UNIVERSITY OF CALIFORNIA, SAN DIEGO

Coordinated Regulation by Shp2 Tyrosine Phosphatase of Multiple Signals Controlling Insulin Biosynthesis in Pancreatic beta-cells

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Molecular Pathology

by

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The dissertation of Sha Zhang is approved, and it is acceptable in quality and form for publication on microfilm:

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To my husband, parents and sister

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ABBREVIATIONS

AIR	acute insulin response
Akt	protein kinase B
AML	acute myeloid leukemia
β-gal	β-galactosidase
bHLH	basic-helix-loop-helix
BMP	bone morphogenetic protein
BrdU	5-bromo-2'-deoxy-uridine
CamK2a	calcium/ calmodulin-dependent protein kinase II alpha gene
CDK	cyclin-dependent kinase
CMV	cytomegalovirus
CNTF	ciliary neurotrophic factor
CPH	peptidylprolyl isomerase A/cyclophilin A
Cre	corticotrophin-releasing hormone
Csw	Corkscrew
DBD	DNA-binding domain
DOS	daughter-of-sevenless
E	embryonic day number
ECM	extracellular matrix
EGF	epidermal gowth factor
ELISA	enzyme-linked immunosorbent assay
Erk	extracellular signal-regulated kinase

FAS	fatty acid synthase
FBS	fetal bovine serum
FGF	fibroblast growth factor
floxed	loxP-flanked
FOXO	forkhead transcription factors
FRS	FGF receptor substrate
Gab1	Grb2 associated binder 1
GAP	GTPase activating protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gck	glucokinase
GEF	guanine nucleotide exchange factor
Grb2	growth factor receptor bound protein 2
hGH	human growth hormone
HNF	hepatic nuclear factor
IGF	insulin-like growth factor-1 receptor
IFN	interferon
IL	interleukin
IR	insulin receptor
IRES	internal ribosomal entry site
IRS	insulin receptor substrate
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
JMML	juvenile myelo-monocyte leukemia

lacZ	Janus kinase
LIF	leukemia inhibitory factor
loxP	locus of excising-over in phage P1 sequences
MAPK	mitogen-activated protein kinase
MEF	myocyte enhancer factor
NFAT	Nuclear factor of activated T-cells
NS	noonan syndrome
ObRb	obese receptor b form (long form of leptin receptor)
PBS	Phosphate Buffered Saline
PDGF	platelet-derived growth factor
Pdx-1	pancreatic-duodenal homeobox 1
PH	pleckstrin homology
PI3'K	phosphatidylinositol 3-OH kinase
PTB	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PTPN11	none-receptor protein tyrosine phosphatase number 11 (human SHP-2 gene locus)
PY	phosphor-tyrosine
R26R	Rosa 26 reporter
RTK	receptor tyrosine kinase
SAB	standard assay buffer
SH2	Src homolog 2
SHPS-1	SH2 domain-containing tyrosine phosphatase substrate-1

SMAD	Small Mothers Against Decapentaplegic
Sos1	son-of-sevenless homolog 1
SOCS3	suppressor of cytokine signaling 3
STAT	signal transducers and activators of transcription
TGF	transforming growth factor
TK	thymidine kinase
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling

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ABSTRACT OF THE DISSERTATION

Coordinated Regulation by Shp2 Tyrosine Phosphatase of Multiple Signals Controlling Insulin Biosynthesis in Pancreatic beta-cells

by

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The pancreatic β cell plays essential role in glucose homeostasis by secretion of the hormone insulin to meet metabolic demand. Previous studies revealed that the insulin signaling pathway is important for β-cell growth and function. Shp2 is a ubiquitously expressed protein tyrosine phophatase (PTP) that possesses two Src-homology 2 (SH2) domains at the N-terminus and a classic PTP domain. This phosphatase is demonstrated to mediate the insulin signaling through interaction with insulin receptor (IR) and insulin receptor substrate (IRS) in insulin responsive tissues. However, the function of Shp2 in pancreatic β cells is still enigmatic. Here we show that Shp2 acts to coordinate multiple signaling events for insulin biosynthesis in β cells. Mice with conditional ablation of the *Shp2/PTPN11* gene in the pancreas exhibited defective glucose-stimulated insulin

secretion and impaired glucose tolerance. Consistently, siRNA-mediated Shp2 knockdown in INS-1 832/13 cells resulted in decreased insulin production and secretion, despite an increase of cellular ATP content, in response to glucose challenge. Shp2 modulates strength of signals flowing through the Akt/FoxO1 and Erk pathways, culminating in the control of Pdx1 expression and its activity on the *Ins1* and *Ins2* promoters, and forced expression of Pdx1 rescued attenuated insulin production in Shp2 knockdown β-cells. Therefore, this study identifies Shp2 as a novel signal coordinator in β-cells, orchestrating multiple pathways controlling insulin biosynthesis for maintaining glucose homeostasis.

I. Introduction

Tyrosine phosphorylation is an important signaling mechanism controlling multiple physiological processes, including cell proliferation, differentiation, adhesion, metabolism and migration (Hunter and Eckhart, 2004). Abnormalities of tyrosine phosphorylation appear to trigger several inherited and acquired human diseases (Hunter, 1998; Hunter, 2000; Ostman et al., 2006). Reversible tyrosine phosphorylation is regulated by two distinct classes of enzymes: protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (Tonks and Neel, 1996). These enzymes function in a coordinated manner to control the strength and duration of the signaling responses (Mustelin et al., 2005). Understanding the physiological roles of these enzymes and identifying the direct targets of these enzymes are critical issues of current signal transduction research.

PTPs constitute large, separate families of tightly regulated enzymes that are structurally and mechanistically diverse (Mustelin et al., 2002; Van Vactor et al., 1998). Recent findings revealed that there was a total of 107 human genes in the PTP superfamily, 105 of which have a mouse ortholog (Tonks, 2006). These enzymes contain the active-site signature motif HCX_5R, in which the invariant cysteine residue functions as a nucleophile in catalysis (Alonso et al., 2004).

Based on the amino acid sequences of their catalytic domains, the PTPs can be classified into four families (Alonso et al., 2004; Neel and Tonks, 1997). Class I cysteine-based PTPs comprise 38 classical PTPs and 61 dual specific protein phophatases (DSPs) (Alonso et al., 2004). Class II cysteine-based PTP contains a low-molecular-weight enzyme, which is tyrosine specific. Class III cysteine-based PTPs are

tyrosine/threonine-specific phophatases and comprise three CDC25 cell cycle regulatory genes. The fourth family of PTPs is aspartic acid-based PTPs and contains 4 EyA genes, which are tyrosine- or dual tyrosine and serine-specific phosphatases. The classical PTPs can be further categorized into receptor-like (R) and non-transmembrane (NT) PTPs (Neel and Tonks, 1997). Most RPTPs contain two PTP domains: the membrane-proximal (domain 1) PTP domain with significant catalytic activity and the membrane-distal (domain 2) PTP domain, which is inactive but maintains a PTP fold and mediates protein-protein interaction. RPTPs mainly function in cell-cell or cell-matrix adhesion (Alonso et al., 2003; Han et al., 2000; Saxena and Mustelin, 2000). Non-transmembrane (NT) PTPs are featured by the their different regulatory domains controlling the enzymatic activity or the substrate specificity of their catalytic domain (Rivard et al., 1995). The Src homology-2 (SH2) domain-containing PTPs (Shps) are a subfamily of NTPTPs that possess two N-terminal SH2 domains (N-SH2 and C-SH2) (Feng, 1999). There are two vertebrate Shps-Shp1 and Shp2, whereas *Drosophila* and *Caenorhabditis elegans* only have single Shp ortholog, Corkscrew (*Csw*) and Ptp-2, respectively (Neel et al., 2003; Zhang et al., 2000). Shp2 and its orthologs are ubiquitously expressed, whereas Shp1 expression is restricted to hematopoietic cells (Nagaishi et al., 2006). Notably, gain-of-function mutations in the Shp2/*PTPN11* gene were found to be linked to Noonan Syndrome (NS) and LEOPARD syndrome (LS) (Tartaglia and Gelb, 2005). Moreover, genetic mutations of Shp2/*PTPN11* were reported in patients with juvenile myelomonocytic leukemia (Bentires-Alj et al., 2004; Chan et al., 2007).

1. Protein tyrosine phosphatase Shp2 in cell signaling

1.1 structure and regulation of Shp2

Shp2 phosphatase possesses at their N-terminus two tandom SH2 domains, a classic PTP domain, a C-terminal tail with tyrosyl phosphorylation sites (Y542 and Y580), and a proline-rich motif (Feng et al., 1993; Hof et al., 1998). The two tyrosines on the C-terminal tail can be phosphorylated by receptor tyrosine kinases (RTKs) and non-RTKs, and regulate downstream signaling events (Neel et al., 2003). The proline-rich domains of Shp2 might associate with SH3 domain-containing proteins.

The resolution of the crystal structure of Shp2 protein (1-527) lacking the 66-residue C-terminal tail revealed a broad interaction surface between the N-SH2 and the PTP domain (Hof et al., 1998). In the structure, the backside of the N-SH2 domain (the surface opposite to the Tyr-P peptide-binding pocket) is directly inserted into the catalytic cleft at the base of the phosphotyrosine-binding pocket. Several hydrogen bonding interactions between the N-SH2/PTP domains occupy the critical sites in catalysis. Two well-studied hydrogen bonding interactions are between D61 (in N-SH2) and C459 (in PTP) and between E76 (in N-SH2) and S502 (in PTP) (O'Reilly et al., 2000). The autoinhibited structure is further stabilized by several polar interactions outside the catalytic center. The C-SH2 domain has minimal interaction with the PTP domains (Takai et al., 2002). Binding of C-SH2 to a phosphotyrosine peptide could enhance the local concentration of the ligand and present it to the N-SH2 domain. Therefore, C-SH2 contributes to the substrate specificity and binding affinity. On the other hand, Shp2 can be activated by phospholipids which may be mediated by lipid interaction with the PTP domain, suggesting that membrane anchorage of Shp2 may regulate its enzymatic activity.

Shp2 plays an important role in signaling events downstream of growth factors,

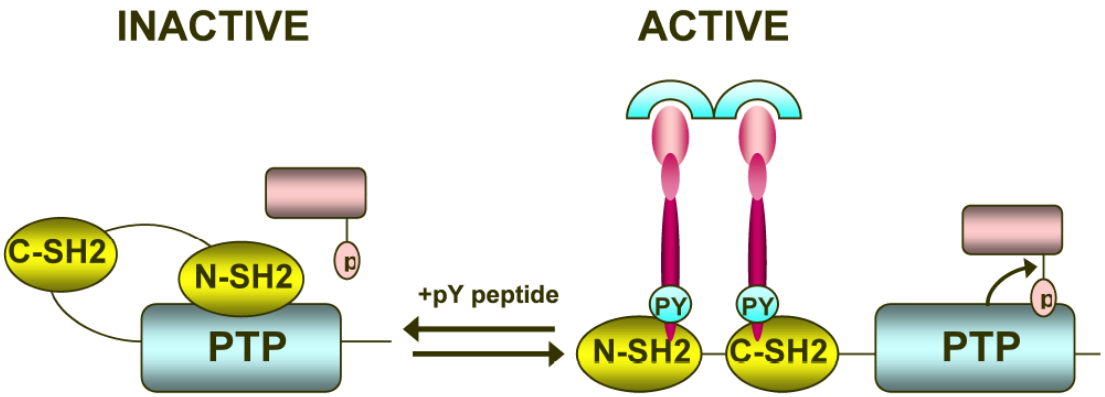


Figure 1. Structure and regulation of Src homology-2 (SH2) domain-containing phosphatases Shp-2.
Basal enzymatic activity of Shp2 is low, whereas addition of a phosphotyrosyl peptide to bind the N-SH2 domain can stimulate catalysis strikingly. Occupation of the two SH2 repeats of Shp2 by the tethered pTyr-containing peptide is much more potent in stimulating the enzymatic activity *in vitro* than monophosphorylated peptides. Adapted from TRENDS in Biochemical Sciences. Vol.28 No.6 June 2003. Neel et al "The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling".

cytokines, hormones, antigens and extracellular matrixes (Allard et al., 1996; Pan et al., 2008; Shi et al., 2000). It is recruited, via SH2 domains, to phosphorylated tyrosine residues on receptors (RTKs and cytokine receptors), and/or adaptor proteins such as insulin receptor substrate, fibroblast growth factor receptor substrate, or GRB2-associated binders (GAB) proteins (Lima et al., 2002; Mussig et al., 2005; Staubs et al., 1994). In addition, most of the protein binding to Shp2 SH2 domains possess one or more immuno-receptor tyrosine-based inhibitory motifs (ITIMs) (Walter et al., 2008). Basal enzymatic activity of Shp2 is low, whereas addition of a phosphotyrosyl peptide to bind the N-SH2 domain can stimulate catalysis strikingly (Figure 1). Occupation of the two SH2 repeats of Shp2 by the tethered pTyr-containing peptide is much more potent in stimulating the enzymatic activity *in vitro* than monophosphorylated peptides. Shp2 is reported to be transiently inactivated by reactive oxygen intermediates (ROI) in platelet-derived growth factor (PDGF)-stimulated rat 1 cells and the inactivation is necessary for PDGF receptor function, indicating that Shp2 can also be regulated by ROI (Wu et al., 2006).

1.2 Modulation of signal strength of RTKs

Numerous experimental data from *C. elegans, Drosophila, Xenopus,* and mammals indicated that Shp2 positively regulate the signaling strength initiated from RTKs (Gutch et al., 1998; Johnson Hamlet and Perkins, 2001; Schutzman et al., 2001). The first genetic evidence was gained from the studies of the Csw function in controlling the expression of terminal genes and formation of *Drosophila* embryonic terminal structures (Freeman et al., 1992; Perkins et al., 1992). Loss of function mutations in Csw, cooperated with D-raf, inhibited the signaling of a hyperactive mutant of Torso, a RTK

gene. On the other hand, Csw plays a crucial role as downsteam of Sevenless RTKs in development of the R7 photoreceptor cell in the ommatidium of *Drosophila* eye (Allard et al., 1996). PTP-2, a homolog of Csw and Shp2, is required for normal oogenesis in C. elegans and functions together with LET-60 Ras during oogenesis (Gutch et al., 1998). Shp2 was also demonstrated to be necessary for FGF-induced MAPK activation, mesoderm induction and elongation of *Xenopus* animal caps induced by basic fibroblast growth factor or activin A (O'Reilly and Neel, 1998; Tang et al., 1995). Genetically engineered deletion of N-SH2 domain of murine Shp2 ($Shp2^{\Delta 46\text{-}110}$) leads to embryonic lethality in homozygous mutant, with multiple defects in mesodermal patterning, which is phenotypic similar to *FGF-R1*-deficient mice (Saxton et al., 1997). Analysis of chimeric animals and *in vitro* ES cell differentiation revealed that Shp2 works downstream of stem cell factor receptor C-kit in hematopoietic cell development and mediates signaling from FGF-R in leading mesodermal cell migration and limb development (Chan et al., 2003). Moreover, biochemical studies showed that there is no Erk activation upon insulin-like growth factor-1 (IGF-1) stimulation in fibroblasts in the absence of Shp2 (Noguchi et al., 1994; Wu et al., 2001). In most cases, Shp2 is required for sustained Erk activation, but not for initiation of the activation (Feng, 1999). In contrast, $Shp2^{\Delta 46\text{-}110}$ mutant cells exhibited enhanced Jnk activity in response to stress condition such as UV irradiation and heat shock protein treatment, suggesting that Shp2 might play an opposite role in regulating Erk and Jnk pathways in signaling events (Shi et al., 1998).

Although positive regulation by Shp2 of Ras/MAPK activation has been much appreciated, the underlying mechanism is still unclear. The first, but also the most controversial model is that Shp2 acts as an adaptor protein through the two SH2 domains

at its N-terminus and a potential phospho-tyrosine site (Y^{542}TN1 of human Shp2) for Grb2/SOS interaction. The supportive evidence is that Shp2 promotes Ras activation following interaction with the Gab1 adaptor protein in $Shp2^{\Delta 46\text{-}110}$ mutant cells (Shi et al., 2000). Additionally, Csw association with the *Drosophila* Gab ortholog Dos is crucial for embryogenesis (Bausenwein et al., 2000; Herbst et al., 1999). However, dominant negative mutant $Shp2^{C459S}$ was sufficient to suppress the insulin-stimulated Erk activation and c-fos promoter-dependent gene transcription in CHO/IR cells (Servidei et al., 1998). In addition, the C-terminal tyrosine residues are dispensable in *Xenopus* FGFR and *Sevenless* signaling. In contrast, the PTP domain is essential for Shp2 positive regulation of Ras/Erk activation, although the direct target of Shp2 remains elusive.

Another proposed model is that Shp2 dephosphorylates the p120-RasGAP-binding site on RTKs (Cleghon et al., 1998). Studies of Torso signaling reveal that Shp2 (Csw) can dephosphorylate tyrosine 918 of Torso, the predominant binding site of the *Drosophila* homolog of RasGAP, and subsequently promote Ras/MAPK activation (Dance et al., 2008). Although this model appears to be attractive, it cannot explain Shp2 action in all signaling pathways. Previous studies using fibroblasts derived from Gab1-/- and FRS2-/- mice indicate that scaffolding Shp2-adaptor protein complexes mediates the positive regulation of Shp2 on MAPK activation (Hadari et al., 2001; Itoh et al., 2000). Moreover, similar to Shp2 Ex3-/- cells, Gab1-/- fibroblasts have impaired PDGF-induced Erk activation (Itoh et al., 2000). Therefore, neither “adaptor model” nor Shp2/Csw dephosphorylating RasGAP can entirely explain the Shp2 function in Ras/MAPK activation.

The third proposed model of Shp2 positive action of Ras/MAPK activation is that Shp2 directly dephosphorylates Sprouty protein, a negative regulator of RTK-mediated Ras/Erk activation (Mason et al., 2006). Prior studies in cultured *Drosophila* cells and developing eye showed that Shp2 can promote RTK signaling by inactivating Sprouty 1, suggesting that Sprouty1 is indeed an *in vivo* target of Shp2 (Jarvis et al., 2006). However, Sprouty4, another member of Sprouty family, suppresses vascular epithelial growth factor (VEGF)-induced Raf1 activation through direct interaction with Raf1 via its carboxy-terminal cysteine-rich domain, which is dispensable for the conserved Sprouty4 tyrosine residue (Sasaki et al., 2003). This evidence argues against Shp2-mediated dephosphorylation of Sprouty model and requires further investigations.

