n = 11-16) STZ studies. Relative expression levels were normalized to 18S rRNA expression. *p<0.05 by Student's t-test.

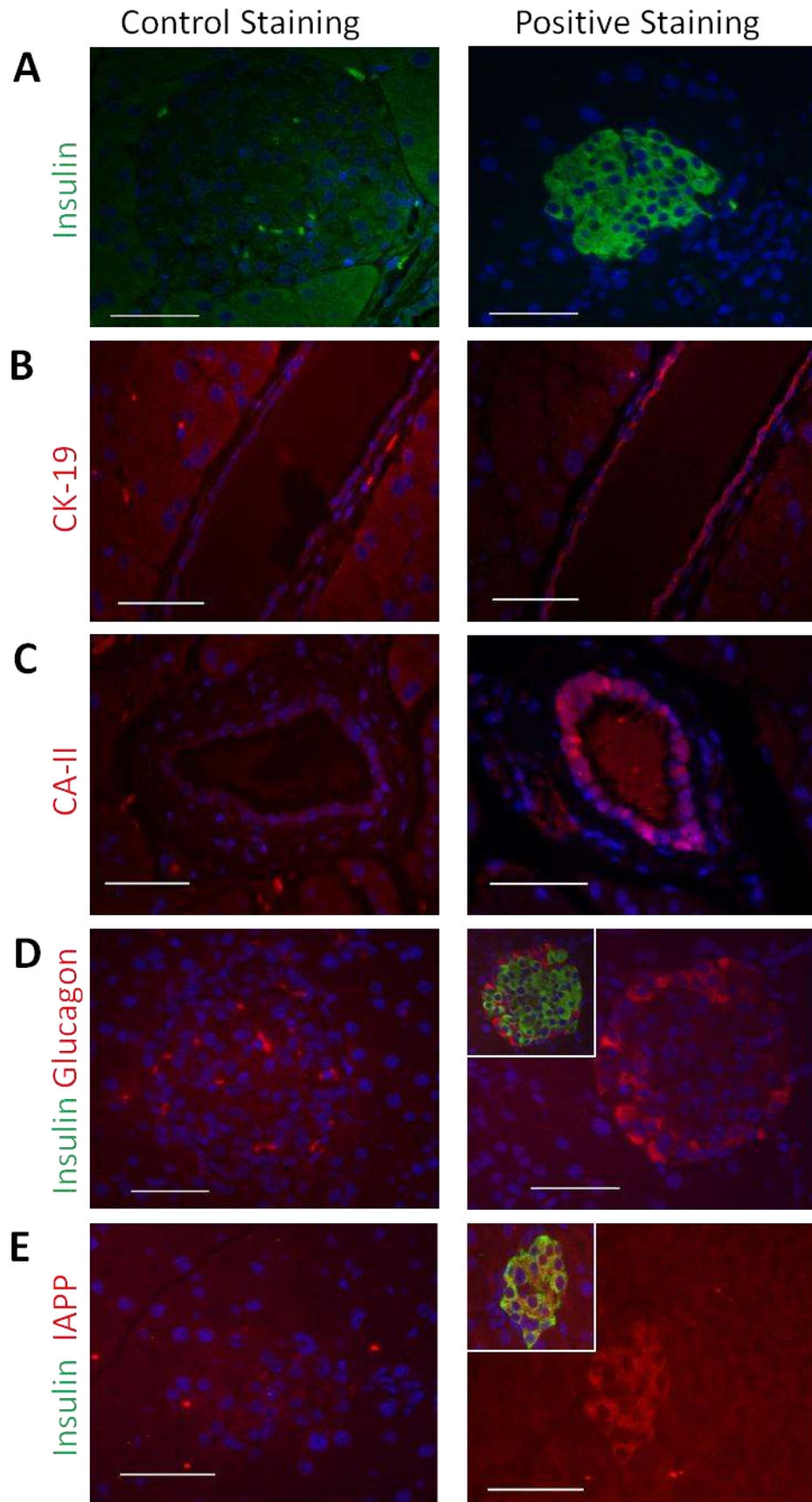
3.4 Ductal insulin-positive cells express markers of β -cell maturity.

To analyze the developmental patterns of insulin-positive ductal cells, immunofluorescence co-staining was performed with a variety of antibodies/antisera (control and positive staining are shown in Figure 3.12). To delineate whether the ductal insulin-positive cells arise from duct cells or another pancreatic cell type, co-immunofluorescence staining for insulin and the duct-cell specific markers cytokeratin-19 (CK-19) and carbonic anhydrase II (CA-II) was performed in pancreatic sections of both *Rspo1*^{+/+} and *Rspo1*^{-/-} mice from the 32-d STZ studies. A lack of co-expression was seen in all insulin-positive ductal cells from both 6-d and 32-d STZ studies (Figure 3.13), suggesting that these β -cells have progressed past the duct cell phenotype and towards a more mature β -cell phenotype.

To examine this further, we next probed the differentiation stage of the duct cell-derived insulin-positive cells. Several studies have demonstrated that transdifferentiation of α -cells to β -cells can be a source of insulin-positive cells in the adult mouse (45-47;137). If this was the case in the present study, we would expect immature, bi-hormonal glucagon- and insulin-positive cells within the ducts. We found no evidence for either glucagon-positive or co-expression of glucagon within insulin-positive ductal cells of *Rspo1*^{+/+} and *Rspo1*^{-/-} mice from the 32-d STZ study (Figure 3.14). This data indicates that the insulin-positive cells seen within the ducts are not bi-hormonal and have not transdifferentiated from an α - to β -cell state.

To further assess if the insulin-positive cells within ducts resembled adult β -cells, we examined key markers of functional, mature β -cells (i.e. GLUT2, Nkx6.1, MafA and IAPP). In general, both 6-d and 32-d STZ-induced $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice had normal islet expression of these markers. No GLUT2 expression was evident in insulin-positive ductal cells from 6-d STZ-induced $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice (Figure 3.15A-B), although Nkx6.1 expression was seen in 6-d STZ-induced $Rspo1^{-/-}$ mice only (Figure 3.15E-F). However, by the end of the long-term STZ study, GLUT2 expression was seen in some insulin-positive ductal cells in $Rspo1^{-/-}$ mice, although its expression was still absent from $Rspo1^{+/+}$ mice (Figure 3.15C-D). Similarly, $Rspo1^{-/-}$ mice from the 32-d STZ study demonstrated increased expression of Nkx6.1 (Figure 3.15G-H) and MafA (Figure 3.15I-J) in insulin-positive ductal cells of $Rspo1^{-/-}$ mice, although no expression was seen in $Rspo1^{+/+}$ mice from the same studies.

Finally, preliminary studies demonstrated that $Rspo1^{-/-}$ mice had greater expression of IAPP in small islets (containing 1-5 β -cell nuclei) than $Rspo1^{+/+}$ mice from the 32-d STZ studies (Figure 3.16A-B), suggesting that these small islets are more differentiated in the knockout animals compared to their wild-type littermates. However, there was no marked difference in the expression of GLUT2, Nkx6.1 or MafA in small islets between $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice in both 6-d and 32-d STZ studies.