The fourth proposed model is that Shp2 operates upstream of the Src family kinase (SFK) to promote Ras/MAPK activation. A recent study reported that Shp2 was targeted to the plasma membrane and stimulated Src activation, subsequently activate Ras/MAPK signaling (Cunnick et al., 2002). Another supportive experimental data showed that Src activation upon upstream stimulation was largely attenuated in the absence of Shp2, which is indicated to dephosphorylate Cbp/PAG (Csk binding protein, or phosphoprotein associated with glycosphingolipid-enriched microdomains) and suppress Csk inhibition of Src-containing complex activation (Ren et al., 2004; Zhang et al., 2004). The most recent studies of trophoblast stem cells revealed that Shp2 operates upstream of Src and Ras to positively regulate cell survival upon FGF-4 stimulation (Yang et al., 2006).

1.3 Involvement in cytokine signaling

Shp2 also mediates cytokine receptor signaling, such as receptors for leukemia inhibitory factor (LIF), growth hormone, interferons, interleukins and erythropoietin (Qu, 2002; Wang et al., 2005). Whereas Shp2 obviously promotes Ras/MAPK activation upon growth factor stimulation, its effect in cytokine-stimulated JAK/STAT activation is indistinct. Shp2 directly binds to the interleukin 6 (IL-6) family cytokine receptor subunit gp130 (Ohtani et al., 2000). Previous studies of Shp2 signal-deficient mice, generated by mutating the Shp2 binding site (Tyr-759) in gp130 receptor, showed splenomegaly and lymphadenopathy, an enhanced acute phase reaction and autoimmune arthritis (Atsumi et al., 2002; Ohtani et al., 2000). The mechanistic studies revealed an enhanced gp130-activated STAT3 activation in mutant mice, suggesting that Shp2 plays a negative role in JAK/STAT3 signaling (You et al., 1999). Besides its negative regulation of IL-6 signaling, Shp2 also inhibits STAT activation upon interferons α and γ stimulation. Initial studies of mouse fibroblast cells lacking a functional Shp-2 revealed that deletion of Shp2 markedly augments the sensitivity of fibroblasts to the cytotoxic effect of IFN-α and IFN-γ, which was resulted from excessive activation of STATs by IFNs in $Shp2^{-/-}$ cells (Du et al., 2005). *In vivo* experimental data showed that the recruitment of PKCβ to the IFN-α receptor and interaction with Shp2 are required for PKC suppression of IFN-α-mediated gene activation and antiviral responses. In the absence of Shp2, PKC-mediated inhibition of IFN-α signaling was abolished, indicating an essential role of Shp2 in the inhibitory pathway (Du et al., 2005).

Not only is Shp2 involved in regulating Ras/MAPK and JAK/STAT3 signaling pathways, Shp2 also functions in other aspects of cytokine signaling events. Shp2 can

mediate the protective effect of interleukine-3 and interleukine-6 against apoptosis (Wheadon et al., 2003; Yu et al., 2003). In the IL-3 signaling pathway, Shp2 negatively regulates Jun N-terminal kinase (JNK) activation, without affecting STAT phosphorylation (Wheadon et al., 2003). Another study confirmed that Shp2 suppresses JNK activation upon heat shock (Shi et al., 1998). Moreover, Shp2 was demonstrated to activate NF-kappa B increase production of interleukin (IL)-6 in response to IL-1 alpha or tumor necrosis factor (TNF)-alpha in fibroblasts, whereas it exerts no impact on the activation of the three types of mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (Erk), c-Jun NH(2)-terminal kinase (Jnk) or on activation of p38 upon IL-1/TNF stimulation (You et al., 1999). Collectively, Shp2 plays both positive and negative roles in cytokine signaling pathways, thereby precisely controlling amplitude and duration of the cytokine signaling events.

1.4 The role of Shp2 in other signaling pathways

In addition to its regulatory role in RTK and cytokine signaling, Shp2 is indicated to control integrin-induced cell spreading, migration, focal adhesion and Erk activation (Yu et al., 1998). Reduced cell motility and enhanced focal adhesion were reported in fibroblasts with Shp2 N-SH2 domain deletion and MCF-7 breast adenocarcinoma cells with Shp2CS dominant negative mutation (Manes et al., 1999). Shp2 defective fibroblasts displayed impaired integrin-induced activation of Src family PTKs, tyrosine phosphorylation of several focal adhesion proteins, MAPK activation, and the ability to spread on fibronectin (Oh et al., 1999).

Shp2 is also reported to interact with cytoplasmic tails of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), killer cell immunoglobulin (Ig)-like receptors (KIRs), B- and T-lymphocyte attenuate (BTLA), programmed death-1 (PD-1) and CD31 (platelet endothelial cell adhesion molecule; PECAM-1) in lymphocytes (Henshall et al., 2001; Leibson, 2004).

1.5 The association of Shp2 with scaffold proteins

Shp2, via its SH2 domains, can interact with several scaffolding proteins such as GAB proteins, FRS2 and SHPS1 (Neel et al., 2003). These scaffolding proteins possess multiple protein-protein interaction motifs as well as tandem tyrosine motif at their C-terminal tails that facilitate the recruitment of Shp2.

GAB proteins compose a distinct family of large adapters that are conserved from worms to mammals, including Gab1, Gab2, Gab3, *Drosophila melanogaster* DOS (Daugther of Sevenless) and *Caenorhabditis elegans* Soc-1 (Suppressor of Clear) (Neel et al., 2003). Gab1 (also called Grb2 associated binder-1), a wildly expressed multiadapter, was discovered during a search for proteins associated with Grb2 SH3-domain (Holgado-Madruga et al., 1996). Gab1 possesses a conserved amino-acid pleckstrin homology (PH) domain, multiple potential tyrosine and serine/threonine phosphorylation sites, and a proline-rich motif that recruits SH3 domains.

Upon activation of some receptor tyrosine kinases (RTKs), the scaffold Gab1 becomes tyrosyl phosphorylated and interacts with SH2 domain–containing signal relay molecules, including the tyrosine phosphatase Shp2, the p85 subunit of phosphoinositide-3-kinase (PI3K) (Montagner et al., 2005). Gab1 contains at least 12 tyrosine motifs that

can be phosphorylated in response to kinase stimulation. These phosphotyrosines specifically interact with SH2-domain-containing proteins or its *Drosophila* or *C. elegans* orthologs [Corkscrew (Csw) and Ptp2, respectively]. Numerous studies have explored the important role of the Gab1 phosphotyrosines in promoting PI3K and Shp2 signaling in multiple pathways (Liu and Rohrschneider, 2002). The binding of Gab1 to Shp2 N-SH2 domain can relieve the allosteric inhibition of the PTP domain by the N-SH2 domain and promotes Shp2 activation. Previous studies of chimeric receptors demonstrated that Gab1 with a Shp2 binding mutation could not mediate EGF-initiated Ras/Erk activation. Gab2–Shp2 complexes appear to play different roles in signaling events. Gab2 containing mutations at the Shp2 binding site suppress IL-3 induced early gene activation with no effect on Erk activation, indicating that Gab2-Shp2 complexes operate at different levels of Ras/Erk signaling in comparison with the Gab1-Shp2 complex (Zhao et al., 1999). The distribution pattern of Gab family members is different: Gab1 is ubiquitous; Gab2 is expressed in brain, lung and testis; Gab3 is limited to hematopoietic tissues such as spleen and thymus (Wolf et al., 2002). In cells expressing 'substrate trapping' mutants of Shp2, Gab1, Gab2 and Dos tyrosine phosphorylation is increased, indicating that Shp2 can target these Gab proteins. The function of Shp2 and Gab interaction is complex and needs to be investigated in individual signaling pathways.

Another category of Shp2 interaction protein is fibroblast growth factor receptors (FGFRs). In FGF receptor signaling, the adapter protein Grb2 was demonstrated to link receptor tyrosine kinases with the Ras signaling pathway by interacting with the guanine nucleotide-releasing factor SOS through its SH3 domains and with tyrosine-phosphorylated receptors or docking molecules via its SH2 domain. FGF receptor

substrate 2 (FRS2) was found to be tyrosine phosphorylated and form a complex with Grb2, SOS and Shp2 upon FGF stimulation (Lax et al., 2002; Ong et al., 2000). The experimental data showed that an FRS2 mutant defective in Grb2 and Shp2 binding suppresses MAPK activation and fails to induce neuronal differentiation of PC12 cells (Hadari et al., 1998). Additionally, brain-derived neurotrophic factor (BDNF) was demonstrated to induce association of Shp2 with FRS2 (Yan et al., 2002). These results imply that FRS2 recruits the Grb2-SOS/Shp2 complex and leads to sustained Ras/MAPK activation and cell differentiation.

In early events of integrin signaling, Shp2 was revealed to mediate integrin-induced cell spreading, migration and Erk activation through forming a complex with SH2 domain-containing tyrosine phosphatase substrate-1, SHPS-1 (Takai et al., 2002). SHPS-1 is a transmembrane glycoprotein, which belongs to SIRP (Signal Regulatory Protein) family. SHPS-1 can be tyrosine phosphorylated and bind to Shp2 upon different stimuli, such as serum, Insulin-like growth factor I (IGF-I), LPS or cell adhesion. The regulatory role of SHPS-1 in Ras/MAPK signaling remains controversial. Previous studies of fibroblasts homozygous for SHPS-1 mutant displayed increased formation of actin stress fibers and focal adhesions, implicating that SHPS-1 negatively modulates integrin-mediated cytoskeletal reorganization, cell motility and growth factor-induced MAPK activation (Inagaki et al., 2000). On the other hand, SHPS-1 was suggested to be positive regulator of IGF-1 initiated MAPK activation. IGF-I stimulates phosphorylation of at least two tyrosine residues of SHPS-1 and leads to recruitment of SHP-2 to SHPS-1, which is essential for IGF-1 stimulated mitogenic signaling (Lieskovska et al., 2006; Maile and Clemmons, 2002). The function of SHPS-1 differs in individual signaling

pathway, nevertheless the formation of Shp2/SHPS-1 is crucial for SHPS-1 regulation. SHPS-1 is implicated to facilitate Shp2 interaction with other signaling modulators, such as kinases. Through the interaction, the autoinhibition of Shp2 enzymatic activity may be alleviated and Shp2 is subsequently activated. Once activated, Shp2 can dephosphorylate SHPS-1 and release the Shp2/SHPS-1 complex, thereby targeting other downstream molecules and promoting MAPK signaling pathway (Oh et al., 1999). Full understanding of the function of SHPS-1 and Shp2 interaction requires further investigation.

2. *PTPN11* mutations in human disease

It is well documented that germ line mutations in *PTPN11* lead to Noonan and LEOPARD syndrome (Tartaglia and Gelb, 2005). On the other hand, somatic mutations in *PTPN11* cause certain types of leukemia, most notably juvenile myelomonocytic leukemia (JMML) (Loh et al., 2004; Tartaglia et al., 2005). Noonan syndrome (NS) is an autosomal dominant disorder, which is characterized by the following features: facial abnormalities, proportionate short stature, skeletal malformation, webbed neck, mental retardation and a wide spectrum of congenital heart defects (Tartaglia and Gelb, 2005). Most of NS mutations reside in either N-SH2 or PTP domain and are predicted to relieve the autoinhibition of PTP activity, indicating that gain-of-function mutations of Shp2 are a common cause of NS. On the other hand, recent studies revealed germ line missense mutations in HRAS and KRAS (around 1-2% overall) and in genes encoding molecules that regulate Ras/Erk activation can lead to Noonan syndrome without *PTPN11* mutations (Schubbert et al., 2007). Germ line gain-of-function mutations in SOS1 (around 10% overall) and RAF1 (around 5% overall) were also reported to cause Noonan

syndrome (Razzaque et al., 2007; Roberts et al., 2007; Tartaglia et al., 2007). These findings implicate that increased RAS-mitogen-activated protein kinase (MAPK) signaling due to germline gene mutations can profoundly change human development and cause Noonan syndrome.

LEOPARD syndrome shares certain overlapping features with Noonan syndrome, including cardiac abnormalities, short stature and facial dysmorphia. Enhanced Ras/MAPK activation due to PTPN11, SOS1 and KRAS mutations leads to approximately 60% of Noonan syndrome cases, and germ line missense mutations in PTPN11 cause 90% of LEOPARD syndrome cases (Legius et al., 2002). Enzymologic, structural, cell biological, and mouse genetic studies indicate that Noonan syndrome is caused by gain-of-function *PTPN11* mutations, whereas LEOPARD syndrome is caused by catalytically defective and dominant negative mutations of *PTPN11* that inhibit growth factor evoked ERK activation in transient and stable transfection assays (Hanna et al., 2006; Kontaridis et al., 2006). Therefore, the pathogenesis of Noonan syndrome and LEOPARD syndrome is different and can be defined by genetic analysis of missense mutations.

A small proportion of Noonan syndrome patients with germ line missense mutations in *PTPN11* also develop juvenile myelomonocytic leukemia (JMML) (Loh et al., 2004; Tartaglia et al., 2005). Juvenile myelomonocytic leukemia (JMML) is a relentless myeloproliferative disorder of young children characterized by overproduction of myeloid cells that infiltrate hematopoietic and nonhematopoietic tissues (Arico et al., 1997; Emanuel et al., 1996). Somatic mutations in *PTPN11* were reported to account for 34% of non-syndromic JMML (Tartaglia et al., 2003). Moreover, somatic mutations in

*PTPN1*1 were detected in a small percentage of individuals with myelodysplastic syndrome (MDS) and de novo acute myeloid leukemia (AML) (Chan et al., 2008). Somatic mutations in *PTPN11* are not random and are functionally equivalent to oncogenic mutations in RAS (Schubbert et al., 2007). According to the crystal structure of SHP-2, each of these mutations is prone to release the autoinhibition between N-SH2 domain and the PTPase domain and promote the enzymatic activity of Shp-2.

3. Shp2 in association with *Helicobacter pylori* carcinogenesis

Shp2 is also reported to correlate with *Helicobacter pylori* carcinogenesis. *H. pylori* is a micro-aerophilic spiral-shaped bacteria, which infects approximately half of the human population and is reported to be associated with gastric carcinoma, which is the second most common cause of cancer-related death worldwide (Hatakeyama, 2004). *H. pylori* primarily infects individuals during childhood, and can cause gastric diseases such as chronic atrophic gastritis and peptic ulcers after certain latent period. A large-scale prospective study reported that the risk for development of gastric carcinoma was largely elevated in the population with *H. pylori* infection than the population without *H. pylori* infection. CagA is a 120–145-kDa protein and was firstly identified as a virulence factor of H. pylori that causes peptic ulcers. Epidemiological studies have shown that the cagA-positive H. pylori strains are correlated with higher rates in the development of atrophic gastritis, peptic-ulcer disease and gastric carcinoma (Hatakeyama, 2004).

Once phosphorylated, the membrane-integrated CagA can function as a scaffold to recruit Shp2 to the plasma membrane and then activate Shp2 in a tyrosine-phosphorylation-dependent manner (Higashi et al., 2002; Tsutsumi et al., 2003). CagA

can cause the hummingbird phenotype in gastric epithelial cells, which is characterized by increased cell spreading and elongation (Higuchi et al., 2004). Induction of the hummingbird phenotype is suppressed by inhibiting CagA activation. Disruption of CagA–Shp2 complex formation, as well as constitutive or conditional Shp2 knockdown, can diminish the hummingbird phenotype induced by CagA (Higashi et al., 2004). These experimental data suggested that deregulated activation of Shp2 is necessary for CagA-induced morphogenetic changes of gastric epithelial cells. Moreover, CagA can enhance the duration of Erk activation upon mitogenic stimulation and Erk activation is necessary for the induction of the hummingbird phenotype by CagA (Higashi et al., 2004). On the other hand, CagA-induced hummingbird phenotype is dispensable for Ras activation. Since Shp2 can promote ERK activation, Shp2 may signal to Erk upon CagA initiation in RAS-independent manner. During CagA-positive H. pylori infection of gastric epithelial cells, CagA deregulation of Shp2 causes constitutive activation of its enzymatic acitivity. However, the elevation of Shp2 activity by CagA promotes cell apoptosis instead of cell proliferation in cultured gastric epithelial cells, which may result from unbalanced mitogenic signals generated by the CagA–SHP2 interaction (Tsutsumi et al., 2003). Consequently, increased cell apoptosis by the CagA–SHP2 interaction leads to chronic loss of gastric epithelial cells and induce the pathogenesis of H. pylori-associated atrophic gastritis.

4. Shp2 in regulation of leptin signaling and obesity

The adipokine leptin activates the leptin receptor long form (LepRb, also called ObRb and LRb) in the hypothalamus to control food intake, metabolism and

neuroendocrine responses to nutritional alteration. Upon leptin stimulation, LepRb can activate JAK2, which in turn phosphorylates the receptor on tyrosine residues in the cytoplasmic domain, including Tyr985 and Tyr1138. These two tyrosine sites of LepRb can bind to Shp2/Socs3 and STAT3, respectively (Flier, 2004). Affinity chromatography using a phosphotyrosine peptide modeled on Tyr985 of LepRb identified Shp2 as the major LepRb interaction protein (Li and Friedman, 1999). Moreover, expression of wild-type Shp2 suppresses phosphorylation of JAK2 induced by leptin, whereas expression of an enzymatically inactive Shp2 could not lower phosphorylation of JAK2. Mutation of tyrosine residue 985 of LepRb to phenylalanine disrupts Shp2 interaction with the receptor and suppresses Shp2 tyrosine phosphorylation in response to leptin stimulation (Carpenter et al., 1998). The regulatory role of Shp2 in leptin signaling is still unclear. In vitro study by transfecting a catalytically inactive (C459S) mutant of Shp2 revealed an inhibited leptin-induced Erk activation through tyrosine residue 985 of LepRb (Bjorbaek et al., 2001). On the other hand, Shp2 mutant containing deletions of two C-terminal tyrosine residues showed decreased inhibitory effect on Erk activation. Collectively, both the catalytic activity and tyrosine phosphorylation of Shp2 account for a positive regulation of leptin-induced-Erk activation.

Besides *in vitro* biochemical studies, genetic evidence further confirmed Shp2 as an important molecule of leptin signaling pathway. Physiological studies revealed the obese and leptin-resistant phenotype of CaSKO mice, in which Shp2 is specifically deleted in postmitotic forebrain neurons (Zhang et al., 2004). CaSKO mice displayed early-onset obesity and accelerated increase of body weight, both found in male and female mice. Increase of body weight in CaSKO mice is associated with elevated

white/brown adipose tissue, higher serum triglyceride levels and marked increase of serum leptin level, indicating leptin resistance in mutant mice. The obese and leptin-resistant phenotype of CaSKO mice is correlated with the inhibited leptin signaling in the forebrain neurons. More specifically, Shp2 plays a positive role in regulating Erk activation, whereas a negative role in JAK2/STAT3 signaling pathway. In CaSKO animal model, deletion of Shp2 leads to suppressed leptin signaling, indicating that the positive effect of Shp2 on Erk activation overrides its negative regulation of JAK2/STAT3 signaling. Metabolic studies revealed that the total food intake of CaSKO mice is normal in spite of the increased body weight (Zhang et al., 2004). Moreover, the body temperature of CaSKO mice is markedly decreased, suggesting that the elevated body weight of CaSKO mice might be due to lower energy consumption. Pathological studies showed that CaSKO mice develop fatty liver with increased size, as evidenced by Oil-Red-O staining, indicating that deletion of Shp2 in forebrain neurons disrupts hepatic lipid metabolism. Previous experimental data reported that leptin treatment of *ob/ob* mice and human subjects can ameliorate insulin sensitivity and lower lipid levels in liver and adipose tissues. In consistence, impaired leptin signaling in CaSKO mice leads to defective peripheral insulin sensitivity, as described by hyperglycemia and hyperinsulinemia.

Collectively, both biochemistry and genetic studies indicated that Shp2 promotes leptin signaling in the hypothalamus by regulating leptin-stimulated Erk activation and ObRb-Jak2/Stat3 pathway. CaSKO mice share common characteristic features with ob/ob and db/db animal model, including fatty liver, glucose/lipid metabolic alterations and early-onset obesity. These findings suggest that pharmaceutical augmentation of Shp2

activity in the brain may potentially be an efficient therapeutic strategy for alleviation of leptin resistance in obese patients. To testify whether constitutive activation of Shp2 can improve leptin signaling and alleviate high-fat-diet induced metabolic disorder, I generated Shp2 gain-of-function (Shp2GOF) transgenic mouse model, which carried Shp2 D61A dominant active mutations in postmitotic forebrain neurons. The metabolic studies of Shp2GOF transgenic mice revealed that augmentation of Shp2 activity in forebrain neurons prevents the onset of high fat diet-induced obesity in female mice and promotes leptin sensitivity.

5. Shp2 involvement in regulation of glucose metabolism

Shp2 was demonstrated to modulate insulin signaling of insulin responsive tissues and therefore regulate glucose homeostasis (Andreozzi et al., 2004; Maegawa et al., 1999; Mussig et al., 2005). This phosphatase is demonstrated to mediate the insulin signaling through interaction with insulin receptor (IR) and insulin receptor substrate (IRS) (Staubs et al., 1994; Sun et al., 1995). However, the role of Shp2 in insulin signaling pathway remains controversial under different circumstances. The evidence supporting the negative role of Shp2 is that overexpression of Shp2 in fibroblasts suppresses insulin signaling by decreasing IRS-1 tyrosine phosphorylation and PI3K activation (Ouwens et al., 2001). On the other hand, Shp2 is demonstrated to promote MAPK family members extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation downstream of insulin receptor in hepatocytes *in vitro* (Yamauchi et al., 1995). *In vivo* studies of Shp2 dominant negative transgenic mice revealed an insulin-resistant phenotype, indicating a positive role of Shp2 in insulin signaling events and glucose homeostasis (Maegawa et al., 1999).

However, other studies of Shp2 knockout animal model could not affirm its regulatory role in insulin signaling. Mutant mice with hemizygous deletion of Shp2 survived to adulthood with no defects in insulin signaling and glucose metabolism (Arrandale et al., 1996). Collectively, the function of Shp2 in insulin signaling might differ among different cell types, tissues, age and background of the mutant mice. Therefore, generation of tissue-specific knockout mice would allow us to spatially/temporally define physical function of Shp2 in insulin signaling pathways.

6. Overview of pancreatic β cells

6.1 Stimulus–secretion coupling of insulin release from β cells

The pancreatic β cell plays an essential role in glucose homeostasis by secretion of the hormone insulin to meet metabolic demand. Insulin is synthesized as preproinsulin in the ribosomes of the rough endoplasmic reticulum (Maechler and Wollheim, 2001). The preproinsulin is then cleaved to proinsulin, which is transported to the Golgi apparatus and is packaged into secretory granules located close to the cell membrane. Although many nutrients, hormones, and neural stimuli can promote insulin secretion, glucose must be regarded as the major regulator of insulin synthesis and release (Nesher et al., 2001). Rapid and sustained stimulation of β-cells with glucose induces biphasic insulin secretion, including a first phase that occurs promptly after exposure to glucose and a prolonged second phase.

Numerous studies have documented that, in individuals with type 2 diabetes, β-cells do not sense glucose properly and therefore do not release appropriate amounts of insulin. GLUT2 is the major glucose transport isoform expressed in the plasma

membrane of β-cells. It has a high Km for glucose (17mM), which may be required for the normal function of the β-cell glucose sensor (Unger, 1991). It was reported that decreased response to glucose challenge is associated with decreased expression of GLUT2. In rat models of type II diabetes such as the diabetic Zucker rat, GLUT2 expression is reduced in glucose-unresponsive β-cells and the extent of reduction is proportional to the severity of the hyperglycemia (Unger, 1991). Thus, decreased expression of GLUT2 is associated with β-cell dysfunction of rodent models of type 1 and type 2 diabetes and decreased GLUT2 levels may impair normal glucose uptake and metabolism thereby preventing glucose sensing. Decreased expression of GLUT2 appears the first morphological marker for β cells from type 2 diabetic animals. In islet β-cells, glucokinase also plays a crucial role in the regulation of glucose secretion (Postic et al., 1999). It catalyzes the high Km conversion of glucose to glucose 6-phophate. This is the rate-limiting step in glucose utilization and insulin secretion in pancreatic β-cells. Oxidative mitochondrial metabolism is essential for glucose-stimulated insulin secretion (Figure 2). Glucose sensing requires oxidative mitochondrial metabolism, leading to the generation of ATP. This increases the ratio of ATP to ADP in β-cells, and then initiates the following chain of events: promoting the closure of ATP-sensitive K^+ channels (K_{ATP}) and subsequently depolarizing the plasma membrane (Bratanova-Tochkova et al., 2002). The membrane depolarization leads to calcium influx through voltage-gated calcium channels and increase of intracellular calcium concentration, which results in discharge of the granules from a "readily releasable pool" and the rapid first-phase insulin secretion. It follows that the second phase requires the preparation of granules for release,

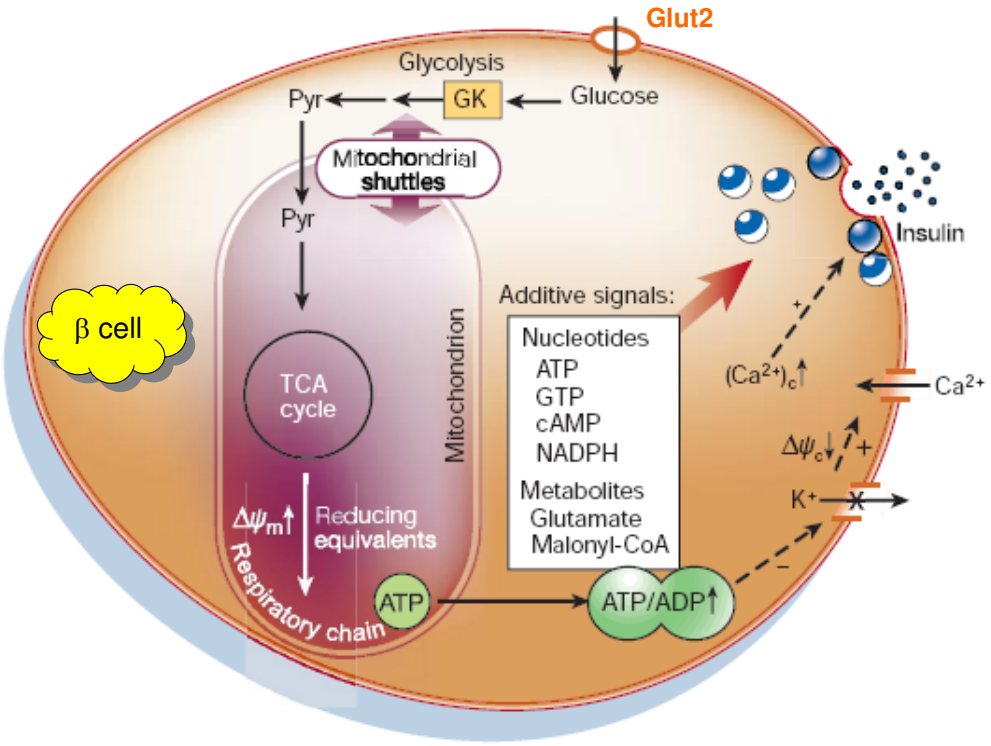


Figure 2. A working model for glucose stimulated insulin secretion of pancreatic β cells.
Adapted from NATURE, Vol 414, Maechler et al "Mitochondrial function in normal and diabetic β-cells", pp 807-812 Copyright (2001).

perhaps including granule translocation to the plasma membrane, morphological docking, preparation for release, priming, and exocytosis. The messengers responsible for the second phase of release include increased ATP/ADP ratio, membrane depolarization, increased intracellular calcium concentration, and additional glucose-induced signals that may include citrate and malonyl CoA, long-chain acyl-CoAs, diacyl glycerol, PKC isoforms, phospholipases, and phosphoinositides (Maechler and Wollheim, 2001).

6.2 Insulin signaling in β cells

Given the importance of the β cell, substantial efforts have been made to define factors and pathways that govern glucose-stimulated insulin secretion. One proposed mechanism underlying β cell dysfunction is altered insulin signaling pathways. Insulin can bind to the insulin receptor (IR) and lead to autophosphorylation of IR. Then phosphorylated IR can induce tyrosine phosphorylation of IRS proteins. Furthermore, insulin also plays a positive role in the regulation of transcription of its own gene and in stimulation of vesicle exocytosis from isolated β-cells (Leibiger et al., 1998).

Previous studies showed mice with a tissue-specific knockout of insulin receptor in β-cells (βIRKO) exhibited inadequate response to glucose stimulus, progressive glucose intolerance and reduced β-cell mass in the later age (Kulkarni et al., 1999). In contrast, mice with β-Cell-specific deletion of the IGF1 receptor manifest hyperinsulinemia and glucose intolerance but does not affect β-cell mass (Kulkarni et al., 2002). Therefore, insulin/IGF signaling is not crucial for early development of pancreatic β cells. Notably, double knockout mice with both IR and Igf1 receptor deletion in β cells

develop early-onset diabetes, which is due to reduced β-cell mass (Ueki et al., 2006). Most recent studies evaluated the insulin/IGF1 receptor function in β-cell compensatory growth by feeding βIRKO and βIGFRKO with high-fat diets (Okada et al., 2007). βIRKO, instead of βIGFRKO, displayed defective islet growth with high-fat diet challenge, indicating that insulin signaling is critical for compensatory β-cell growth instead of early β-cell growth.

Besides the important role of IR and IGF receptors in β cells, mice with β-cell specific deletion of PDK1, a signaling molecule downstream of both insulin receptor and IGF receptor, also develop progressive hyperglycemia as a result of reduced islet mass (Hashimoto et al., 2006). The reduced islet mass is a sum of lower islet density, the number and size of β cells. Moreover, IRS-1 deficiency leads to a decrease in insulin content of β-cells and decreased insulin secretion following glucose and arginine challenge (Kulkarni et al., 1999). It was reported that IRS-$2^{-/-}$ mice displayed reduced β-cell mass and relative hypoinsulinemia, leading to overt diabetes by 10 weeks (Lin et al., 2004). Collectively, these data suggest that severity of the phenotype due to alteration of insulin and IGF-1 signaling pathways are proportional to the extent of signaling defects. A complete disruption of the signaling leads to dramatically reduced β−cell mass and early-onset diabetes, while a less severe alteration of signaling components manifests only a defect in glucose sensing.

Numerous studies demonstrated that IRS2 signaling through PKB/Akt phosphorylation and nuclear exclusion of the forkhead-O transcriptional factor 1 (FOXO1), and subsequently promotes expression of the homeodomain protein pancreas

duodenum homeobox-1 (Pdx1) gene, an important β cell proliferation and survival factor. Studies of transgenic mice carrying a constitutively active Akt1/PKB in β cells exhibited improved glucose tolerance, increased serum insulin level and a marked increase of islet mass as a result of enhanced β-cell proliferation (Bernal-Mizrachi et al., 2001; Tuttle et al., 2001). On the other hand, transgenic animals expressing a kinase-dead mutant of Akt in beta cells displayed impaired glucose tolerance and defective insulin secretion, which is due to alteration of distal components of the secretory pathway. Foxo1, the downstream target of Akt, links insulin signaling to Pdx1 regulation by exhibiting mutually exclusive nuclear localization patterns with Pdx1 in β cells and acting as a repressor of Foxa2-dependent (Hnf-3β–dependent) expression from the Pdx1 promoter (Kitamura et al., 2002).

Another crucial signaling pathway downstream of IRS-2 in β-cells is Grb2/SOS/Ras/Raf/MEK-1/ERK pathway. It was found that mitogen-activated protein kinase ERK1/2 is essential for glucose-dependent transcription from both the full-length rat insulin I promoter and the glucose-sensitive isolated E2A3/4 promoter element in intact islets and beta cell lines (Khoo et al., 2003). Glucose stimulation can induce phosphorylation of ERK1/2 and consequently activate a variety of transcription factors including NeuroD1 and PDX-1, which promotes their binding affinity to elements in the promoter. Beta2, Pdx-1, MafA, E2A and NFAT are identified as direct ERK1/2 substrates using in vitro phosphorylation with purified active ERK2 (pERK2) and expression of mutant factors in functional assays (Lawrence et al., 2008). ChIP assays implicate that ERK1/2 activity is necessary for the DNA binding of all five proteins (Lawrence et al., 2007). It was observed that the majority of ERK1/2 serine

phosphorylation induced by glucose stimulation remains in the cytoplasm and phosphorylates synapsin I, a cytoplasmic protein implicated in exocytosis of insulin granules (Longuet et al., 2005). Collectively, ERK1/2 operates at multiple levels to mediate a glucose signal to insulin gene transcription.

6.3 Other signaling pathways in β cells

Besides the well documented insulin signaling pathway in β cells, leptin signaling was demonstrated to regulate β–cell growth and insulin secretion (Figure 3) (Niswender and Magnuson, 2007). Pancreas-specific leptin receptor (ObR) knockout mice exhibited enhanced glucose tolerance, elevated early-phase insulin secretion, and a greater β-cell mass related to increased β-cell size (Morioka et al., 2007). Phosphorylation of Akt, FoxO1 and p70S6K were promoted by both knockout and knockdown of the ObR gene, indicating crosstalk between leptin and insulin signaling pathways. In contrast, the knockout mice challenged with high-fat diet displayed severer defect in first phase insulin secretion, glucose tolerance and compensatory islet growth compared with littermate controls, providing a clue for obesity-associated diabetes. Another study using knockout mice with leptin receptor attenuation in β cells and hypothalamus revealed fasting hyperinsulinemia, defective glucose-stimulated insulin release, glucose intolerance and obese phenotype in mutant mice (Covey et al., 2006). Collectively, these experimental data provide direct genetic evidence for a crucial role for leptin signaling in regulating β-cell function and implicate that abnormalities in adipoinsular axis lead to diabetes associated with obesity.

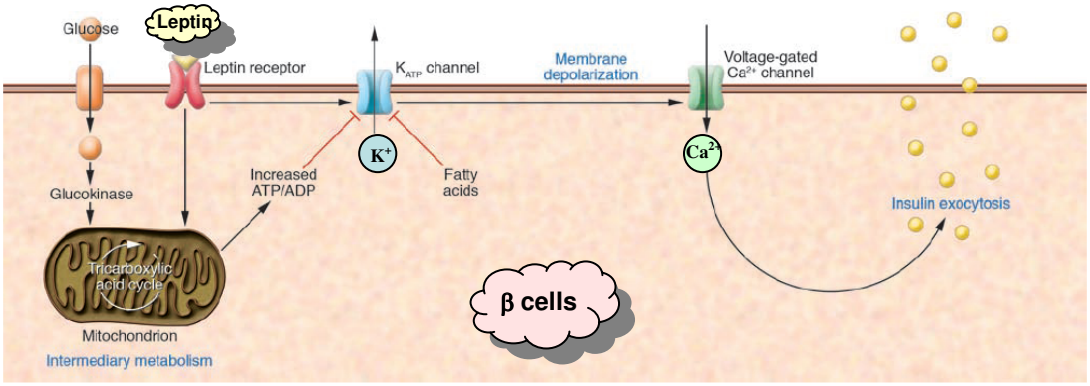


Figure 3. Leptin signaling of the pancreatic β cell.
Leptin suppresses insulin secretion of β cell through retaining the opening status of the K_{ATP} channel. On the other hand, leptin may sustain flux by ensuring the proper allosteric and transcriptional regulation of key metabolic enzymes.
Adapted from Niswender et al. **"**Obesity and the beta cell: lessons from leptin". J Clin Invest. 2007 Oct;117(10):2753-6.

In addition, Wnt signaling pathway is identified to modulate endocrine pancreatic β-cell proliferation by targeting Pitx2 and Cyclin D2, an essential regulator of beta cell cycle progression (Rulifson et al., 2007).

6.4 Pancreatic development and β-cell formation

The vertebrate pancreas derives from the foregut of the endoderm and is composed of exocrine, endocrine and ductal cells (Murtaugh and Melton, 2003). During embryogenesis, the pancreas develops from distinct dorsal and ventral anlagen. Before specification toward a dorsal pancreatic fate, midline endoderm in posterior foregut is a single layer of epithelial cells in contact with notochord, which is an axial mesoderm-derived structure (Wilson et al., 2003). Laterally, endoderm destined to form ventral pancreas is adjacent to both splanchnic mesoderm and aortic endothelial cells, whereas not contiguous with notochord (Wilson et al., 2003). By embryonic day 8.5 (E8.5) in the mouse, 24 hours before the dorsal pancreatic bud formation, cells in the endoderm in the region of the foregut/midgut junction are already destined to pancreatic fate (Kim and Hebrok, 2001). From E9.5 to E12.5, the majority of the endocrine compartment is formed. Within the endocrine pancreas, a pluripotent cell expressing a number of islet hormones appears to undergo progressive restriction until each of the individual islet cell populations is formed (Kim and Hebrok, 2001). The endocrine compartment eventually differentiate into four populations of pancreatic endocrine cell subtypes (α, β, δ and PP), which secret glucagon, insulin, somatostatin and pancreatic polypeptide, respectively (Murtaugh and Melton, 2003). Immediately after birth, there is a burst of islet cell proliferation. During weaning, there is a short burst of neogenesis that supplements the

increased β-cell proliferation (Murtaugh and Melton, 2003). Therefore, in the early stage, there is an increase of β-cell growth and expansion of β-cell mass. In the adulthood, the turnover rate of β-cells remains very slow. The overall life span of β-cell is approximately 60 days. Taken together, the most active neogenesis of β-cells occurs in the early stage, which determines the β-cell mass for the whole life span.