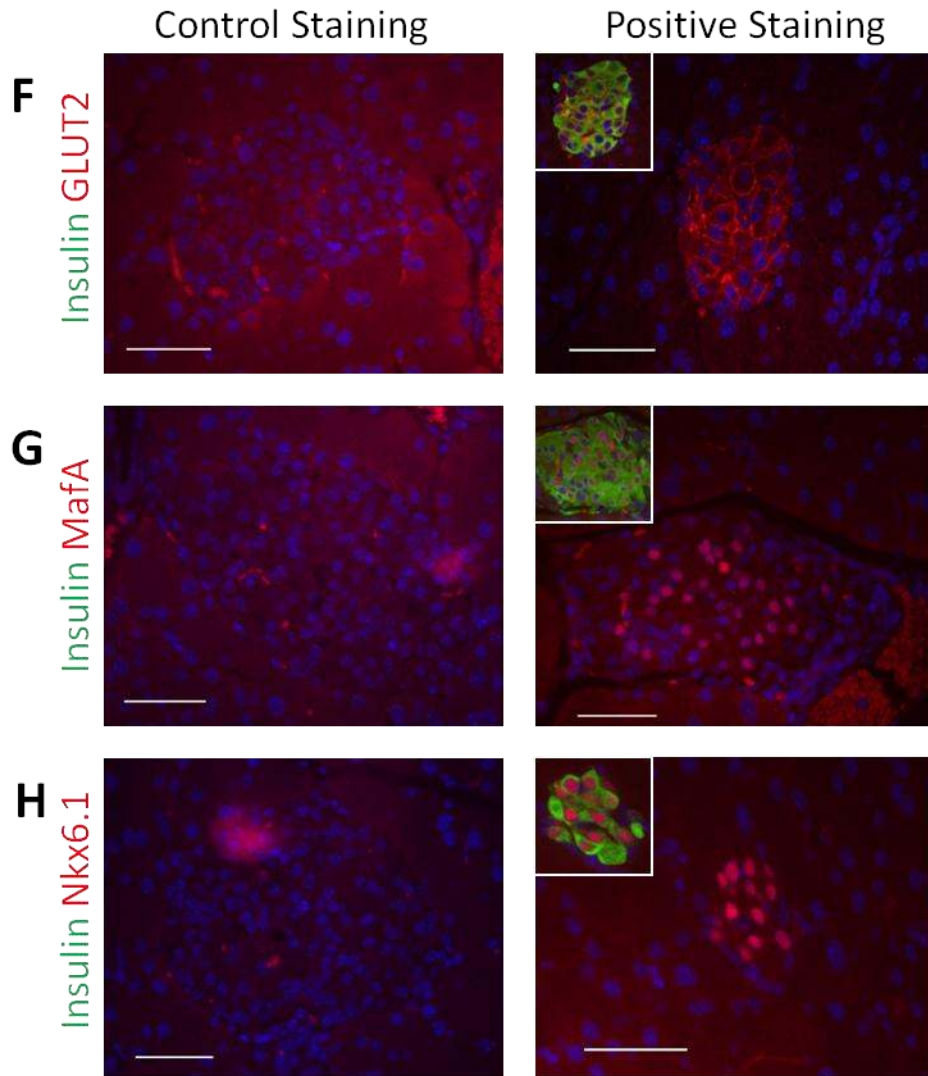


Figure 3.12 Validation of immunofluorescence staining. Negative (control; left-hand panels) and positive (right-hand panels) immunofluorescence staining for insulin (A), cytokeratin-19 (CK-19; B), carbonic anhydrase II (CA-II; C), glucagon (D), islet amyloid polypeptide (IAPP; E), GLUT2 (F), MafA (G) and Nkx6.1 (H). Insets show representative islets. Scale bars represent 50 μm .

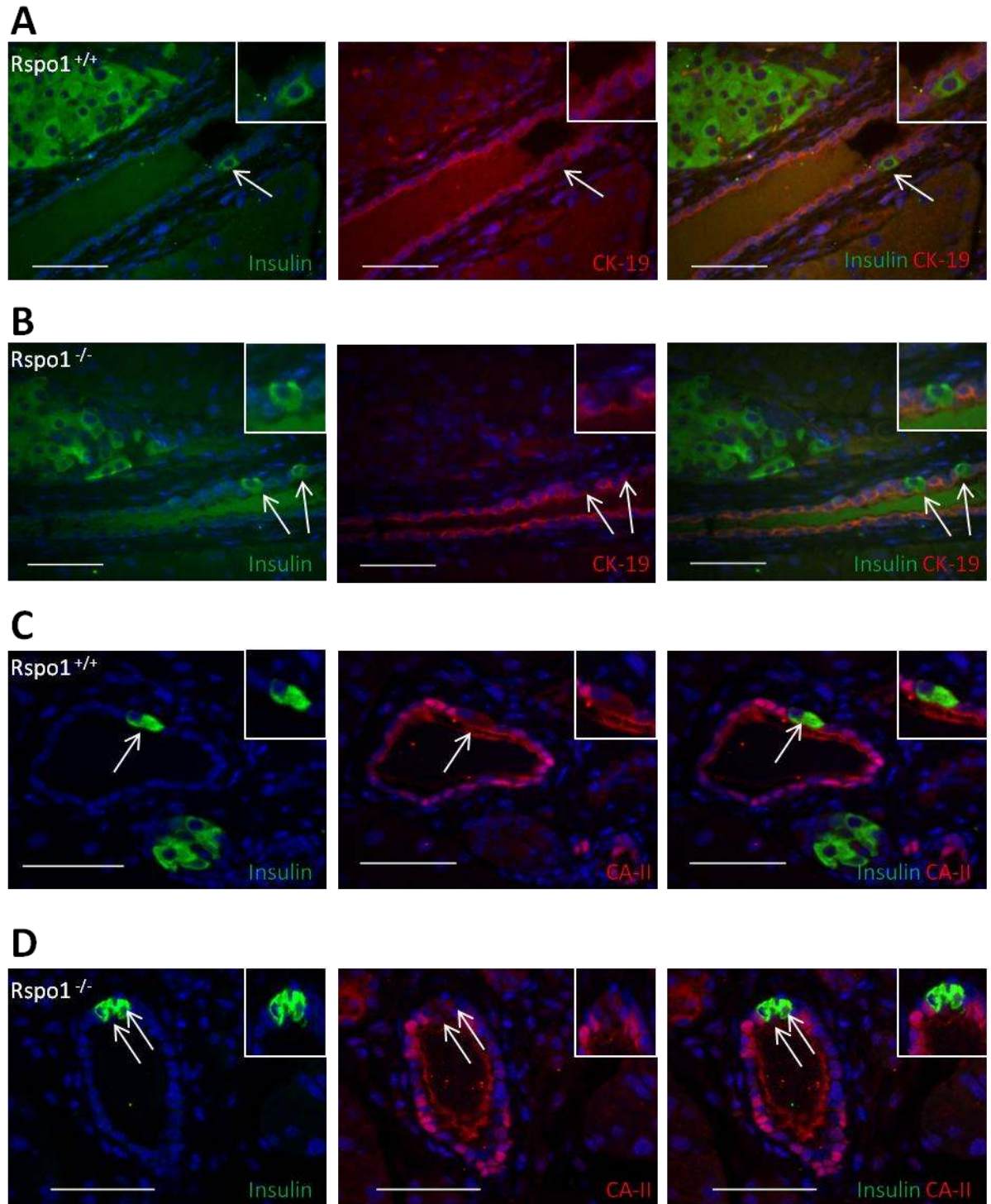


Figure 3.13 Insulin-positive cells arise from ductal epithelium. Immunofluorescence with insulin (green) and the duct-cell specific markers CK-19 (red; **A-B**; $n = 5-6$) and CA-II (red; **C-D**; $n = 3$) in 32-d STZ *Rspo1*^{+/+} and *Rspo1*^{-/-} mice. Insets show representative β -cells; arrows indicate positive cells. Scale bars represent 50 μm .