In contrast to the endocrine pancreas, development of exocrine cells into acini and ducts is detectable by E14.5 (Wilson et al., 2003). At this stage, endocrine cells are found embedded as individual cells in ducts or in small cell clusters distinct from ducts. The exocrine pancreas is comprised from a relatively homogeneous population of dense epithelial acinar cells, which arise from the pancreatic ducts (Wilson et al., 2003). Exocrine cells synthesize digestive enzymes, such as proteases, amylases, lipases, and nucleases, which are secreted into the acinar lumens and released into duodenum via pancreatic ducts. Secretion of these exocrine enzymes is regulated by hormonal stimulation, mainly by secretin, cholecystokinin, and gastrin, as well as neural stimuli (Johansson and Grapin-Botton, 2002).

A group of genes encoding essential transcription factors that play important roles during development and differentiation of the pancreas have been identified. Ipf1/PDX1 is a member of the ParaHox group of homeodomain transcription factors detected in endoderm and is necessary for pancreatic development in mice and humans (Jonsson et al., 1994). Embryonic tissue recombination studies suggest that there is an endodermal cell-autonomous requirement for Ipf1/PDX1 to elevate competence to growth signals from pancreatic mesenchyme (Offield et al., 1996). During bud outgrowth, PDX1

expression shifts from uniform to biphasic, at high levels in differentiated β-cells, whereas lower levels in undifferentiated precursors (Ashizawa et al., 2004). In adult animals, Ipf1/PDX1 is expressed in pancreatic β-cells and δ cells and is required for maintaining normal function of the mature β-cell (Ashizawa et al., 2004). Thus, Ipf1/Pdx1 plays distinct roles at different developmental stages. It contributes to pancreatic formation during embryogenesis and then regulates the pancreatic islet cell physiology in adult pancreas. Another crucial regulator of endocrine development is the bHLH protein Neurogenin3 (Ngn3), which is expressed restrictly in endocrine precursor cells, and subsequently downregulated in differentiated cells (Gradwohl et al., 1996). It is suggested that Ngn3 promotes endocrine cell fate in pancreatic progenitors with Pdx1 expression (Gradwohl et al., 2000).

It has been demonstrated that cell-cell interaction, such as pancreatic epithelium and midline mesoderm-derived tissues, is crucial for normal pancreatic development (Kim et al., 1997). Many of the signal transduction pathways, including the transforming growth factor-β (TGF-β), Notch, Hedgehog, epidermal growth facor (EGF) and fibroblast growth factor (FGF), have been defined to regulate cell interactions in the developing pancreas (Kim and Hebrok, 2001). These signaling pathways regulate the ratio of exocrine to endocrine pancreatic cell mass, which is approximately 100:1 in mice and humans (Murtaugh and Melton, 2003). FGF, activin and Hedgehog signaling pathways are indicated to govern the morphogenesis of pancreatic structures (Kim and Hebrok, 2001). FGF signaling regulates proliferation of endocrine and exocrine cells, and inhibition of FGFR2 leads to a significant decrease of acinar cells and loss of islets (Celli et al., 1998).

6.5 Transcription factors regulating β-cell function

It was well-documented that mutations in Pdx-1, glucokinase, Hnf4α, Hnf1α, Hnf1β or Beta2 can lead to monogenic forms of type 2 diabetes known as maturity onset of the young (MODY) (O'Rahilly et al., 2005). MODY is characterized by autosomal dominant inheritance, non-ketotic diabetes mellitus, an age of onset of <25 years and a major defect in pancreatic β-cell function (Butler et al., 2003). During pancreas organogenesis, several transcription factors are crucial to ensure proper development and differentiation pattern of the endocrine and exocrine compartment. In mature pancreas, transcription factors manipulate proliferation and stimulus coupling insulin secretion of islet β cells by targeting downstream genes, most importantly the insulin gene.

In mature β cells, Pdx-1 is required for maintaining the pancreatic islet function by regulating the transcription of insulin, GLUT-2 (Waeber et al., 1996), GK (Watada et al., 1996), somatostatin (SST), islet amyloid polypeptide and Nkx 6.1 genes (Pedersen et al., 2005; Shih et al., 2002). Pdx-1 directly modulate insulin transcription through interacting with transcriptional co-activators, p300, on the proximal insulin promoter, thereby activating components of the basal transcriptional machinery and histone/protein acetylation (Mosley et al., 2004; Qiu et al., 2002). Previous studies showed that Pdx-1, MafA, and NeuroD1/E47 bind to the insulin promoter proximal A1, C1, and E1 sites respectively, which is critical for sustaining basal human insulin promoter activity. Among these transcription factors, Pdx-1 possesses the most potent stimulatory effect on the insulin promoter, as compared with the weaker effects of MafA, NeuroD1, and E47.

NeuroD1 (Sharma et al., 1997), Hnf-1a and Hnf-3b (Ben-Shushan et al., 2001) may positively regulate Pdx-1 gene transcription. On the other hand, Pdx-1 binds to its own promoter elements and thereby transactivating its gene transcription (Cerf, 2006).

MafA, a β-cell specific basic leucine zipper transcription factor, can also bind to the conserved C1/RIPE3b element of the insulin promoter (Zhao et al., 2005). The triple infection with adenovirus for MafA, Pdx-1, and NeuroD1 was more potent to elevate insulin promoter activity than single or double infection, suggesting that MafA acts synergistically with Pdx1 and Beta2 to promote insulin gene transcription and insulin promoter activity.

Recent studies reported that MafA knockout mice develop progressively impaired glucose-stimulated insulin secretion and abnormal islet architecture. MafA regulates not only insulin gene transcription but also other genes modulating β-cell function, including potassium channel subunits *Kir6.2* and *SUR1*, *Glut2*, *Glucokinase*, *Glucagon-like peptide 1 receptor (GLP1-R)* and *Pyruvate carboxylase*. Moreover, MafA also modulates the gene expression of other transcription factors, such as *Pdx-1, Beta2* and *Nkx6.1* (Matsuoka et al., 2004). Collectively, MafA plays important role in the regulation of key β-cell genes and pathogenesis of type 2 diabetes.

NeuroD1 (Beta2) is another key transcription factor involved in regulating insulin gene transcription and maintaining normal β-cell function. Mutations of NeuroD1 lead to MODY6 in humans (Malecki et al., 1999). NeuroD1 can bind and activate the Sur1 promoter, which is important for glucose stimulated insulin secretion. NeuroD1 represses somatostatin promoter and consequently relieve the inhibition of insulin secretion by somatostatin. Moreover, NeuroD1 can form a heterodimer with E47, a wildly expressed

E2A family member, and then the heterodimer transactivates insulin E-boxes and promote insulin gene transcription in β-cells. The heterodimer can regulate both insulin and glucagon gene transcription and activate the upstream glucokinase promoter.

HNF-1α is expressed in both endocrine cells (α, β, δ and PP cells) and exocrine cells in the pancreas from the early development stage. Heterozygous mutations in gene encoding HNF-1α leads to MODY3, which is characterized by impaired insulin secretion by pancreatic β cells (Yamagata et al., 1996). HNF-1α can activate GLUT2 and L-type pyruvate kinase (PKL), a rate-limiting enzyme of glycolysis, through association with their promoter sequences. HNF-1α can also modulate mitochondrial metabolism through regulating gene expression of the mitochondrial 2-oxoglutarate dehydrogenase (OGDH) E1 subunit. Moreover, HNF-1α manipulates β-cell growth and death through affecting gene transcription of cyclin E, p27KIP1, Bcl-XL and IGF-1. HNF-1α can upregulate gene expression of HNF-4α in pancreas, implicating HNF-4α as a target of HNF-1α. HNF-1α can regulate the gene expression of E-cadherin, indicating the involvement of HNF-1α in cell adhesion and organization within pancreatic islets.

HNF-4α is a member of the steroid hormone receptor superfamily and can form a homodimer to target downstream genes. Loss of HNF-4α mutaions leads to MODY1, which is characterized by defective insulin secretion from pancreatic β cells (Yamagata et al., 1996). HNF-4α, as well as HNF-1α, can regulate GLUT2, PKL, OGDH E1genes.

HNF-1β, another homeodomain-containing transcription factor, is associated with MODY5 (Ek et al., 2001). HNF-1β can form homodimer itself or heterodimer with HNF-

1α to regulate downstream gene expression. The clinical features of MODY5 include diabetic symptom, and nondiabetic renal dysfunction.

Collectively, transcription factors play critical roles in control of glucose homeostasis by regulating the expression of several key genes involved in maintenance of normal β-cell function. Understanding the network of transcription factors as well as the molecular basis for regulation of target gene expression will provide deeper insight into the mechanisms leading to β-cell dysfunction.

7. Development of type 2 diabetes

Type 2 diabetes is the most common metabolic disease in the world. In the United States, it is the leading cause of blindness, end-stage renal disease, and nontraumatic loss of limb (Muoio and Newgard, 2008). Of even greater concern, type 2 diabetes is rapidly becoming a global pandemic and is projected to affect more than 300 million individuals worldwide by the year 2020 (Muoio and Newgard, 2008). Type 2 diabetes is characterized by defective pancreatic beta cell insulin release in response to glucose and by impaired insulin action on its target tissues (Weyer et al., 1999). Although the primary cause of the disease is unknown, it is clear that insulin resistance plays a role in the early stage of pathogenesis and that defects in insulin secretion by pancreatic β-cells are instrumental in progression to hyperglycemia (Weyer et al., 1999). In human type 2 diabetes, loss of glucose-sensitive insulin secretion is an early pathogenetic event and crucial in the development of the diabetes since correction of this defect by, for example, allosteric activators of glucokinase in islets can reverse the diabetic phenotype in rodents (Postic et al., 1999).

Insulin resistance is a common feature of several metabolic diseases, such as obesity, hypertension and glucocorticoid excess, but many patients with marked insulin resistance do not develop frank diabetes (Prentki and Nolan, 2006). Pancreatic β cells initially compensate for the insulin resistence associated with obesity and aging by increasing secretion of insulin. This adaptation involves expansion of β-cell mass, as well as maintenance of normal responsiveness of β-cells to glucose. Conversely, in individuals destined to develop type 2 diabetes, β-cells do not secret enough insulin to compensate for the increased demand. This β-cell failure is likely caused by inadequate expansion of the β-cell mass and/or failure of the existing β-cell mass to respond to glucose. Multiple factors, including free fatty acid (FFA) signaling, increased islet glucose metabolism, sensitivity to the incretin hormone GLP-1 and elevated parasympathetic neurons innervation, are indicated to promote islet compensation (Prentki and Nolan, 2006). In addition, upregulation of insulin biosynthesis is required to ensure improved β-cell function for islet compensation. Insulin biosynthesis is governed by complex interaction between downstream signaling molecules initiated from nutrient and hormonal stimuli (e.g., phospho-Erk1/2 and phospho-Akt), transcription factors, and binding elements on the insulin promoter (e.g., A1, C1, and E1 sites) (Prentki and Nolan, 2006).

Among the total population of type 2 diabetic patients, maturity onset diabetes of the young (MODY) accounts for only 1-2%. Inherited susceptibility to type 2 diabetes is believed to be accumulation of a cluster of genetic defects in either transcriptional factors important for β-cell development and function or fundamental components of insulin granule secretory machinery.

8. Combination of *in vivo* knockout model with *in vitro* gene silencing methodology

Gene-targeting technology through homologous recombination provides a powerful means to dissect the physiological function of a specific gene in the whole organism (Evans, 2001; Sauer, 1998). Gene targeting use targeting vectors in which the gene of interest has been disrupted by selectable markers to identify cells that are correctly targeted. Once selected, targeted cells are subsequently injected into blastocysts and injected embryos are implanted into pseudopregnant foster mothers to identify ES-cell-derived gametes. The problem of the conventional gene targeting technology is that null mutations in the germ-line are lethal in most cases, which precludes further analysis of gene function in adult tissues or cells. This problem was resolved with the generation of the first knockout mice by Thompson and colleagues. The strategy for making conditional gene knockout animals has taken advantage of the Cre/loxP system from bacteriophage P1 and the Flp/FRT system from S. cerevisiae. The site specific DNA recombinases, Cre and Flp, catalyze the deletion of gene sequences flanked by two loxP or frt sites, the recognition sequences for Cre or Flp, respectively (Sauer, 1998). The controlled recombinase expression driven by a tissue-specific promoter will allow inactivation of a gene of interest spatially in a tissue-specific manner and/or temporally by using Cre under the control of drug-inducible promoters. This Cre/loxp conditional knockout technology has been proven efficient and become increasingly popular in the field of transgenic study (Rossant and McMahon, 1999). There are over 100 transgenic Cre mouse lines generated (http://www.mshri.on.ca/nagy/Cre-pub.html). In addition, it is

practical to provide Cre by adenovirus infection, which is particularly applicable for deletion of alleles in the liver.

To delineate the precise expression pattern and action of the Cre recombinase in individual conditional knockout animal model, a reporter line was generated with reporter genes, β-galactosidase gene (lacZ), knocked into a defined genomic location of Rosa26 and flanked by two loxP sites. By breeding reporter line with Cre transgenic mice, a specific lacZ expression mode can be formed and detected by the X-Gal staining to reveal the Cre recombinase expression pattern.

Although conditional gene knockout technique has been traditionally used to analyze signaling pathways *in vivo*, it still remains a laborious method and its application is limited by the availability of Cre transgenic mice and potential leakage of Cre expression. RNA interference (RNAi) RNA interference has evolved into a powerful research tool for specific gene silencing of target genes in which 21-23 nucleotide small interfering RNAs work as sequence-specific RNAi mediators. RNAi was originally discovered in *Caenorhabditis elegans* as a means to interfere gene expression by transfection of gene-specific double-stranded RNA sequences (Fire A, et al. 1998 nature). Nowadays, RNAi methodology has been developed to apply in mammalian cells *in vitro* and whole animals, and also as a potential variant of gene therapy (Czauderna et al., 2003; Gaither and Iourgenko, 2007).

Combination of *in vivo* conditional gene knockout technology with *in vitro* gene silencing methodology appears to be powerful research tool to dissect function of specific gene in the pathogenesis of disease and holds promising therapeutic potential to develop gene therapy of diseases.

9. Objectives of the dissertation

Multiple signaling pathways are involved in mediating glucose-stimulated insulin production and secretion in β-cells. However, one critical and unanswered question is how these different pathways are coordinately regulated in β-cell response to glucose and insulin signals. Shp2 has been implicated in various pathways activated by receptor tyrosine kinases (RTKs) or cytokine receptors as a positive or negative regulator in different cell types. In particular, Shp2 has been found to bind tyrosine-phosphorylated IRS proteins and is involved in insulin-regulated glucose metabolism in insulin responsive tissues (Kuhne et al., 1993). The objective of my thesis research is to investigate whether Shp2 plays a biological role in insulin-producing pancreatic β cells. Inactivation of Shp2 in a spatially- and/or temporally controlled manner was achieved by crossing $Shp2^{flox}$ allele with mice carrying Cre under the control of the pancreatic-specific pancreatic and duodenal homeobox 1 (Pdx-1) promoter.

In this study, I initially used the well-known rat insulin 2 promoter (RIP) Cre driven transgenic line to generate β-cell specific Shp2 knockout mice (Gannon et al., 2000; Kulkarni et al., 1999; Lee et al., 2006), but the RIP-Cre expression is also detectable in hypothalamus and leads to partial hypothalamic knockout of the Shp2 gene. Shp2 deletion in hypothalamus was reported to induce early-onset obesity and hyperinsulinemia, which precludes the assessment of β-cell Shp2 deletion on insulin secretion and glucose homeostasis. The application of Pdx-1 Cre enables deletion of Shp2 in the pancreas without affecting its expression in the brain. Pdx-Cre mediated recombination exhibits a unique expression pattern (Gannon et al., 2000). In the early

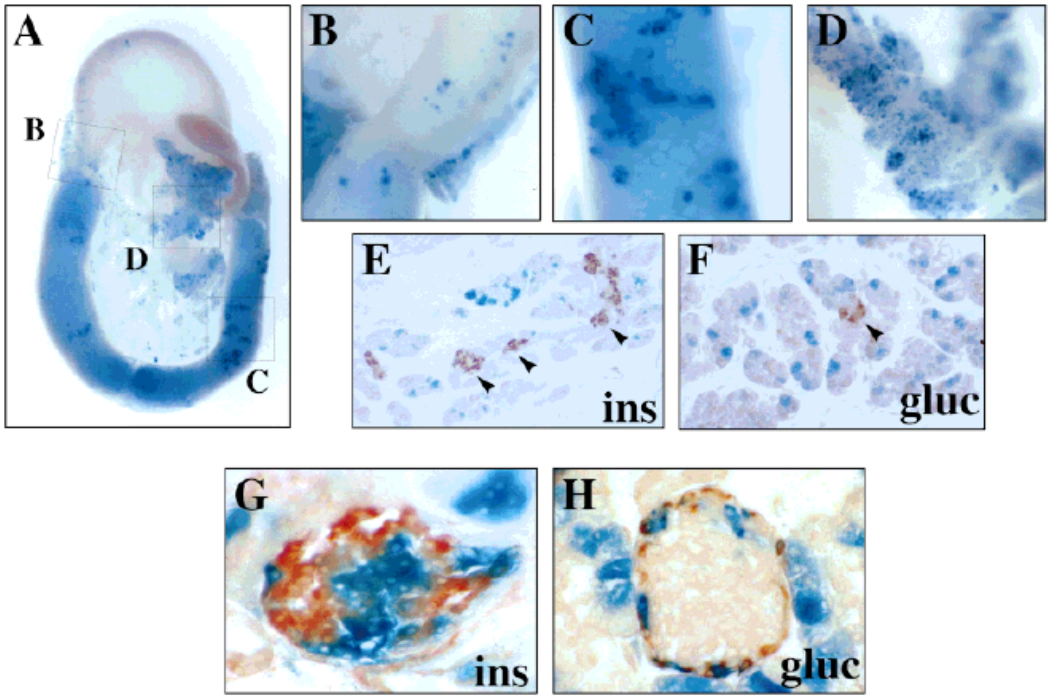


Figure 4. Cre-mediated recombination within the pdx-1 expression area.
(A) Neonatal digestive tract stained with X-gal in whole mount. Boxes indicate regions magnified in B–D. Dark blue shows regions of β-gal activity. (B) antral stomach. (C) Duodenum. (D) Splenic lobe of pancreas. (E,F) Sections of neonatal pancreas immunostained (brown) with insulin (E) or glucagon (F) to highlight islets (arrowheads). (G,H) Sections of islets from 3-week-old mouse.
Taken from Gannon et al. **"**Mosaic Cre-mediated recombination in pancreas using the pdx-1 enhancer/promoter". Genesis. 2000 Feb;26(2):143-4.

stage of embryonic development, Pdx-1 promoter driven Cre recombinase is detected in the entire pancreatic epithelium (Figure 4A), including antral stomach (Fig. 4B), duodenum (Fig. 4C) and pancreas (Fig. 4D). The β-galactosidase (β-gal) activity is extremely low in endocrine cells, whereas slightly higher in exocrine (Figure 4E and 4F). In adult pancreas, the recombination is elevated and can be detected in both center (Figure 4G) and boundary islet area (Figure 4H) (Gannon et al., 2000).

To dissect Shp2 function specifically in pancreatic β cells, I take advantage of recently developed gene silencing technology to interrupt Shp2 gene expression in INS-1 832/13, a robustly glucose-responsive rat insulinoma cell line. Combination of conditional gene targeting approach with gene silencing methodology enables us to explore a novel and crucial role of Shp2 in the regulation of β-cell insulin secretion and glucose homeostasis, as reported in this thesis.

II. Methods and Materials

1. Animals and genotyping.

Mice were maintained in a virus-free, normal 12-hour light/dark cycle with free access to water and standard mouse chow food (4% fat, RM1; Special Diet Services) and housed in specific pathogen-free barrier animal facility. All protocols for animal use and euthanasia were approved by Burnham institutional animal committee. All results presented were obtained from knockout mice and appropriate littermate controls. Generation of Shp2flox mice was reported previously (Zhang et al., 2004). Mice deleted for Shp2 selectively in pancreas ($Shp2^{panc-/-}$) were generated by crossing Pdx-1 Cre transgenic mice (kind gift of F. Levine) to Shp2 flox/flox mice on the mixed genetic background (C57BL/6 X ICR) (Gu et al., 2002).

Genotyping of mice was performed by PCR analysis on tail genomic DNA. For quick genotyping, tail samples of 3- to 4-week-old mice were boiled in basic denaturing reagent A (25mM NaOH and 0.2 mM EDTA) at 100 °C for one hour, and then neutralized in reagent B (40mM Tris-HCl pH 4.5). After brief vortex and Spin in microfuge at top speed 5 minutes, the supernatant was transferred to new microfuge tube and ready for PCR analysis. For higher quality of tail DNA sample, genomic DNA was extracted from tail biopsy using DNA digestion buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl and 1% SDS) with proteinase K added to 0.5 mg/ml final concentration. Tails were digested overnight at 50-55 °C with gentle shaking, and the lysates were ready for PCR analysis after adding 0.7 ml neutralized phenol/chloroform/isoamyl alcohol (25:24:1). For $Shp2^{flox}$ genotyping, we used primers that flank the exon 4 loxP site: loxP forward, 5'-ACGTCATGATCCG CT- GTCAG-3'

and loxP reverse, 5'- ATGGGAGGGACAGTGCAGTG-3'. For Cre detection, we performed genotyping with a pair of primers as following: CreA, 5'-GCC-TGCATTACCGGTCGATGCAACGA-3' and CreB, 5'-GTGGCAGATGGCGCGGCA-ACACCATT-3'. The PCR parameters include an initial denaturation step at 95°C for 5 min, followed by 30-40 amplification cycles of 94 °C for 30 sec, 55-70°C for 30 sec and 72 °C for 30-60 sec.

2. Metabolic studies.

All blood glucose measurements were determined on whole venous blood by using an automatic glucose monitor (One touch basic, Lifescan) (Zhang et al., 2004). Blood collection was restricted from 10 am to 11 am. For serum collection, the blood samples were put at room temperature for 30 minutes and then spun in centrifuge at 3400rpm for 20-30 minutes. The supernatant containing pure serum was used immediately for experiments or stored at -80°C in aliquots.

Body weights were measured by using a Sartorius BP610 balance. Serum insulin levels were determined by ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) using a mouse insulin standard (Crystal Chem Inc.) (Ueki et al., 2006). The Rat Insulin ELISA is a solid phase two-site enzyme immunoassay. ELISA is based on the direct sandwiched-complex technique in which two monoclonal antibodies directly bind to separate antigenic determinants on the insulin molecule. During incubation, insulin in the sample reacts with peroxidase conjugated anti-insulin antibodies and anti-insulin antibodies bound to the microplate well, and the unbound materials were removed by washing. After addition of horse radish peroxidase (POD)-conjugated secondary (anti-

guinea pig IgG) antibody and removal of excess POD-conjugate, the bound conjugate is detected by reaction with the enzyme substrate (3,3', 5,5'-tetramethylbenzidine). The reaction is stopped by adding acid to give a colorimetric endpoint result by using spectrophotometer.

Glucose tolerance tests were performed on animals after 16-hour overnight fasting. We injected intraperitonealy (IP) with D-glucose (2 g/kg body weight), and then obtained blood samples before and 15, 30, 60, 120 minutes post IP glucose injection. For acute insulin release measurement, blood samples were collected in heparinized tubes before (time 0) and 2 and 5 minutes after glucose (3g/kg body weight) IP injection. Statistical analysis of the data was performed by using a two-tailed unpaired student's *t*-test.

3. Islet isolation and insulin content measurement.

Mice were sacrificed, the common bile duct was cannulated with 27mm needle in the anterograde direction, and its duodenal end occluded by clamping (Kulkarni et al., 2002). We subsequently injected collagenase P (2-3mg/ml) into the duct to distend the pancreas. The pancreas was removed and incubated at 37°C for 15-24 minutes, then put in 10 ml of HBSS to dilute enzyme and shaken vigorously to break tissue completely. Cellular components were obtained by centrifugation (220 g for 1 minute) and resuspended in 10 ml of RPMI 1640 medium. We handpicked islets under light microscope and washed the islets once in RPMI 1640 medium. Islets were spun in centrifuge at 15,600 g speed for 5 minutes and cultured in RPMI 1640 supplemented with 10% FBS overnight. After incubation, islets can be used for experiments immediately or stored at –80°C for DNA or protein extraction.

We measured insulin content in islets isolated from knockout mice and littermate controls in acid-ethanol extracts using ELISA kit (Crystal Chem Inc.).

4. Islet morphology and Immunohistochemistry.

Mice were sacrificed and pancreas were dissected, cleared of fat tissues and lymph nodes immediately. After rinsed in PBS, pancreas were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) for 18 hours and embedded in paraffin. Consecutive 5-µm-thick sections were cut from the tissue and mounted on glass slides. The sections were stained with hematoxylin and eosin stain (H&E) to observe pancreatic structure and islet morphology (Kulkarni et al., 1999).

For immunohistochemistry of paraffin-embedded sections, the slices were dewaxed by xylene and rehydrated by sequential treatment of ethanol with different concentration (100%, 95%, 70% and 50%) and ddH2O, followed by blocking in PBS containing 5% normal goat serum and 0.1% Triton X-100. Afterwards, the sections were incubated with primary antibody for 2 hours at room temperature or overnight at 4°C and secondary antibody for one hour at room temperature. Imaging was captured under a Leica fluorescence microscopy.

Sections of pancreas were stained with Glut2 (Chemicon, Temecula, CA) 1:200; Insulin (Santa Cruz Biotechnology, Santa Cruz, CA) 1:500; Shp2 (Santa Cruz Biotechnology, Santa Cruz, CA) 1:300; Pdx-1 (gift of C.V. Wright, Vanderbilt University) 1:500. The secondary antibodies included Alexa Fluor594 anti-rabbit IgG anf Alexa Fluor488 anti-mouse IgG (1:200 dilution, Molecular Probes).

5. Insulin+ β-cell quantification in pancreatic sections.

For quantitation of β cell area, the whole pancreas was serially sectioned to generate 5 μm-thick tissue sections and 25 representative sections per pancreas were used (Rulifson et al., 2007). At least 4 animals of each genotype were analyzed per genotype. Sections of paraffin-embedded pancreas were immunostained at 200-μm intervals to avoid measurement of the same islets twice. The deparaffinized sections were stained for insulin and a mixture of antibodies to glucagon, somatostatin, pancreatic polypeptide and β-amylase to detect insulin positive β-cells and non-β cells in pancreas. Images of β cells and of the entire pancreas were obtained under Leica fluorescence microscopy. For each section, the total area occupied by insulin-positive β cells and non-β cells was analyzed by using NIH ImageJ software. The β cell area was expressed as the percentage of the total pancreatic area surveyed.

6. RT-PCR and Quantitative real-time RT-PCR.

After isolation, total RNA was extracted from islets using RNeasy Mini Kit (Qiagen) following the standard protocol (Heit et al., 2006). For *in vitro* study, total RNA was isolated from INS-1 832/13 cells using Trizol (Invitrogen). In detail, Trizol was added to each sample based on the sample size, and homogenized at room temperature for 10 minutes. Then chloroform was added and mixed by inverting the tubes, followed by a brief centrifugation at 4°C. The upper aqueous phase including RNA was separated from the lower organic phase including DNA. To obtain high-quality RNA, the upper aqueous phase was filtered through RNAeasy spin column and washed with

Table 1. List of qRT-PCR Primers Used for Mouse Islets.

Glut2-F	CTGTCGGTAATTGGCATCCG
Glut2-R	GGCATCGACTGAGCAGAAGGT
Pdx1-F	ACCCGTACTGCCTACACCCG
Pdx1-R	GGGCCGGGAGATGTATTTGT
INS1-F	GCTGGTAGAGGGAGCAGATG
INS1-R	CAGAGACCATCAGCAAGCAG
INS2-F	GAAGTGGAGGACCCACAAGT
INS2-R	AGTGCCAAGGTCTGAAGGTC
GcK-F	AGTTCCTCCTGGAGTACGACCG
GcK-R	CCCATGTACTTTCCGCCAATG
Beta2-F	CGTCAGTTTCACTATTCCCG
Beta2-R	GCCTTCTGTAAACAGGACAGTCAC
Ccnd1-F	CTCTCCTGCTACCGCACAACG
Ccnd1-R	GCAGTCCGGGTCACACTTGAT
Ccnd2-F	CAAGCTGAAAGAGACCATCCCG
Ccnd2-R	CAGTTCCCACTCCAGCAGCTC
Cdk4-F	GCACAGACATCCATCAGCCG
Cdk4-R	CGTGAGGTGGCCTTGTTAAGGA
Shp2-F	CATGGCTGTCCAGCACTACA
Shp2-R	TGTCCTTTTCTTTTGCTTTTCTG
c-Myc-F	TTTGTCTATTTGGGGACAGTGTT
c-Myc-R	CATCGTCGTGGCTGTCTG

Table 2. List of qRT-PCR Primers Used for INS-1 832/13 Cells.

Glut2-F	TTTCTGTGCCGTCTTCATGT
Glut2-R	CCGTCATGCTCACATAACTCA
Pdx1-F	CTCTCGTGCCATGTGAACC
Pdx1-R	TTCTCTAAATTGGTCCCAGGAA
INS1-F	GACCTTGGCACTGGAGGTT
INS1-R	CCAGTTGGTAGAGGGAGCAG
INS2-F	CGAAGTGGAGGACCCACA
INS2-R	TGCTGGTGCAGCACTGAT
GcK-F	GCCCAGTTGTTGACTCTGGT
GcK-R	CATCACCTTCTTCAGGTCTTCC
Beta2-F	ACGCAGAAGGCAAGGTGT
Beta2-R	TTTGGTCATGTTTCCACTTCC
Hnf1α-F	CTCAGCACCAGTCCCACAG
Hnf1α-R	CGTTGGAGTCAGAACTCTGGT
Hnf4α-F	CAAGAGGTCCATGGTGTTCA
Hnf4α-R	CCGAGGGACGATGTAGTCAT
NFATc1-F	AGTTATGGCCAGACAGCACCATCT
NFATc1-R	TGTGCAGCTACACGGTTACTTGGA
MafA-F	CGAGTACGTCAACGACTTCG
MafA-R	AAGAGGGCACCGAGGAGCAG
Ccnd1-F	GCACAACGCACTTTCTTTCC
Ccnd1-R	TCCAGAAGGGCTTCAATCTG
Ccnd2-F	CACCGACAACTCTGTGAAGC
Ccnd2-R	CCACTTCAGCTTACCCAACAC
Cdk4-F	GTCAGTGGTGCCGGAGAT
Cdk4-R	GGATTAAAGGTCAGCATTTCCA
c-Myc	GCTCCTCGCGTTATTTGAAG
c-Myc	GCATCGTCGTGACTGTCG

ethanol-containing buffer and eluted in RNase-free water.

The extracted RNA was reverse-transcribed using SuperScript II reverse trancriptase (Invitrogen). Expression of knockout or WT allele of *Shp2* gene in islets was detected by RT-PCR by using DNA Engine® PTC-200 (MJ Research Corp). Quantitative real-time RT-PCR was performed using SYBR Green dye (Applied Biosystems) in a MX3000P Thermal cycler (Stratagen). Each reaction was carried out in triplicate and gene expression was normalized against Cph. Results are expressed as the mean +/- S.E.M. The primers used for real-time RT-PCR of mouse islet samples and INS-1 832/13 cells extraction are listed in Table 1 and Table 2, respectively.

7. Western blot analysis.

For protein isolation, mouse islets or INS-1 832/13 cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% Deoxycholate and 0.02% Sodium Azide) supplemented with protease and phosphatase inhibitors (1mM Na3VO4, 1mM PMSF, 10µg/ml aprotinin and leupeptin) and protein was quantified by standard techniques. For protein extraction of tissue samples, we used ice-cold tissue lysis buffer, containing 50 mM Tris–HCl, 0.15 M NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 µg/ml leupeptin, 2 µg/ml pepstatin A, 10 µg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, 1mM benzamidine, 0.1 mM benzethonium chloride, pH 8.0. 20 µg of protein was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane, blocked in 5% milk in TBST buffer (25mM Tris-HCl, Ph7.4, 137mM NaCl, 3mM KCl ad 0.1% Tween-20) for 1 hour, and probed for appropriate primary antibody at

4 °C overnight and HRP-conjugated secondary antibody at room temperature for 1 hour. Antibody signals were detected by enhanced chemiluminescence (ECL analysis kit, Amersham Corp.). For primary antibody, protein lysates were blotted with antibodies against phospho-Foxo1 (Ser256), Foxo1, phospho-Akt (Ser473), Akt, phospho-Erk1/2, Erk1/2 (Cell signaling Technology Corp.), Pdx-1 (gift of C.V. Wright, Vanderbilt University), Shp2 (Products of our laboratory), phosphorthreonine 172 AMPK (cell signaling Technology Corp.), AMPK (cell signaling Technology Corp.), Glut2 (Millipore), p-Stat3 (cell signaling Technology Corp.), Stat3, p-Stat5 (cell signaling Technology Corp.), Stat5, p-Smad 1/5/8, Cytochrome C (BD biosciences) and α-Tubulin (Sigma).

8. Immunoprecipitation.

After measurement of protein concentration, INS-1 832/13 cell lysates was diluted to 1μg/μl in HNTG buffer (20mM Hepes, pH7.5, 150mM NaCl, 0.1% Triton X-100, 10% glycerol, 1mM Na3VO4, 0,1mM ZnCl2, 1mM PMSF, 1 μg/ml leupeptin and 10 μg/ml aprotinin). Antibody for immunoprecipitation was subsequently added into 1mg cell lysates and incubated at 4°C overnight. Then protein A/G plus agarose (Santa Cruz) was added at 4°C for another 1-1.5 hours, and were washed three times with cold HNTG buffer. After centrifugation, 10μl 1 X SDS loading buffer was added to each immunocomplex. Samples of immunoprecipitates were immunoblotted with antibodies to analyzing the associated protein. The antibodies used for immunoprecipitation were Shp2 (Santa Cruz Biotechnology) and Sprouty 1 (Santa Cruz Biotechnology). The antibodies for subsequent immunoblots were IRS2 (Upstate), P85 (Cell signaling Technology Corp.),

Shp2 (Santa Cruz Biotechnology), Anti-Phosphotyrosine and Anti-Phosphotyrosine, clone 4G10 (Millipore).

9. Cell culture, Transfection and Immunocytochemistry.

INS-1 832/13 cells were cultured in regular RPMI containing 10% FBS, 10Mm HEPES, 1 mM soldium pyruvate, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, and 100 ug/ml streptomycin at 37 ^{o}C in a humidified (5% CO_2) atmosphere (Pagliarini et al., 2005). Shp2 expression was knocked down by using 200 pmol of Qiagen siRNA (sense: 5'-GGACUAUGACCU- CUAUtt-3', antisense: AUAGAGGUCAUAGUAGUCCtt-3') in an Amaxa nucleoporator with solution T on program T-20, which was tested to be the most efficient way to knockdown *Shp2* gene expression in INS-1 832/13 cell line. Oligofectamine and FuGENE 6 transfection reagent were also tested to transfect Shp2-siRNA, but the knockdown efficiency was very low.

We performed all the assays approximately 72 hours after siRNA treatment. For immunocytochemistry, cells were washed with PBS and fixed in 4%PFA for 15 min. After 3 washes with PBS, cells were blocked in 5% normal goat serum in PBS/Triton for 60 minutes and then incubated overnight with Insulin (Santa Cruz Biotechnology, Santa Cruz, CA) 1:500; Pdx-1 (gift of C.V. Wright, Vanderbilt University) 1:500; Shp2 (Santa Cruz Biotechnology, Santa Cruz, CA) 1:300; Foxo1 (Chemicon, Temecula, CA) 1:500 . The secondary antibodies included Alexa Fluor594 anti-rabbit IgG anf Alexa Fluor488 anti-mouse IgG (1:200 dilution, Molecular Probes).