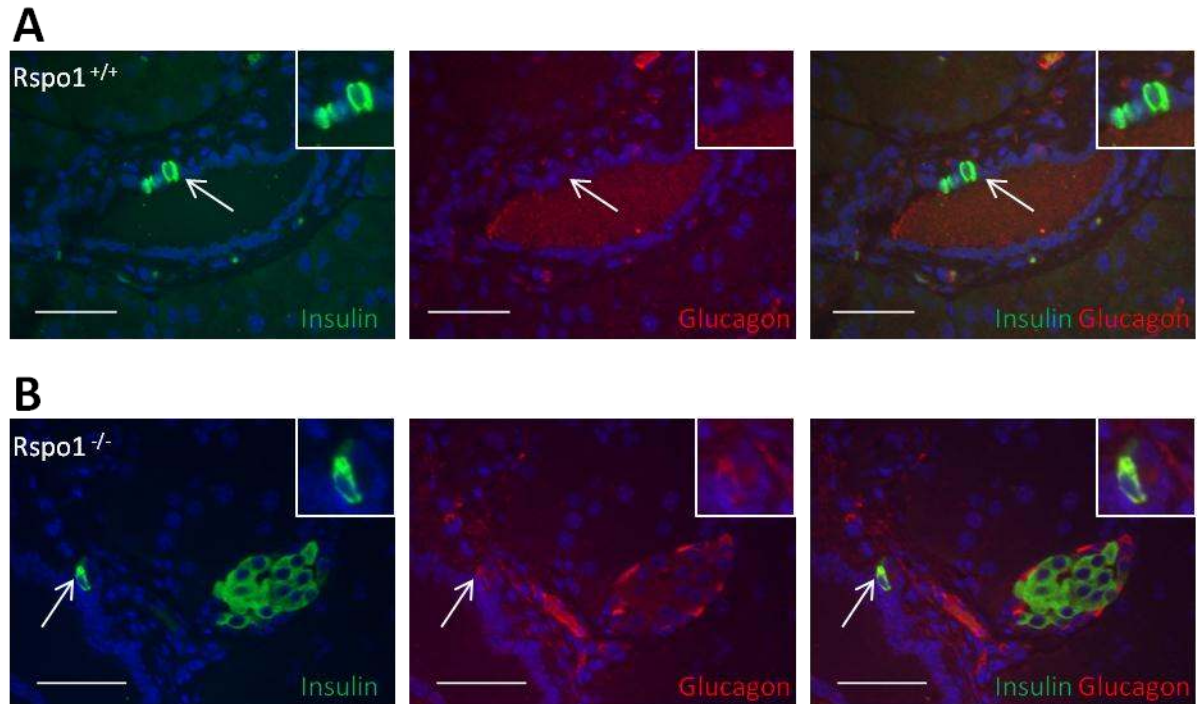
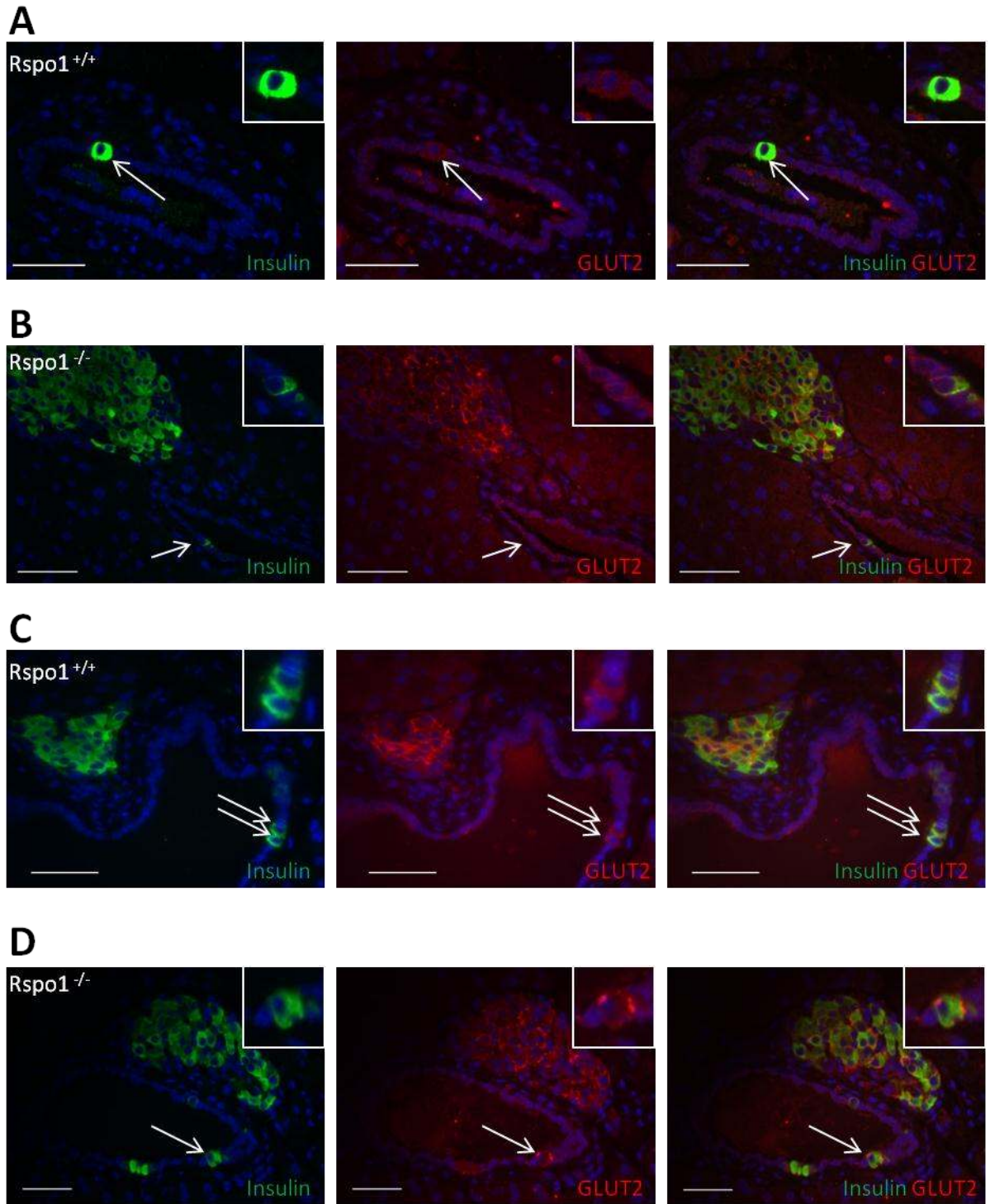
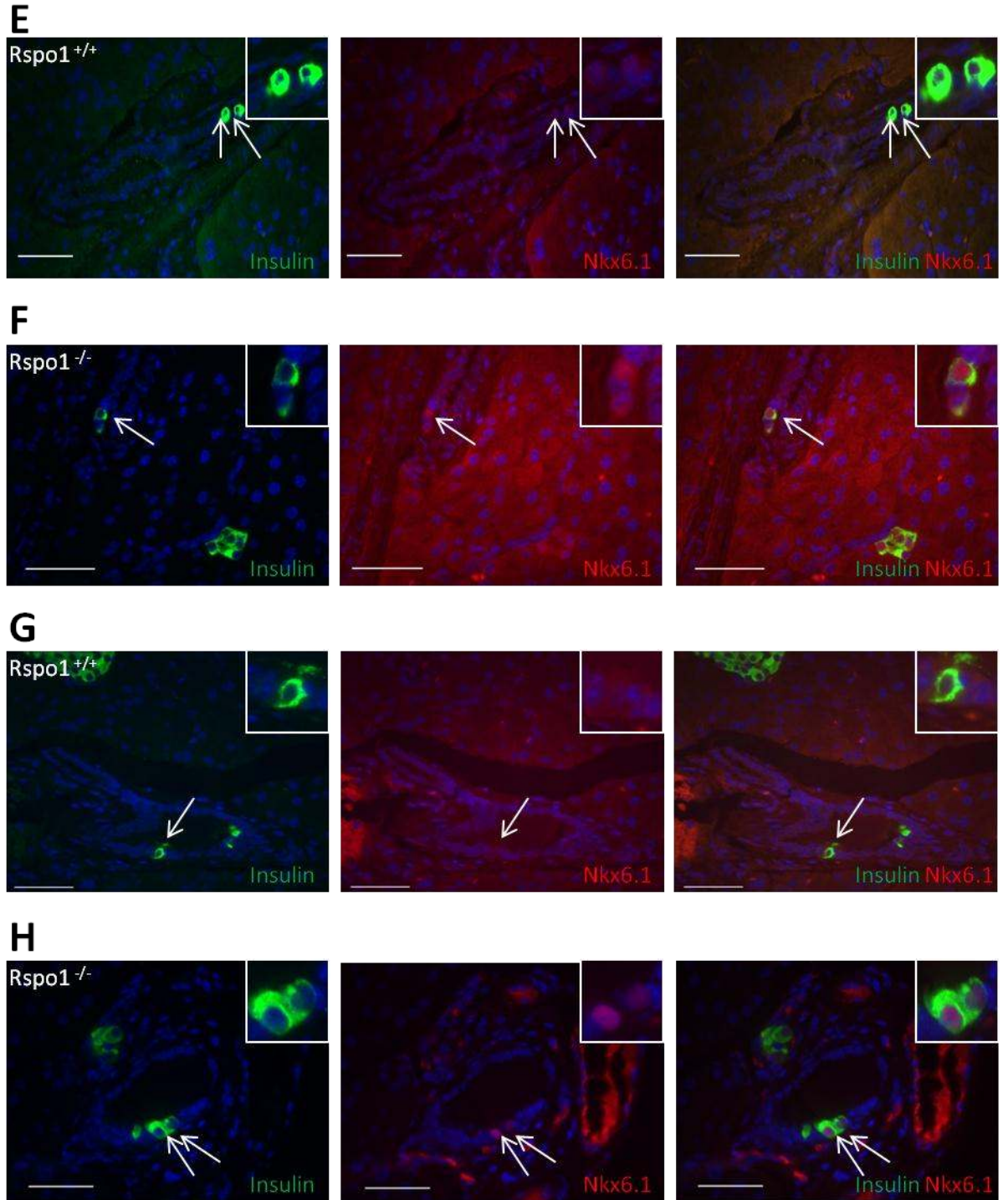