10. In vitro insulin secretion assay.

INS-1 832/13 cells were seeded in 12-well culture plates after 72 hours siRNA treatment. After two washes with PBS, cells were starved for 2 hours with standard assay buffer (SAB: 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.16 mM MgSO4, 20 mM HEPES [pH 7.2], 25.5 mM NaHCO3, 10 mM CaCl2, and 0.2% BSA) to normalize insulin release (Pagliarini et al., 2005). Subsequently, the medium was replaced by SAB containing 3 mM glucose (for basal secretion) or high glucose (15 mM) to measure insulin secretion during 2 hour incubation. The supernatants were removed for measurements of secreted insulin, and the attached cells were extracted by acid-ethanol for determination of insulin content. Insulin was assayed by an RIA kit (Linco Research, St. Charles, MO). The results of insulin secretion were standardized for cell number.

11. Radioimmunoassay.

Rat Insulin Radioimmunoassay (RIA) Kit Linco Research, St. Charles, MO) is for the quantitative determination of Insulin levels in serum, plasma, and culture media. Radioimmunoassay is based on the antigen-antibody reaction in which tracer amounts of the radio-labeled antigen competes with the native antigen for limited binding sites of the specific antibody against the same antigen. For rat insulin RIA kit, ^{125}I is used as radio-isotope. Other important components of RIA are the specific guinea pig anti-rat insulin serum and pure rat insulin as the standard or calibrator.

In radioimmunoassay, a certain concentration of ^{125}I-labeled insulin in trace amounts is incubated with a fixed amount of insulin antibody. When unlabeled insulin, either as standard or test sample, is added to this system, there is competition between ^{125}I -labeled insulin and the endogenous insulin antigen for the limited constant number of

binding sites on the guinea pig insulin antiserum. After optimal incubation, ^{125}I-labeled antigen bound to antibody is separated from unbound ^{125}I-labeled insulin. After separating antibody-bound ^{125}I-labeled insulin from free ^{125}I-labeled insulin, the bound or free fraction is counted for ^{125}I using a γ counter with appropriate settings. A standard curve is set up with increasing concentrations of standard unlabeled ^{125}I and from this curve the amount of antigen in tested samples can be calculated.

12. Mitochondrial fraction.

INS-1 832/13 cells were washed with PBS and lysed in ice-cold mitochondrial isolation buffer (65 mM sucrose, 215 mM mannitol, 5 mM KH2PO4, 5 mM KHCO3, 3 mM MgCl2, 5 mM HEPES [pH 7.4]). Cells were spun and lysates were made from the resuspended pellet in a 2ml glass Dounce homogenizer. Lysates were centrifuged for 3 min at 1,800 rcf, and the supernatant was centrifuged at 10,800 rcf for an additional 3 min. The mitochondria-enriched pellet was resuspended in isolation buffer, and low- and high-speed spins were repeated (Kibbey et al., 2007).

13. ATP determination assay.

After 72 hours siRNA treatment, cells were starved for 2 hours with standard assay buffer (SAB) containing 3mM glucose and then treated with low (3mM) or high (15mM) glucose SAB for another 2 hours, then lysed in lysis buffer (10 mM Tris pH 7.5, 0.1 M NaCl, 1 mM EDTA and 0.01% Triton X-100). After centrifugation, the supernatant was obtained for quantitative ATP determination using ATP Determination Kit (Molecular Probe). All the results of ATP were standardized for protein.

14. Chromatin immunoprecipitation (ChIP) assay.

INS-1 832/13 cells were fixed with formaldehyde (1%, v/v) (sigma) for 10min at room temperature followed by washing with PBS twice. Stop solution (0.1M Tris.Cl pH 9.4, 0.01M DTT) was then added. The cells were collected and washed with PBS, and then lysed in 300 ul lysis buffer (1% SDS, 0.01M EDTA, 0.05M Tris.Cl pH 7.8) containing protease inhibitor cocktail (Roche) for each 10 cm^2 plate. The samples were incubated on ice for 10 min and sonicated until DNA fragments get to around 500bp in length, which were verified through electrophoresis (Garcia-Bassets et al., 2007). Following centrifugation at 14000rpm for 10 min at 4 °C, the supernatant was divided into two tubes and made up to 1.5 ml with ChIP dilution buffer (1% (v/v) Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris/HCl, pH 7.8) containing protease inhibitors.5 ul of each sample was saved for input DAN. Equal amount of normal rabbit IgG (Santa Cruz Biotechnology) or anti-Pdx1 antibody (Millipore) was added to each tube and the samples incubated at 4 °C overnight with rotation. Protein A slurry (20 µl) (Sigma) was added and the samples were further rotated at 4 °C for 4 h. The agarose was pelleted by centrifugation at 4 °C for 1 min at 1000 *g* and the supernatant was discarded. The agarose pellet was washed once in TSE I buffer (0.1% SDS, 1% Triton X-100, 4 mM EDTA, 20 mM Tris/HCl, pH 8.0, and 150 mM NaCl), once in TSE II buffer (0.1% SDS, 1% Triton X-100, 4 mM EDTA, 20 mM Tris/HCl, pH 8.0, and 500 mM NaCl) and twice in TE buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0). Input samples and the immunoprecipitated DNA-protein complex were de-crosslinked and eluted by incubating the agarose pellet in elution buffer (1% SDS in TE buffer) at 65 °C over night. The DNA

Table 3. List of Primers Used for Anti-Pdx-1 CHIP

INS1-F	ACTGCTTCATCAGGCCATCT
INS1-R	AGGAGGGGTAGGTAGGCAGA
INS2-F	ACCCAGGAGCCCC TATT
INS2-R	ACCCAGGAGCCCC TATT
GAPDH -F	TGAGAGAGGCCCAGCTACTC
GAPDH -R	GAACAGG- GAGGAGCAGAGAG

was then extracted by using QIAquick Spin Columns (QIAGEN) following manufacture standard protocol.

Q-PCR was then performed on a Mx3000P machine from Stratagene. PCR conditions were: 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s, and finally 1 cycle at 95 °C for 1 min, 55 °C for 30 s, 95 °C for 30 s. The primers used were listed in Table 3.

15. CyQuant Cell Proliferation Assay.

INS-1 832/13 cells were seeded in 96-well culture plates after siRNA treatment. At days 2, 3, and 4, cell culture medium was removed and the cells were frozen at -80 °C until the cell-counting assay. Cell growth was analyzed by using the CyQuant cell proliferation assay kit (Molecular Probes), based on the use of CyQuant GR dye, which exhibits strong green fluorescent intensity after association with cellular nucleic acids. Frozen cells were simply thawed and lysed by addition of a buffer containing the CyQuant GR dye (200 μl/well) and incubate at room temperature for 5 minutes. Fluorescence was then measured by using a fluorescence microplate reader (Fluorocount Packard) with 480-nm excitation wavelength and 520-nm emission wavelength. Data were expressed in percentage compared with control.

16. In situ cell death (apoptosis) detection by terminal transferase dUTP nick end labeling (TUNEL) Assay.

To examine β-cell apoptosis in pancreas of Shp2 knockout mice and littermate controls, we performed in situ cell death (apoptosis) detection by TUNEL assay on

paraffin-embedded pancreatic sections with the application of cell death detection kit (Roche). TUNEL assay is for measuring and quantitating cell apoptosis by labeling DNA strand breaks in individual cells by flow cytometry or fluorescence microscopy. In principle, optimized terminal transferase (TdT) is used to label free 3'OH ends in genomic DNA with fluorescein-dUTP or TMR-dUTP.

Firstly, paraffin sections was dewaxed by putting slides in Xylenes for 10 minutes twice and in series of ethanol with different concentration (100% EtOH, 5 minutes, twice; 95% EtOH; 70% EtOH; 50% EtOH), and rinsed in ddH2O and PBS. Then 100µl TUNEL reaction mixture containing TdT and fluorescein-dUTP or TMR-dUTP was applied for each slide and incubated in humid chamber at 37°C for 60 minutes. Fluorescein-conjugates facilitate the analysis by fluorescence microscopy to detect early stage of DNA fragmentation in apoptotic cells.

17. Plasmid construction and tranfection.

Mouse Pdx-1 cDNA was PCR amplified from the plasmid pZL1-Pdx-1 (kind gift of Dr. Christopher Wright, Vanderbilt University) using primers 5'-ACGCGGATCCATGAACAGTGAGGAGCAGTA-3' (BamH I) and 5'-ACCGCTCGAGTCACCGGGGTTCCTGC-3' (Xho I), and subcloned into pCMV-Tag2B (Flag), thus creating the plasmid pCMV-Tag2B-mPDX1. The correct pCMV-Tag2B-mPDX1 recombinant clones were selected and confirmed by sequencing. Three groups of INS-1 832/13 cells were transfected with Shp2-siRNA+empty vector, Shp2-siRNA+ pCMV-Tag2B-mPDX1, and scramble-siRNA+empty vector by an Amaxa nucleoporator with solution T on program T-20. Immunoblot analysis of Flag tag was

performed to determine the efficiency of transfection. After 72 hours transfection, cells were harvested and were extracted by acid-ethanol for determination of insulin content. Insulin content was assayed by ELISA with a rat insulin standard (Crystal chem). The results of insulin content were standardized for protein concentration. Statistical analysis of the data was performed by using a two-tailed unpaired t-test.

18. Statistical analyses.

Experimental data are represented as mean ± SEM. Statistical analysis was performed by the two-tailed Student's *t*-test. Differences were considered significant when $P < 0.05$, very significant when $P < 0.01$, and highly significant when $P < 0.001$.

III. Results

1. Generation of Pdx1-Shp2KO mice

Since the homozygous disruption of Shp2 gene in mice resulted in embryonic lethality at mid-gestation (Saxton et al., 1997), our laboratory has generated a conditional Shp2 knockout allele, $Shp2^{flox/fllox}$, in mice using the Cre-loxP system (Zhang et al., 2004). Success of this work allows us to study Shp2 function in a tissue-specific manner. To produce a conditionally targeted allele, two loxP sites were introduced into introns flanking exon 4, which encodes amino acid 111-176. Additionally, a targeting construct, with neomycin-resistant (neo^R), thymidine kinase (TK), and diphtheria toxin (DT-A) genes are used as selective markers (Figure 5A). Deletion of exon 4 causes a frame-shift mutation and creates a stop codon TAG, which gains an advantage over previously reported Shp2 mutant model with removal of either exon 2 or exon 3 deletion and generation of truncated proteins of Shp2. $Shp^{flox/flox}$ mice were then crossed with mice carrying Cre under the control of pancreas-specific Pdx-1 promoter. The Pdx1-Cre transgenic line was originally from Dr. Douglas A. Melton laboratory. The Pdx-1 promoter driven Cre is exclusively expressed in pancreatic progenitor cells from embryonic day 8.5 and in mature β cells and δ cells (Figure 5B). $Shp^{flox/flox}$ mice were bred with Pdx1-Cre transgenic mice to generate $Shp2^{flox/+}$: Cre/+ animal, which is then mated with $Shp2^{flox/fllox}$ to create pancreas-specific Shp2 knockout mice ($Shp2^{flox/fllox}$: Cre/+). In the following content, these conditional Shp2 knockout animals are designated $Shp2^{panc-/-}$ mice.

Both $Shp2^{panc-/-}$ mice and littermate controls were born at the expected mendelian frequency, survived to adulthood, and were fertile. The protein expression level of Shp2

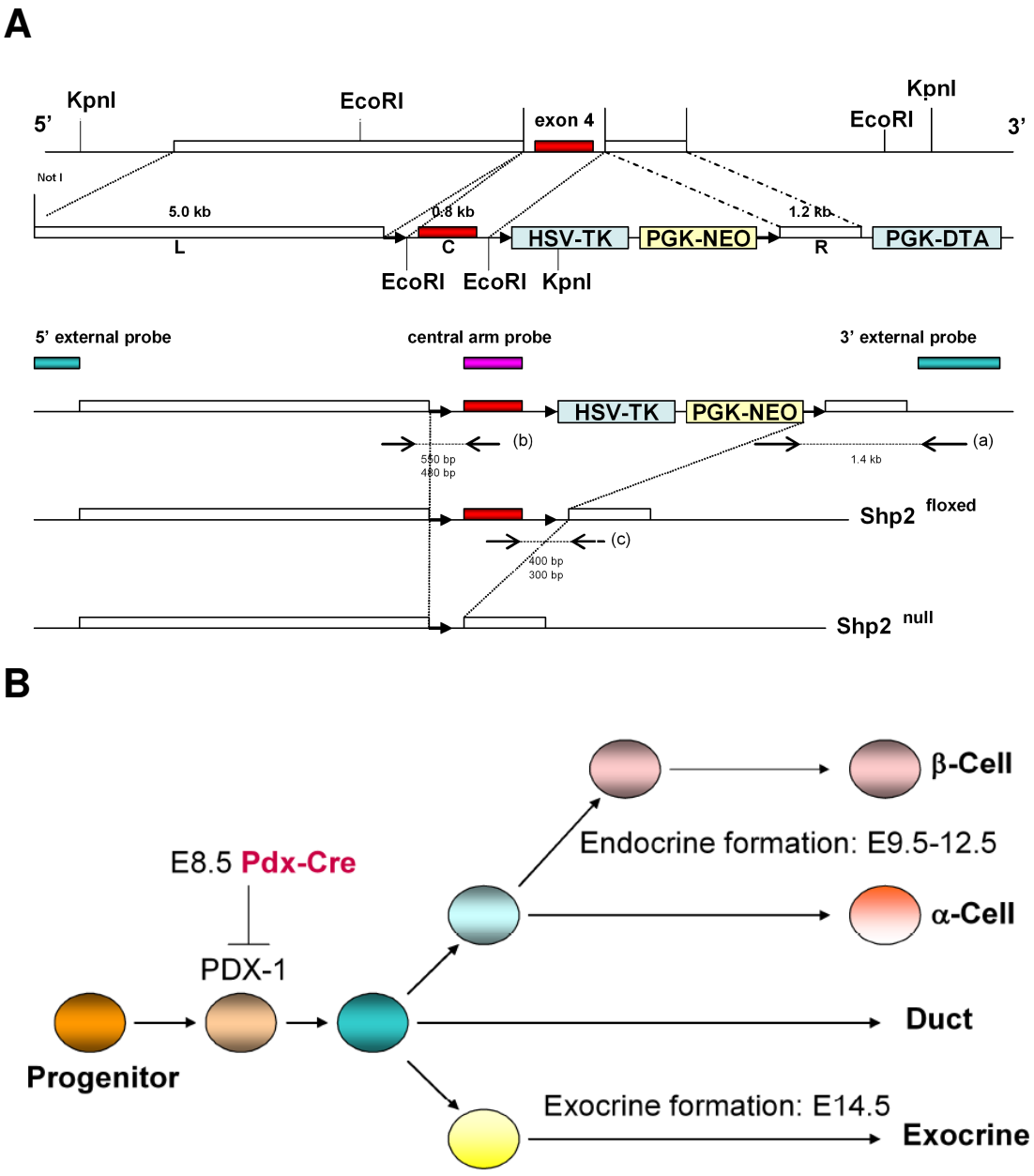


Figure 5. (A) Gene Strategy for generation and detection of the Shp2^{flox} allele. Adapted from Zhang et al. "Neuronal Shp2 tyrosine phosphatase controls energy balance and metabolism". Proc Natl Acad Sci U S A. 2004 Nov 9;101(45):16064-9. Epub 2004 Nov 1. (B) Simplified model of pancreatic development. Shp2 $^{panc-/-}$ animals are derived from Pdx-1 driven Cre transgenic mice, in which Cre expression starts from E8.5.

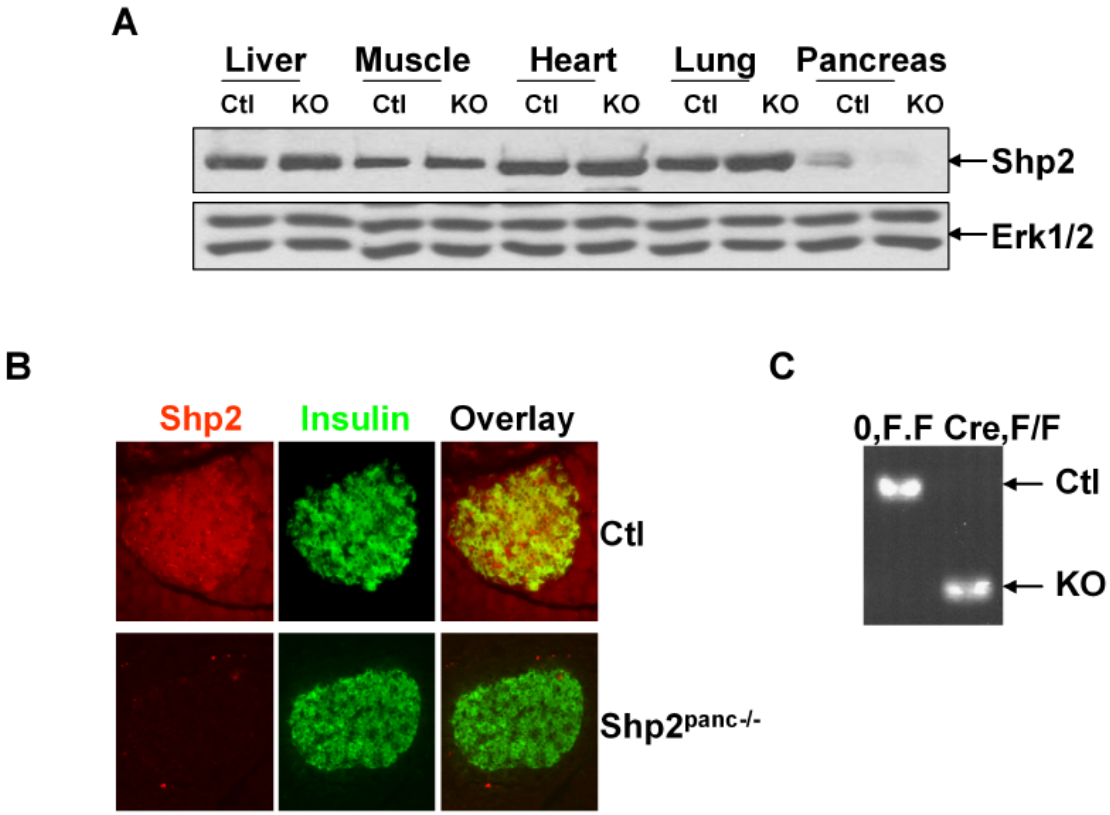


Figure 6. Generation of pancreatic specific Shp2 knockout (*Shp2*$^{panc-/-}$) mice. (A) Immunoblot for expression of Shp2 gene in pancreas, liver, muscle, heart and lung dissected from *Shp2*$^{Panc-/-}$ (Cre/+, Shp2$^{flox/flox}$) mouse and control (Shp2$^{flox/flox}$) mouse. (B) Immunofluorescence for insulin (green) and Shp2 (red) and overlay in pancreatic sections from 3-month-old *Shp2*$^{Panc-/-}$ and control mice. (C) RT-PCR analysis of RNA extraction from handpicked pancreatic islets of 1-month-old *Shp2*$^{Panc-/-}$ and control mice. Shp2 KO allele was detected in islets from *Shp2*$^{Panc-/-}$ mice.