Figure 3.14 Insulin-positive cells within ducts are not bi-hormonal. Immunofluorescence with insulin (green) and glucagon (red) in 32-d STZ Rspo1^{+/+} (**A**; n = 3) and Rspo1^{-/-} (**B**; n = 3) mice. Insets show representative β -cells; arrows indicate positive cells. Scale bars represent 50 μ m.





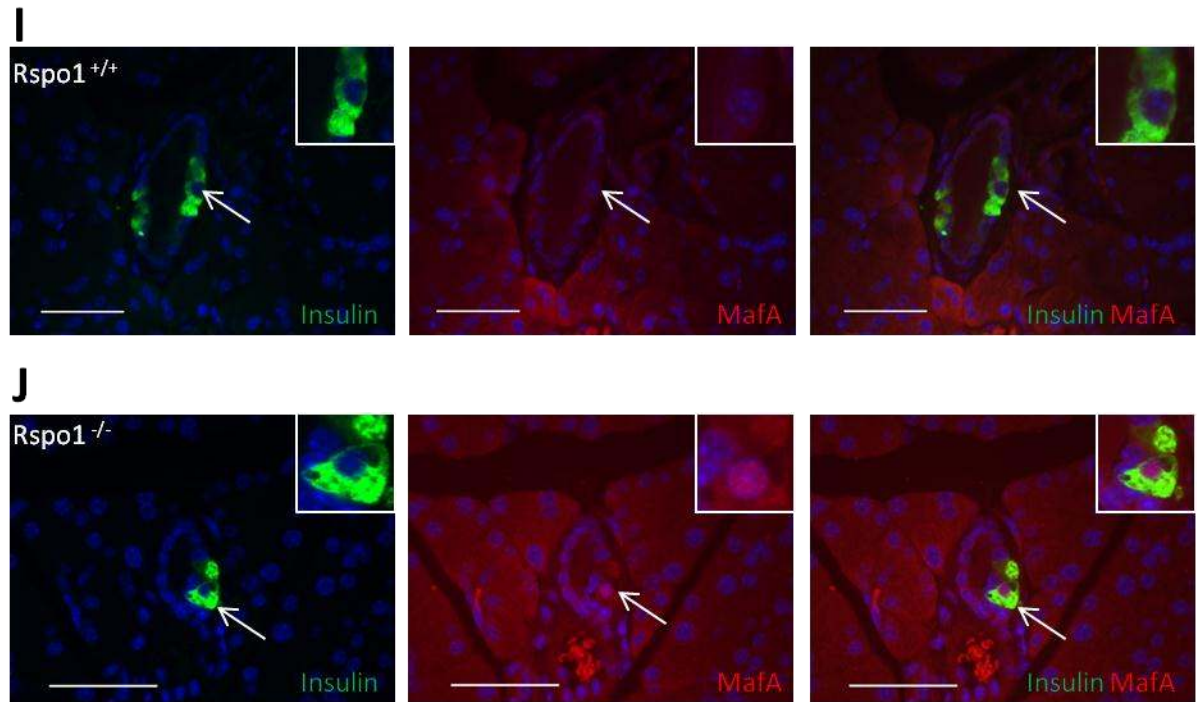


Figure 3.15 Insulin-positive cells in ducts of $Rspo1^{-/-}$ mice express markers of β -cell maturity. **A-B** Immunofluorescence with insulin (green) and GLUT2 (red) in 6-d ($n = 3$) and 32-d (**C-D**; $n = 3$) STZ $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice. **E-F** Immunofluorescence with insulin (green) and Nkx6.1 (red) in 6-d ($n = 3$) and 32-d (**G-H**; $n = 3$) STZ $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice. **I-J** Immunofluorescence with insulin (green) and MafA (red) in 32-d ($n = 3$) STZ $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice. Insets show representative β -cells; arrows indicate positive cells. Scale bars represent 50 μ m.

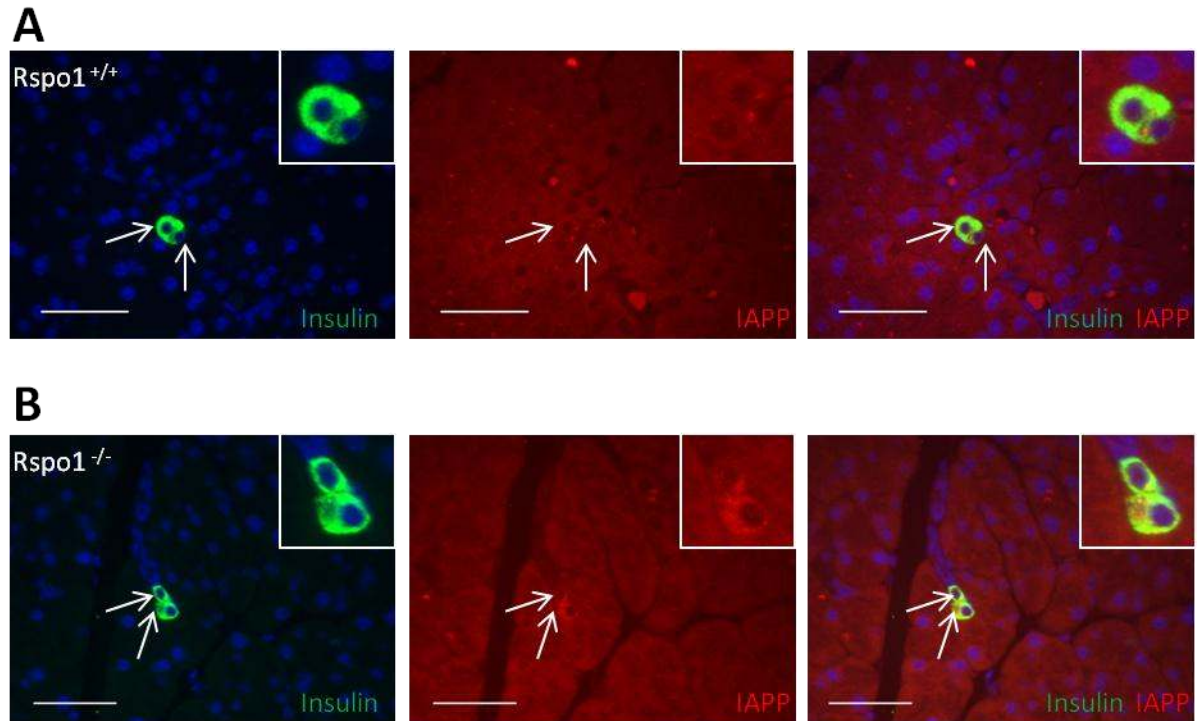


Figure 3.16 Rspo1^{-/-} mice have more mature small islets. **A-B** Immunofluorescence with insulin (green) and IAPP (red) in 32-d (n = 3) STZ Rspo1^{+/+} and Rspo1^{-/-} mice. Insets show representative β -cells; arrows indicate positive cells. Scale bars represent 50 μ m.

4. DISCUSSION

The cWnt activator, Rspo1, has recently been identified as a novel regulator of β -cell growth and function (71;122). Its expression has been reported in mature human pancreas (114), as well as in murine pancreas, islets and β -cell lines *in vitro* (71) and murine pancreas *in vivo* (122). Rspo1 induces cWnt signaling in MIN6 β -cells, leading to elevated levels of nuclear β -catenin in association with increased transcription of *c-myc* and *ins2* (71). In addition, Rspo1 treatment increases MIN6 and murine β -cell proliferation, prevents cytokine-induced apoptosis, and stimulates glucose-independent insulin secretion (71). As expected, mice with global Rspo1 deficiency have reduced levels of islet nuclear β -catenin-positive cells, as well as decreased mRNA expression of cWnt target genes (122). Surprisingly, in marked contrast to *in vitro* findings (71), Rspo1^{-/-} mice have enhanced β -cell mass as a result of increased β -cell proliferation and significantly increased β -cell neogenesis (122). Furthermore, under basal conditions, mice lacking Rspo1 demonstrate better glucose handling in response to an oral glucose load (122). However, whether Rspo1 regulates β -cell function, as well as β -cell growth and neogenesis, under pathophysiological conditions of metabolic stress has not yet been examined. The current study demonstrates that, in STZ-induced diabetes, Rspo1 deficiency increases β -cell neogenesis and survival. Moreover, I show, for the first time, the developmental patterns of newly formed β -cells as a result of β -cell neogenesis in the face of metabolic stress in the presence and absence of Rspo1.

STZ is a widely used model to induce experimental diabetes similar to T1DM and T2DM in rodents (123-126;126). STZ is a toxic glucose analogue that enters the β -cell via GLUT2 transporters, thus causing β -cell death through the alkylation of DNA and subsequent repair-induced depletion of cellular NAD⁺ and ATP stores (126). As a result, insulin synthesis and secretion is inhibited and β -cells undergo destruction through apoptosis,

thereby inducing gradual hyperglycemia and pancreatic insulinitis in certain inbred mouse strains (125;126). A single high-dose injection of STZ causes severe β -cell apoptosis and impaired insulin secretion with resulting hyperglycemia similar to T1DM, whereas multiple low-dose STZ injections have been shown to induce mild hyperglycemia due to a more moderate loss in β -cells and impairment of insulin secretion, which is more similar to T2DM (138;139). In male CD1 mice in particular, a single high-dose of 130 or 150 mg of STZ/kg of body weight is sufficient to produce severe hyperglycemia in all mice by day 7 after injection as a model of T1DM, whereas a minimum of three consecutive days of low-dose STZ (40 mg/kg body weight) induces mild hyperglycemia in mice over a period of 28 days (138). The current study employed the multiple low-dose STZ model of diabetes. Both $Rspo1^{+/+}$ and $Rspo1^{-/-}$ CD1 mice developed progressive hyperglycemia by the end of the 32-d long-term STZ study, with blood glucose levels reaching similar values as those previously reported (132;138), although there were no marked differences in fed glycemia between the two groups of mice. However, pancreatic weights of $Rspo1^{-/-}$ mice were significantly reduced compared to wild-type mice. Since islets constitute a very small percentage of the pancreas (1-2%), this reduction in pancreatic weight is likely due to changes in the exocrine pancreatic mass. Therefore, $Rspo1$ may affect the exocrine pancreas in this global knockout model. Alternatively, $Rspo1^{-/-}$ mice may have less edema in their pancreatic tissue, resulting in a decreased pancreatic mass compared to $Rspo1^{+/+}$ mice. Thus, assessing the dry weight of pancreatic tissue may be a more accurate measure of pancreatic weight.