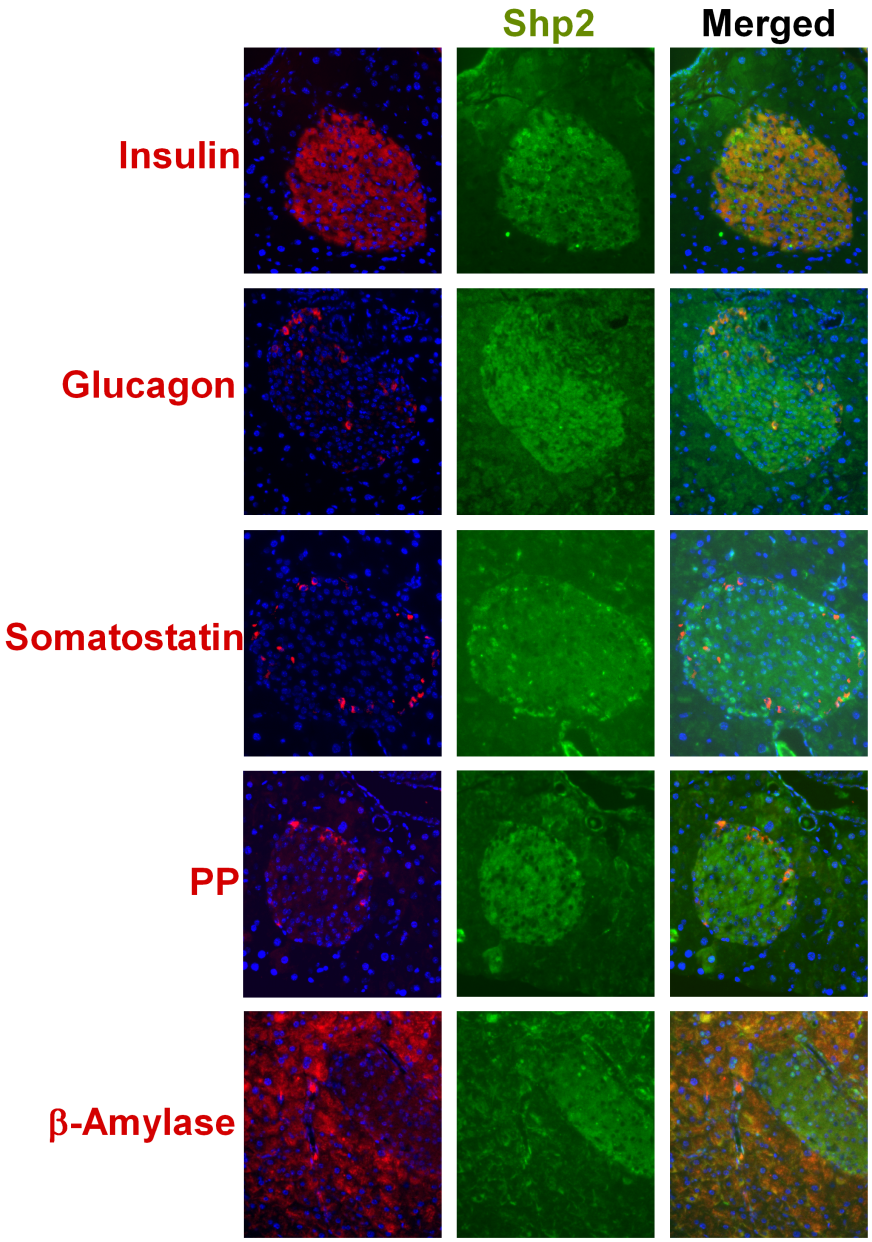


Figure 7. Detection of Shp2 expression in different cell types in the whole pancreas.
Shp2 staining (green) overlapped with staining of insulin (red), glucagons (red) and β-amylase (red), but not with pancreatic peptide (PP) (red) and somatostatin (red).

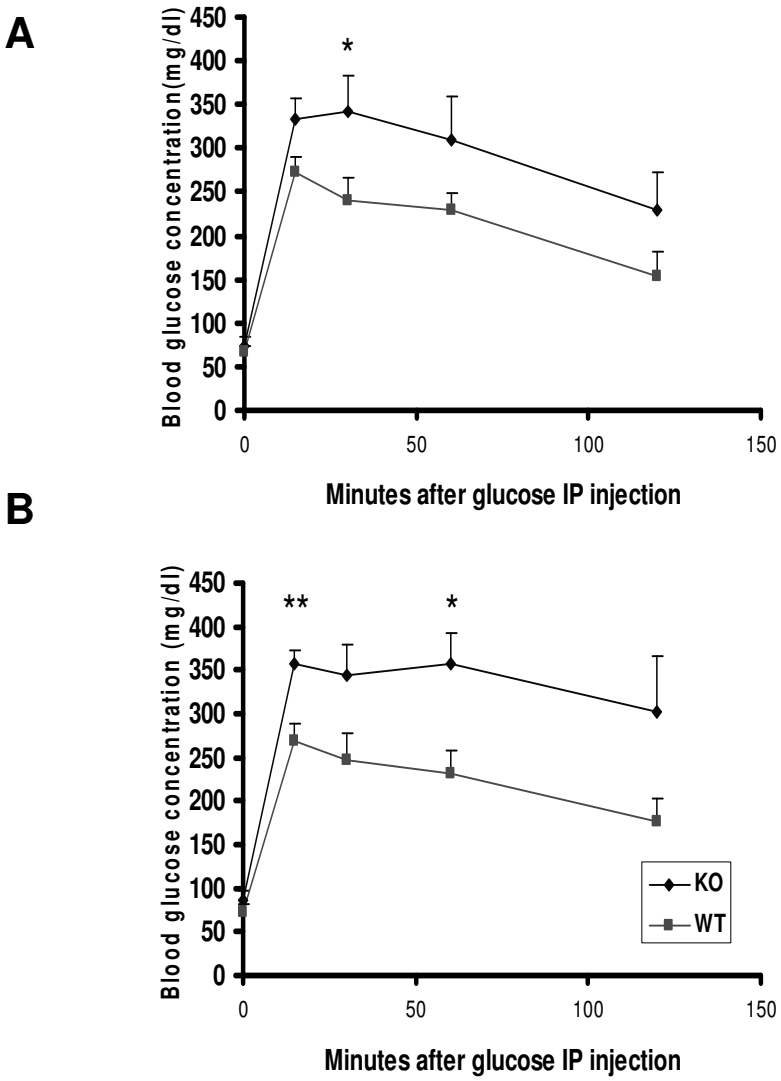


Figure 8. Blood glucose levels after i.p. injection of glucose (2g/kg body weight) in 3-month-old (A) and 10-month-old (B) male *Shp2*$^{Panc-/-}$ and control mice. (n=6 per group).

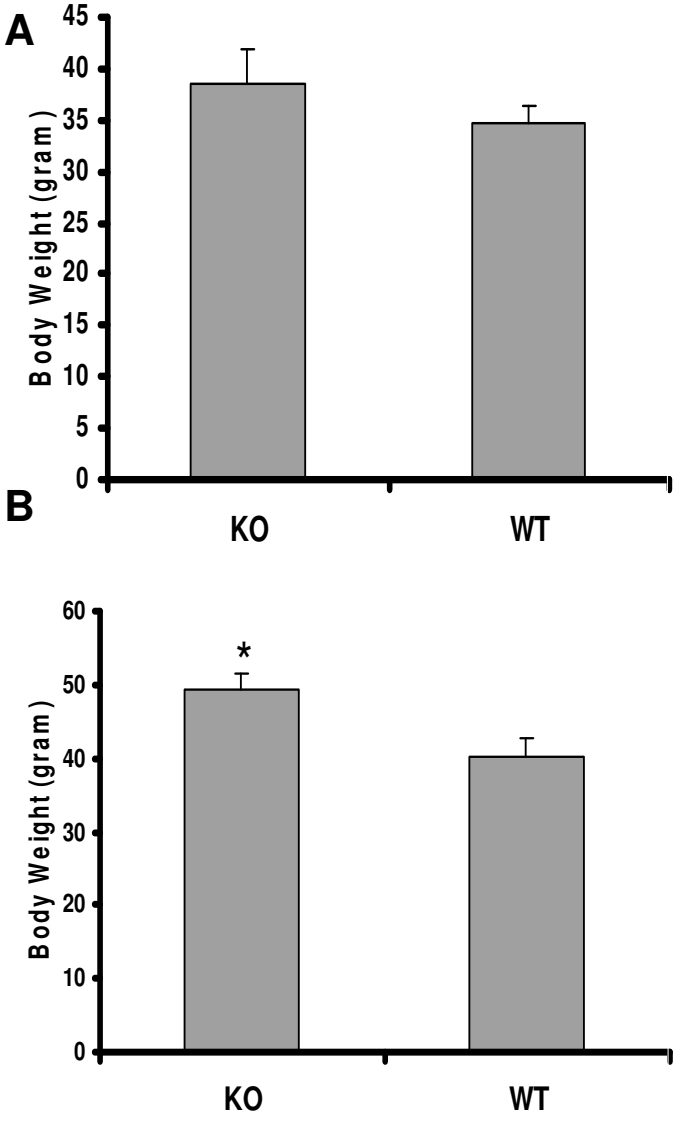


Figure 9. Body weight of 3-month-old (A) and 10-month-old (B) male *Shp2*$^{Panc-/-}$ and control mice. (n = 6 per group).

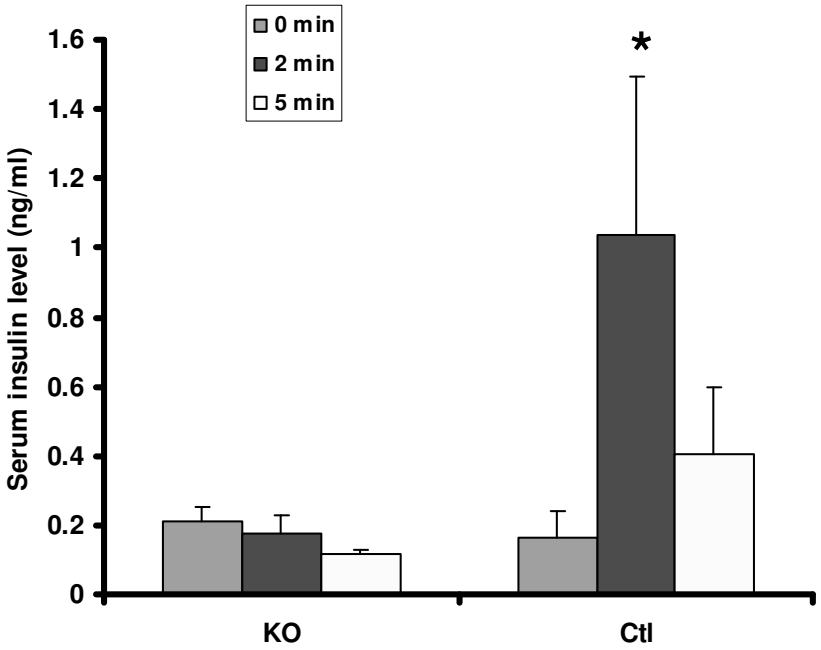


Figure 10. Acute-phase insulin secretion after injecting glucose (3 g/kg body weight) intraperitonally in 8-month-old *Shp2*$^{Panc-/-}$ and control mice.

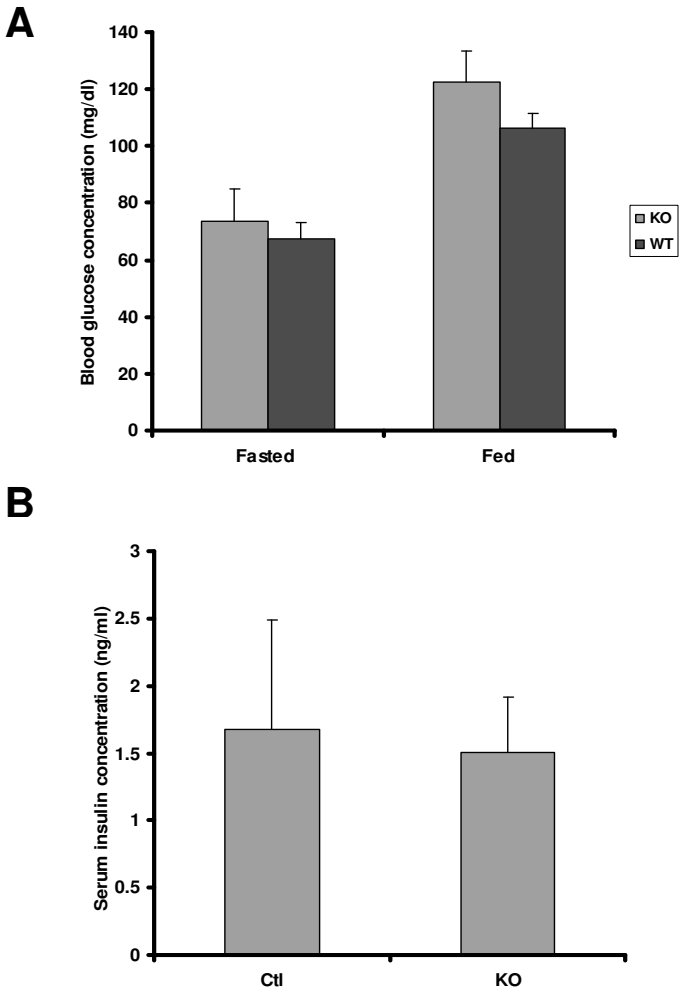


Figure 11. (A) Random-fed blood glucose levels and fasting blood glucose levels in 3-month-old *Shp2*$^{Panc-/-}$ and control mice. (n=8 per group). (B) Serum insulin levels in random-fed 3-month-old *Shp2*$^{Panc-/-}$ and control mice. (n=11 per group).

in pancreas dissected from $Shp2^{panc-/-}$ mice was significantly reduced compared with control, whereas no difference was detected in liver, muscle, heart and lung (Figure 6A).

Shp2 was found to be abundantly expressed in insulin positive β-cells in pancreatic section from control animal by immunofluorescence analysis, whereas the expression level of Shp2 in β-cell was markedly decreased in $Shp2^{panc-/-}$ mice (Figure 6B). Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of mRNA also showed efficient deletion of exon 4 at the *Shp2* locus in pancreatic islets isolated from $Shp2^{Panc-/-}$ mice (Figure 6C). On the other hand, we studied Shp2 distribution in the entire pancreas by immunofluorescence staining and detected that Shp2 is localized in α, β and exocrine acinar cells, but not in δ and PP cells (Figure 7). Notably, the expression level of Shp2 in α and acinar cells is much lower compared with its intensity in insulin producing β cells, which only makes up ~ 1% of the entire adult pancreas. This observation may explain the less abundance of Shp2 in pancreas relative to other tissues (Figure 6A).

2. Metabolic studies in $Shp2^{panc-/-}$ mice

2.1 Impaired glucose tolerance and defective acute insulin secretion in $Shp2^{panc-/-}$ mice

To access the consequences of pancreatic Shp2 deletion on metabolism, we firstly evaluate glucose handling by $Shp2^{panc-/-}$ and control mice. At 3 months of age, the blood glucose levels in $Shp2^{panc-/-}$ mice at 0, 15, 60, 120 minutes are not significantly distinct from control mice, while in contrast, the glucose concentration exhibited in knockout mice at 30 minutes post IP injection was significantly higher than controls at the same

timepoint ($P < 0.05$) (Figure 8A). At 10 months of age, blood glucose level at 15 minutes after IP injection was markedly elevated compared with littermate controls ($P < 0.01$). The significantly increased blood glucose concentration was also detected at 60 minutes ($P < 0.05$) (Figure 8B). Therefore, there is age-dependent progressive glucose intolerance. We also measured the body weight of $Shp2^{panc-/-}$ mice and their age- and sex-matched littermate controls. The experimental data revealed that body weight of $Shp2^{panc-/-}$ mice appears normal at 3 months of age (Figure 9A), whereas the mutant mice displayed a modestly increased body weight at 10 months of age (Figure 9B).

To evaluate the effect of tissue specific Shp2 deletion on pancreatic β-cell function, we examined first-phase insulin secretion of pancreatic β cells, which is 2 minutes after the glucose load (3mg glucose/kg body weight). It has been well documented that lack of early insulin secretion or acute insulin response (AIR) elicited by glucose challenge is the most striking defect of β-cell dysfunction in type 2 diabetes mellitus (T2DM). Consistent with previous report, there is a potent increase of serum insulin level 2 minutes after glucose load in control mice. However, the serum insulin concentration of $Shp2^{panc-/-}$ mice is not elevated at 2 minutes post glucose IP injection, indicating that acute phase insulin secretion was ablated by Shp2 deletion (Figure 10). Blood glucose concentration and serum insulin levels during random feeding or fasting status exhibited certain variation among individuals from the mutant and control experimental group. Elevated blood glucose and decreased serum insulin levels were detected in some of the $Shp2^{panc-/-}$ mice, whereas these parameters were comparable in other mutant mice. Overall, blood glucose and serum insulin levels were statistically insignificantly different between $Shp2^{panc-/-}$ mice and littermate controls, which is similar

to β-cell specific insulin receptor knockout (βIRKO) mice (Figure 11) (Kulkarni et al., 1999), indicating that the mutant mice still holds the capability to maintain glucose homeostasis in the absence of progressive factors, such as high fat diet and oxidative stress.

2.2 Decreased insulin content of islets from Shp2$^{panc-/-}$ mice

The ablation of acute-phase insulin secretion of Shp2$^{panc-/-}$ mice could be a sum of multiple factors such as reduced β-cell mass and defective insulin biosynthesis from individual β cell. To define the factors, we carried out morphometric quantitation of insulin^{+} β-cell area on sections of paraffin-embedded pancreas from Shp2$^{panc-/-}$ mice at 3 months of age and littermate controls. There is no significant difference of total β-cell area versus entire pancreatic area in mutant mice (1.11%) as compared with controls (1.08%) (Figure 12A), which excludes the possibility that defective glucose stimulated insulin secretion is resulted from altered β-cell area.