Fasting plasma levels of insulin did not appear to decrease from control to STZ-induced experimental conditions, and there was no statistical difference in fasting insulin concentrations between $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice in control, 6-d and 32-d STZ studies. In contrast to previous reports (122), control $Rspo1^{-/-}$ mice in the present study failed to

demonstrate an improvement in glucose handling after an oral glucose load as compared to control $Rspo1^{+/+}$ mice. It was interesting to note that although control $Rspo1^{-/-}$ mice followed similar trends as the knock-out mice from previous studies (122), control $Rspo1^{+/+}$ mice showed slightly improved glycemic control in response to the OGTT in the current study. Although the reason for this observed improvement is currently unknown, it may be due to genetic drift over time. Nonetheless, it is likely the reason as to why these results are inconsistent with what has been previously published (122).

By the end of the long-term study, STZ-induced $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice exhibited fasting hyperglycemia and impaired oral glucose handling, as expected. However, there was no difference in glycemic control after an OGTT between $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice. Furthermore, no significant change was observed in plasma insulin levels 10 min following an oral glucose load in either wild-type or knockout STZ-induced animals, although it would have been interesting to see if there was a greater change in plasma insulin concentrations in the last 30 min of the study when the glucose handling appeared to display a trend for improved glycemic control in the knockout animals. .

In STZ-induced diabetes, hyperglycemia can result in the development of progressive insulin resistance of peripheral tissues (140-143) due to decreased autophosphorylation and kinase activity of the insulin receptor (141), as well as a reduction in phosphorylation of Akt, a downstream serine-threonine kinase involved in mediating insulin-regulated metabolic pathways (142). Consistent with these findings, the glycemic responses to ITTs performed in the 6-d and 32-d STZ studies suggest that both $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice have some degree of insulin resistance six days after induction of STZ-induced diabetes versus Wong et al. (122). However, despite this, STZ-induced $Rspo1^{-/-}$ mice had significantly better glycemic responses after insulin administration by the end of the long-term study. These results were

surprising, given the fact that oral glucose handling was not different between $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice. However, this may be due to increased insulin sensitivity in the knockout versus wild-type animals. Therefore, $Rspo1$ may affect peripheral glucose uptake in this global knockout model, with $Rspo1$ deficiency resulting in increased insulin sensitivity by the end of the STZ studies and improved glucose uptake in peripheral tissues such as muscle and/or adipose tissue.

Although there were no observed changes in plasma insulin concentrations in STZ-induced studies, I examined whether $Rspo1$ deficiency would continue to stimulate β -cell growth and neogenesis after STZ-induced diabetes, since $Rspo1$ was suggested to be a negative regulator of β -cell growth in control, non-stressed mice (122). I therefore first confirmed that β -cell mass was significantly enhanced in control $Rspo1^{-/-}$ mice. Although administration of STZ did not decrease β -cell mass in wild-type mice as expected (21;144), these animals were clearly diabetic by the end of the long-term studies, as evident by their presiding hyperglycemia. Furthermore, there was no difference in β -cell mass between wild-type and knockout animals throughout both the short- and long-term STZ studies. In addition, $Rspo1^{-/-}$ mice exhibited a significantly greater decrease in β -cell mass after insult with STZ than $Rspo1^{+/+}$ mice, suggesting that these mice may have increased sensitivity to the effects of the STZ.

Changes in β -cell mass *in vivo* are determined by the rates of proliferation and apoptosis of existing β -cells, as well as by islet neogenesis and β -cell hypertrophy. STZ-induced diabetes abolished the proliferative effects of $Rspo1$ deficiency seen in β -cells of control mice (122). Wong et al. (122) demonstrated extremely low levels of β -cell apoptosis in $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice, consistent with the low levels of β -cell turnover that occur during adulthood (145). In contrast, STZ-induced diabetes induces β -cell apoptosis (132).

Thus, greater numbers of apoptotic β -cells were observed in both $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice after insult with STZ as compared to (122). However, β -cell apoptosis was significantly reduced in $Rspo1^{-/-}$ mice, thus suggesting, for the first time, that $Rspo1$ serves as a negative regulator of β -cell survival *in vivo* under conditions of metabolic stress.

Although $Rspo1^{-/-}$ mice exhibited a much greater reduction in β -cell mass after induction of STZ-diabetes compared to wild-type littermates, these mice had greater protection against β -cell apoptosis. This paradoxical observation may be due to the timing of the β -cell apoptosis analysis. For example, O'Brien et al. (146) demonstrated that the multiple low-dose STZ model is associated with two peaks in the incidence of β -cell apoptosis; the first occurring on day 5 (the last day of STZ injection) and the second occurring on day 11 (6 days after the last STZ injection). Thus, it is possible that the greatest peak of β -cell apoptosis was missed in the current study, since pancreatic sections were analyzed from day 6 and day 32 of the STZ studies. Therefore, the reduced β -cell apoptosis seen in the $Rspo1^{-/-}$ mice may be due to better recovery after early insult with STZ. Furthermore, the levels of both β -cell apoptosis and β -cell proliferation seen in the $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice were extremely low. Hence, although mice with $Rspo1$ deficiency demonstrated reduced β -cell apoptosis compared to wild-type littermates, this may have been unlikely to affect β -cell mass.

Several studies have reported the occurrence of β -cell regeneration after STZ-induced diabetes (127-131). However, whether this regeneration occurs from intra-islet progenitors or duct cells is controversial (128;130). The present study has shown that adult $Rspo1^{+/+}$ control mice and $Rspo1^{+/+}$ mice with STZ-induced diabetes demonstrate the presence of insulin-positive cell ductal cells, suggestive of β -cell neogenesis. Furthermore, similar to the findings of Wong et al. (122), $Rspo1^{-/-}$ mice continued to display significantly increased levels of β -

cell neogenesis in both control animals and following induction of STZ-induced diabetes. Intriguingly, the wild-type mice exhibited no change in the levels of insulin-positive ductal cells between control and STZ-induced mice.

No differences in the total number or size distribution of the islets were apparent between $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice throughout the STZ experimental protocols. Together, these findings demonstrate that, consistent with the lack of difference in β -cell mass, β -cell hypertrophy and hyperplasia are unaltered between STZ-treated $Rspo1^{+/+}$ and $Rspo1^{-/-}$ mice. In contrast, $Rspo1^{-/-}$ mice demonstrated greater protection against β -cell apoptosis and significantly enhanced β -cell neogenesis compared to wild-type mice in the face of metabolic stress. However, this increased β -cell regeneration was insufficient to increase β -cell mass and restore normoglycemia in the knockout animals. It is possible that $Rspo1^{-/-}$ mice have not yet generated sufficient numbers of new islets to completely restore euglycemia in these animals, as it has previously been reported that in STZ-induced diabetic mice, transplantation of a large number (i.e. 200) of islets is required to restore euglycemia (147). Indeed, Wang et al. (148) reported increased numbers of insulin-positive duct cells in mice overexpressing a transforming growth factor α ($TGF\alpha$) transgene, another model of neogenesis. However, these insulin-expressing cells were not sufficient to increase islet mass until gastrin, an additional growth factor, was overexpressed in the pancreas of transgenic mice (148). Therefore, it is possible that, although $Rspo1^{-/-}$ mice have increased levels of insulin-positive cells arising from the ducts, insult with STZ may require an additional stimulus to promote enhanced β -cell mass in these animals.

Neogenesis refers to the differentiation of pancreatic progenitor cells to β -cells (14). Several models of islet neogenesis have been used to demonstrate the induction of β -cell regeneration, including STZ-induced diabetes (127-131), partial pancreatectomy (149),

partial duct ligation (150-155), cellophane wrapping of the pancreas (156), overexpression of pancreatic TGF α (148), interferon-gamma (157), or gastrin (148;158;159), as well as treatment with trypsin inhibitors (160), betacellulin (161), and exendin-4 (54). Although the source of pancreatic progenitors is controversial, numerous studies have suggested that duct cells within the adult pancreas act as precursor cells that give rise to new islets (54;56-59). To examine the potential origins of the single insulin-positive cells that reside among the ductal epithelium, pancreatic sections were co-stained for the duct-specific markers CA-11 (57;162) and CK-19 (56;163) and insulin. A lack of co-expression between these duct cell markers and hormone expression was seen in all insulin-positive ductal cells from both the short- and long-term STZ studies, suggesting that, although these β -cells exist within the ducts, they have likely progressed past the duct cell phenotype and towards a more mature β -cell phenotype. It has also been reported that exocrine acinar cells contribute to β -cell neogenesis (48;49). Thus, amylase staining could be performed in the future to examine whether any insulin-positive cells arise from acinar tissue. The transdifferentiation of α -cells to β -cells has also been reported as a source of insulin-positive cells in the adult mouse (45-47;164). However, the present study failed to demonstrate the presence of either glucagon-positive ductal cells or bi-hormonal glucagon- and insulin-positive cells within either the ducts or islets of Rspo1^{+/+} and Rspo1^{-/-} mice. This data suggests that the insulin-positive cells seen within the ducts are not bi-hormonal and have not transdifferentiated from α -cells.

Previous studies exploring the mechanisms of duct-to- β -cell conversion have hypothesized that mature duct epithelial cells replicate and regress to a less differentiated state before re-differentiating into β -cells with the appropriate external stimuli (56;57;59). Examination of a loss of Hnf6 expression in mature duct cells could demonstrate the loss of the differentiated duct phenotype (59). Although the present study did not detect any insulin-

positive duct cell proliferation, the possibility of duct cell hyperplasia and loss of differentiated phenotype in mature ducts cannot be ruled out, as this is a very early event in pancreatic regeneration (59). Therefore, it is possible that inspection of pancreatic sections from STZ-treated animals earlier in the time course may have demonstrated this effect. Future studies could also examine the expression of Sox9 (10;165) and/or Ngn3 (8), both markers of endocrine progenitors, to determine the presence of de-differentiated duct cells and whether insulin-positive ductal cells are newly formed in *Rspo1* mice after insult with STZ.

As insulin-positive cells ‘bud’ from ducts, the expression of markers of β -cell maturity (i.e. Nkx6.1, GLUT2 and MafA) gradually increases (59). Consistent with this observation, preliminary results from the present study showed that, although insulin-positive ductal cells of *Rspo1*^{-/-} mice expressed Nkx6.1 on the sixth day after STZ-administration, these cells expressed GLUT2 on day 32 only. Conversely, the majority of insulin-positive duct cells from wild-type mice did not show expression of Nkx6.1 or GLUT2 on day 6 or day 32 of the STZ studies. GLUT2 is a marker of mature β -cell function, whereas the expression of Nkx6.1 is observed both during the development of new insulin-positive cells and in mature β -cells (59). Thus, it is possible that by the end of the short-term STZ study, insulin-positive cells located within the ducts of *Rspo1*^{-/-} mice have not yet fully differentiated into a mature β -cell phenotype. However, since Nkx6.1 is involved in both the early and mature stages of β -cell development, co-staining of Nkx6.1 with Sox9 or Ngn3 would be a better indicator of ‘immature’ β -cell status. Unlike wild-type mice, *Rspo1*^{-/-} mice demonstrated a progressive increase in GLUT2 expression within insulin-positive ductal cells from the 6-d to 32-d STZ studies, thereby suggesting that these insulin-positive ductal cells are gradually differentiating into a mature β -cell phenotype. Moreover, by the end of the

long-term STZ study, preliminary results showed that $Rspo1^{-/-}$ mice had increased expression of Nkx6.1, MafA and GLUT2 in insulin-positive ductal cells, whereas wild-type mice failed to demonstrate the expression of these mature β -cell markers in the majority of the insulin-positive duct cells observed. Unfortunately, preliminary studies examining IAPP, another marker of mature β -cell status, did not yield significant numbers of insulin-positive duct cells to conclude whether IAPP expression is observed in these cells in STZ-induced animals. However, these findings suggest that the insulin-positive ductal cells in $Rspo1^{-/-}$ mice represent mature β -cells. Additionally, $Rspo1^{-/-}$ mice were found to have greater expression of IAPP in small islets than wild-type mice by the end of the long-term STZ studies. Together, these preliminary findings demonstrate that after insult with STZ, mice with $Rspo1$ deficiency may have more differentiated insulin-positive ductal cells and small islets, suggesting that $Rspo1$ may serve as a dedifferentiation factor.

Multiple rodent studies have shown that high glucose levels result in β -cell dedifferentiation (42-44;166-169). As a result, it is possible that islets from diabetic animals contain 'empty' β -cells, suggesting cells that have become functionally exhausted (166). The present study suggests that, although insulin-positive cells are located within the ductal epithelium of wild-type mice, these cells are likely immature and have not differentiated towards a mature β -cell phenotype. Furthermore, STZ-induced hyperglycemia may cause insulin-positive ductal cells in wild-type mice to dedifferentiate, as these mice had reduced expression of several markers of β -cell maturity (Nkx6.1, MafA, GLUT2 and IAPP) in these cells. Furthermore, preliminary studies demonstrating reduced expression of IAPP in small islets of wild-type mice suggests that, although the number of islets did not alter between the two groups of mice, β -cells from wild-type mice may have regressed from a mature β -cell phenotype and may constitute 'empty' β -cells that have not yet fully dedifferentiated.

Alternatively, *Rspo1*^{-/-} mice may have increased protection from the glucotoxic effects of hyperglycemia in these STZ-induced studies. Therefore, the more differentiated β -cells in *Rspo1*^{-/-} mice after insult with STZ may not be directly due to *Rspo1* deficiency in duct cells, but due to improved insulin sensitivity in these animals. Finally, a limitation of the present study is that the protein expression as determined by IHC was not quantified; thus future studies using a quantitative analysis (i.e. Western Blot or qRT-PCR) of mature β -cell proteins such as glucokinase and GLUT2 may provide a more reliable measure of protein expression levels. Nevertheless, further examination is required in order to fully elucidate the developmental expression patterns of these new β -cells.

The findings of this thesis suggest that *Rspo1* may not act solely on the β -cell, but may also play a role in the ductal epithelium. Thus, the present study highlights the value of using a whole-body *Rspo1* knockout approach over β -cell- or duct cell-specific *Rspo1* knockout models, as it is uncertain whether *Rspo1* exerts its actions on duct cells through an autocrine or paracrine effect. However, the use of *in vitro* duct cell cultures from *Rspo1* mice would be extremely beneficial in determining whether *Rspo1* directly acts on duct cells to promote the formation of new β -cells. Thus, preliminary studies were performed in an attempt to isolate primary ductal cells from both wild-type and *Rspo1* knockout mice, using previously established protocols from Kitamura et al. (134) and the laboratory of Dr. Bonner-Weir (135). It was hypothesized that, since *Rspo1* appears to act as a negative regulator of β -cell neogenesis *in vivo*, the addition of recombinant *Rspo1* to ductal cells from *Rspo1*^{-/-} mice *in vitro* would result in reduced transdifferentiation to β -cells. Unfortunately, although the presence of ‘cobble-stone’ shaped cells indicative of ductal cells was observed, cell counts were extremely low after several weeks in culture, and this approach was therefore abandoned. Nevertheless, future studies should attempt these existing, or new, protocols in

order to elucidate the potential mechanism of action by which Rspo1 exerts its effects on β -cell neogenesis. It would be interesting to determine whether duct cells demonstrate expression of cWnt signaling molecules and the Rspo1 receptors LGR4/5, and whether the addition of Wnt ligands to ductal cells from Rspo1^{+/+} or Rspo1^{-/-} mice results in the subsequent activation of cWnt signaling and dedifferentiation to β -cells. Wong et al. (71) showed that the addition of Rspo1 and Wnt3a ligands to MIN6 β -cells *in vitro* increased expression of the cWnt target gene c-myc and increased β -cell proliferation. Although increased c-myc expression has been associated with β -cell hypertrophy, it also causes a reduction in nuclear Pdx1 levels and reduced endocrine differentiation (24;170). Thus, perhaps a potential explanation for the increased β -cell neogenesis and differentiation in mice lacking Rspo1 is due to decreased activation of cWnt signaling and a subsequent reduction of c-myc levels. Indeed, loss of c-myc regulatory function has been suggested as an alternative mechanism for β -cell neogenesis (24;170). Consistent with this hypothesis, it would be interesting to note whether c-myc mRNA transcript or protein levels are reduced in ductal and/or β -cells from mice with Rspo1 deficiency. This hypothesis is also consistent with previous studies performed by Wallace et al. (171), as they demonstrated that pancreatic AR42J-B-13 cells transdifferentiate into hepatocyte-like cells through the inhibition of constitutively active cWnt signaling. AR42J cells have also been shown to transdifferentiate into endocrine cells upon treatment with various growth factors such as GLP1, exendin-4, hepatocyte growth factor, betacellulin, and activin A (172-175). It is thus tempting to speculate that these results may also involve repression of cWnt signaling in AR42J cells. β -catenin can also be phosphorylated and subsequently translocated to the nucleus through more than just Wnt-ligand induced cWnt signaling, as GLP-1 has also been found to phosphorylate β -catenin in PKA-dependent and GSK3 β -independent pathways (81). Thus, it

is possible that cWnt signaling acts differently in various tissues, as it has been shown that inhibition of cWnt signaling in adipocytes is required for their differentiation (176;177), whereas other studies have shown that activation of cWnt signaling in pancreatic islets is required for their growth and function.

In summary, future studies should address the limitations described above and examine possible mechanism(s) of action of Rspo1 in increasing β -cell neogenesis. For example, a limitation of the current study is that Rspo1 deficiency in knock-out animals was not analyzed. Thus, studies in the immediate future should demonstrate a lack of Rspo1 gene expression in Rspo1^{-/-} mice by quantitative analysis examining Rspo1 mRNA transcript levels and/or protein levels in both wild-type and knockout mice. Furthermore, Rspo1^{-/-} mice are characterized by a truncated version of Rspo1 rather than a complete deletion of the protein. Consequently, it is possible that a truncated version of the Rspo1 protein may play a confounding role in these studies (i.e. as an antagonist or partial agonist), and further analyses should examine this possibility. Another limitation of the current study is that the co-localization data from IHC studies needs to be quantified in order to make conclusive statements about cell numbers. Furthermore, IHC analyses should be performed in control Rspo1^{+/+} and Rspo1^{-/-} mice in order to examine whether insulin-positive duct cells in these animals also demonstrate co-localization with markers of β -cell maturity and whether hyperglycemic conditions results in dedifferentiation of these cells. As aforementioned, the source of pancreatic progenitor cells is controversial. Thus, further analysis of insulin-positive ductal cells could be done to examine whether any insulin-positive cells arise from transdifferentiation of acinar tissue (i.e.. using amylase staining), whether ducts may have dedifferentiated and proliferated prior to redifferentiation towards β -cells (i.e. using Hnf6 and earlier time points to analyze possible duct hyperplasia), and whether insulin-positive cells

within ducts are newly formed after insult with STZ, or if they have de-differentiated in the presence of hyperglycemia (i.e. using Sox9 and/or Ngn3 staining). Additionally, islet expression of Sox9/Ngn3 could be examined to delineate whether intra-islet progenitors may contribute to β -cell neogenesis after STZ-induced diabetes. Furthermore, since it is unknown whether Rspo1 acts directly on duct cells, cell specific knockout of Rspo1 in the pancreatic ductal epithelium using carbonic anhydrase II as a duct-cell-specific promoter with the Cre-Lox system would allow determination of the role of Rspo1 in regulating β -cell neogenesis. Finally, as mentioned above, the use of primary duct cell cultures from Rspo1 mice or duct cell lines (i.e. Capan-1, ARIP, and PANC-1) would also provide important insight into the possible mechanisms of action of Rspo1 in the regulation of β -cell neogenesis. In conclusion, the findings of the present study indicate a role for Rspo1 as a negative regulator of *in vivo* β -cell neogenesis and β -cell survival in the face of STZ-induced diabetes. Moreover, these findings suggest that duct cells are important contributors of potential pancreatic precursor cells, resulting in the development of differentiated insulin-positive ductal cells in mice with Rspo1 deficiency following insult with STZ. Furthermore, the absence of Rspo1 *in vivo* appears to protect β -cells from dedifferentiating in the presence of STZ-induced hyperglycemia, as Rspo1^{-/-} mice demonstrate increased expression of mature β -cell markers in insulin-positive ductal cells and small islets. Thus, the present data support the role of Rspo1 as a novel regulator of β -cell growth (122). By elucidating the mechanism through which Rspo1 deficiency enhances β -cell neogenesis, the results of the present study will contribute to a more comprehensive understanding of the pathways regulating β -cell regeneration, thereby providing a potential novel target for therapeutic treatment for the preservation or restoration of functional pancreatic β -cell mass in diabetes.

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