We subsequently measured insulin content of isolated islets from Shp2$^{panc-/-}$ mice and the littermate controls. Islet insulin content of mutant mice was decreased by 57% as compared with control ($P < 0.01$) (Fig. 12B), implicating that insulin supply and storage in knockout islets are impaired. Therefore, impaired insulin biosynthesis from individual β cells may have contributed to aberrant glucose stimulated insulin secretion in *Shp2$^{Panc-/-}$* animals.

In addition, TUNEL assay of paraffin-embedded pancreatic sections of Shp2$^{panc-/-}$ and control (WT) animals showed that cell apoptosis are unaltered by Shp2 deletion

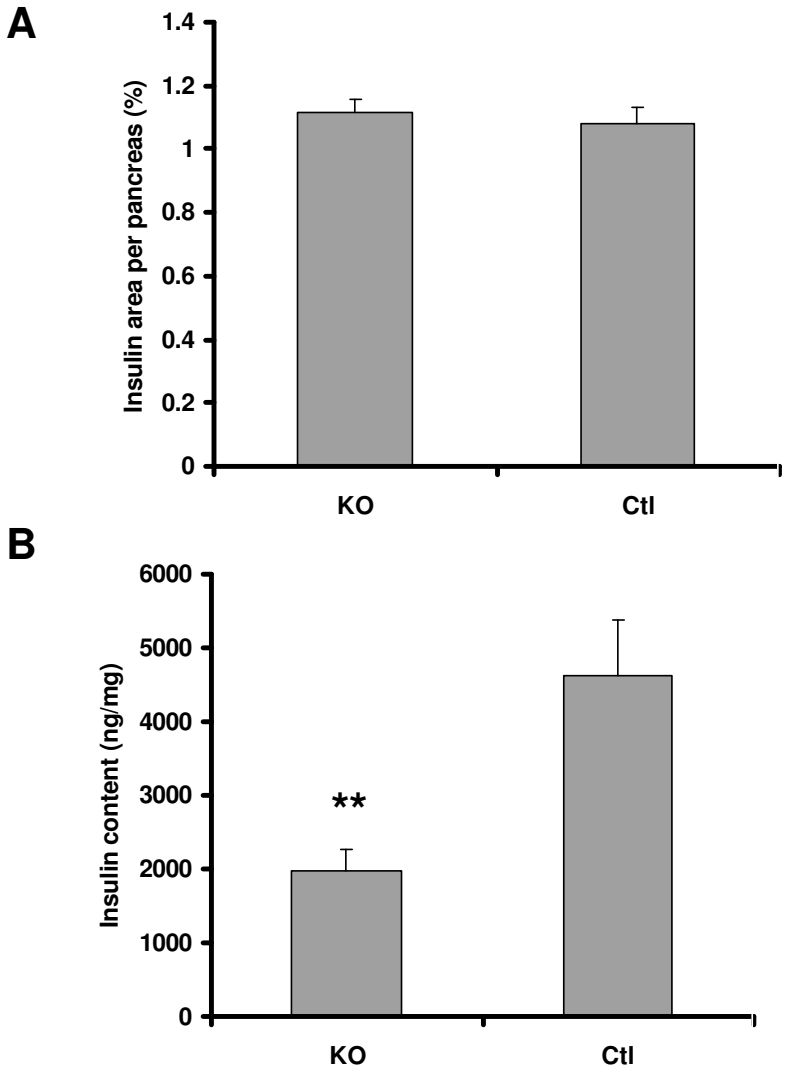


Figure 12. (A) Morphometric analysis of insulin positive area of total pancreatic area from 3-month-old *Shp2*$^{Panc-/-}$ and control mice. (n=4 per group). (B) Islet insulin content in 3-month-old *Shp2*$^{Panc-/-}$ and control mice. (n=5 per group).

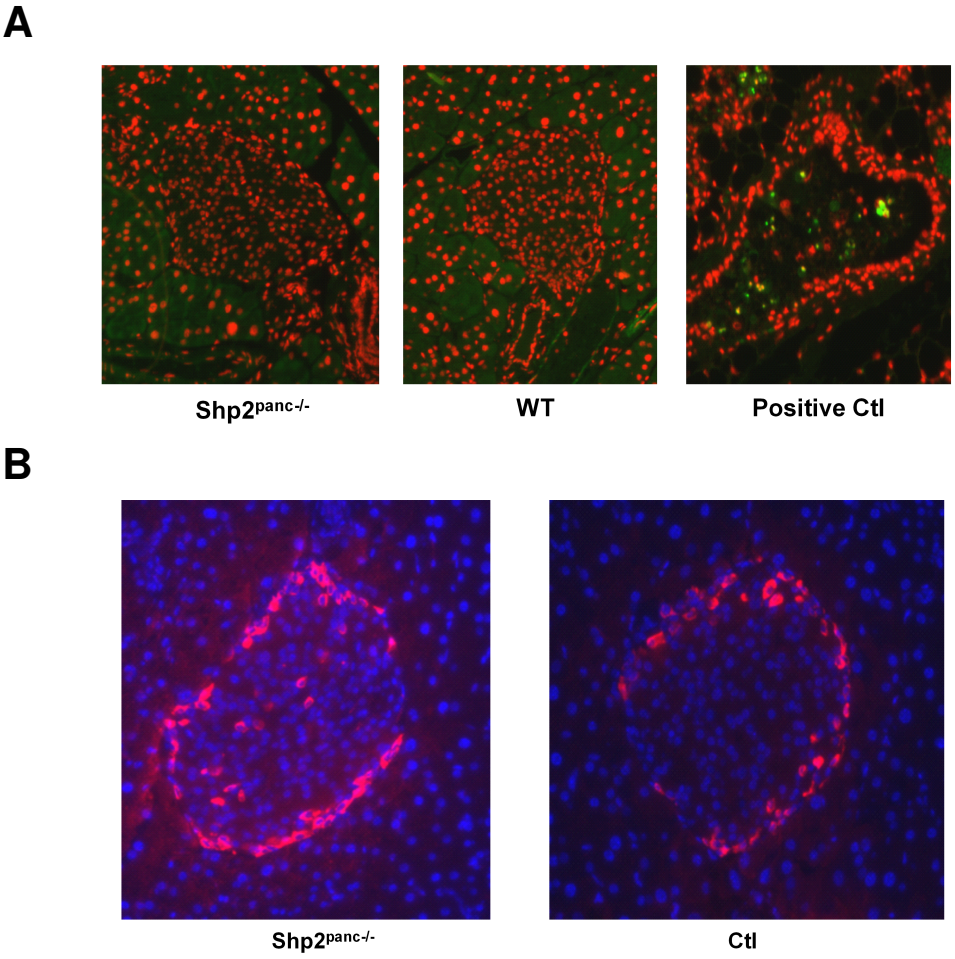


Figure 13. (A) TUNEL assay of paraffin-embedded pancreatic sections of Shp2$^{panc-/-}$ and control (WT) animal. Positive control is included. Red, Propidium Iodide (PI) Staining for nuclei, green, TUNEL positive apoptotic cells. (B) Glucagon staining of pancreatic sections from Shp2$^{panc-/-}$ and control animal.

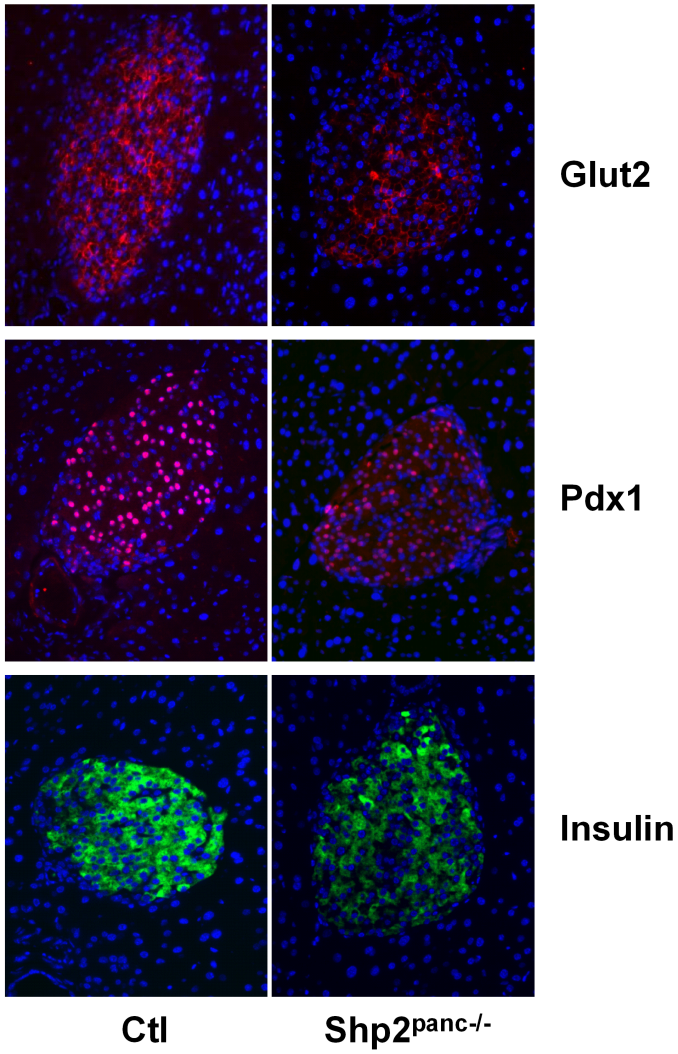


Figure 14. Immunofluorescence detection of Glut2, Pdx1 and insulin in pancreatic sections from 3-month-old *Shp2*$^{Panc-/-}$ and control mice.

(Figure 13A). On the other hand, insulin immunofluorescent staining revealed that β cells are distributed in the center of the islets isolated from both knockout and control mice. There is no spotty distribution of β cells throughout the exocrine pancreas (data not shown). Analysis of pancreatic sections using anti-glucagon antibody showed that the peripheral distribution of α cells is disturbed in $Shp2^{panc-/-}$ mice and there is protrusion of α cells into the core of mutant islets (Figure 13B).

2.3 Disruptive β-cell specific gene profile and altered protein expression of β-cell regulators by Shp2 deletion

To unveil the molecular basis underlying decreased β-cell insulin secretion in $Shp2^{panc-/-}$ mice, we assessed expression levels of several β-cell specific regulators by immunofluorescent staining of paraffin-embedded pancreas dissected from $Shp2^{panc-/-}$ and control mice. The insulin protein expression level in islets is decreased in $Shp2^{panc-/-}$ mice (Figure 14), which is consistent with the reduced islet insulin content detected by acid ethanol extraction and ELISA analysis. We further measured *Ins1* and *Ins2* mRNA levels by quantitative real-time RT-PCR analysis (qRT-PCR). Result shown in Figure 15B indicates significantly reduced amounts of *Ins1* and *Ins2* transcripts in Shp2-deficient islets, compared to control.

Glucose sensing and transport into β cells is the first rate-limiting step for glucose stimulated insulin secretion. Glucose transporter-2 (Glut2) is the major glucose transport isoform expressed in the plasma membrane of β cells, so we carried out immunofluorescence staining of paraffin sections of pancreas dissected from knockout

mice and control mice to examine the Glut2 expression level. The experimental result explored that Glut2 exhibited lower expression level in islets of $Shp2^{panc-/-}$ mice (Figure 15). Immunostaining results also exhibited decreased expression levels of Pdx-1, which is documented to regulate Glut2 and insulin gene expression (Figure 14). Moreover, immunoblot analysis also revealed deceased protein expression levels of Pdx-1 and Glut2 in pancreatic islets isolated from $Shp2^{panc-/-}$ animals (Figure 15A).

We subsequently measured the mRNA levels of Glut2, Pdx-1, Gck, Beta2 (NeuroD) and several β-cell-cycle regulatory genes, such as cyclin D1 (ccnd1), cyclin D2, cyclin-dependent kinase 4 (Cdk4) and c-myc. The experimental data revealed markedly decreased mRNA levels for Glut2, Pdx-1, glucokinase (Gck) and Beta2/ NeuroD, whereas no significant difference of mRNA levels of β-cell-cycle regulatory genes (Figure 16). As mentioned before, the mutations in Pdx-1, glucokinase or Beta2 cause monogenic forms of type 2 diabetes known as maturity onset of the young (MODY), indicating an essential role of these transcription factors to maintain normal β-cell function (Boyer et al., 2006). The altered gene profile of these transcriptional factors by Shp2 deletion can lead to the aberrant glucose sensing and insulin secretion of β cells. On the other hand, the conserved gene profile of several β-cell cycle regulators in Shp2 knockout islets corresponds with the normal β-cell proliferation.

Collectively, both protein expression and transcription levels of Pdx-1, Glut2 and insulin in knockout islets are impaired, which may lead to attenuated glucose-stimulated insulin secretion and consequent glucose intolerance in $Shp2^{panc-/-}$ mice.

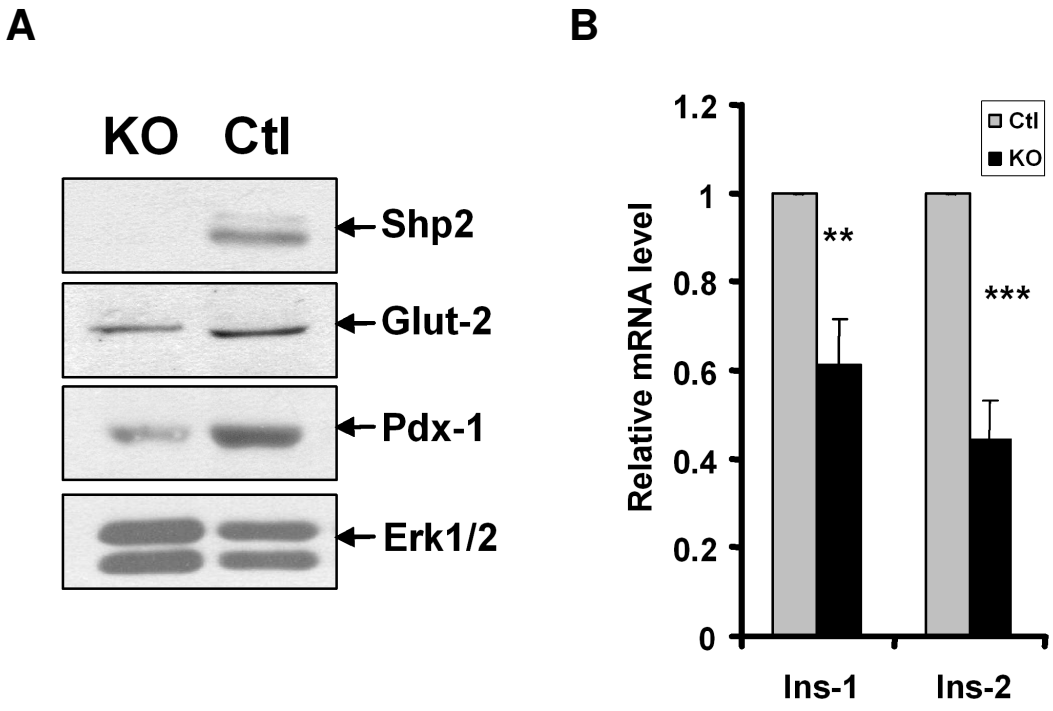


Figure 15. (A) Immunoblot analysis of protein expression of Shp2, Glut2, Pdx1 and Erk in pancreatic islets isolated from *Shp2*$^{Panc-/-}$ and control mice. (B) Real-time RT-PCR analysis of *Ins-1* and *Ins-2* mRNA levels in isolated *Shp2*$^{Panc-/-}$ and control islets. All data are statistically significant. **P < 0.01 versus controls, ***P < 0.001 versus controls (two-tailed Student's t-test). All data are presented as means ± s.e.m.

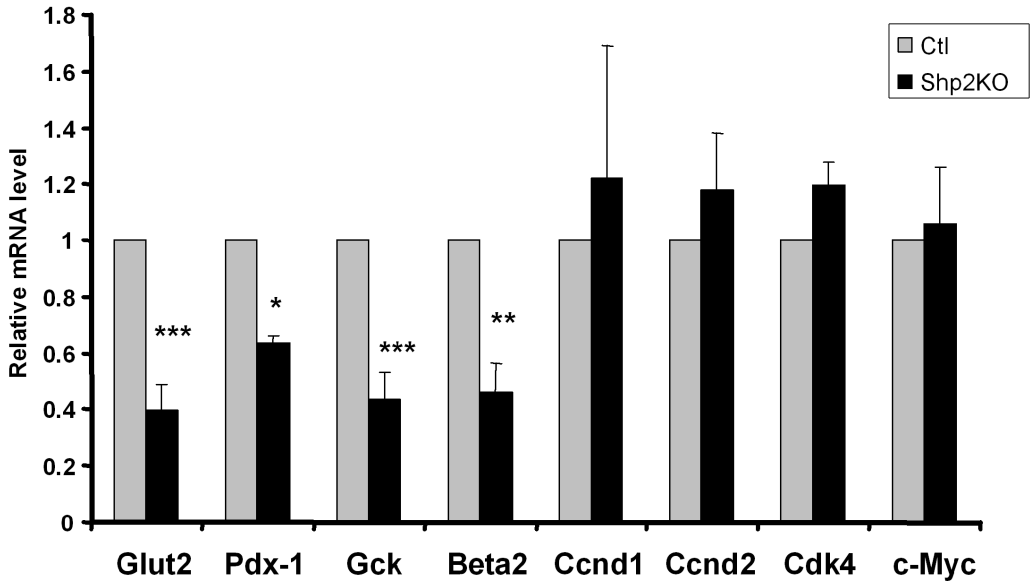


Figure 16. Quantitative real time RT-PCR analysis of expression of characterized β-cell gene products and cell-cycle regulators in Shp2 knockout and control islets. mRNA levels for each gene were normalized to *cph* mRNA. All data are presented as means ± s.e.m. *P< 0.05, * *P < 0.01 * * *P < 0.001 (two-tailed student's t-test).

3. *In vitro* study of Shp2 regulation of insulin signaling and β-cell function

3.1 Decreased insulin secretion in INS-1 832/13 by Shp2 gene silencing

In $Shp2^{panc-/-}$ animal model, Cre transgene driven by Pdx1 promoter also deleted Shp2 gene in cells that secrete glucagon, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), ghrelin, and others, all of which have well-documented roles in insulin secretion and glucose homeostasis. To exclude the possibility that the defective phenotype in knockout mice were resulted from Shp2 deletion in the non–β cells existing in pancreas, we also knocked down Shp2 gene in INS-1 832/13 cells, a rat insulinoma cell line. In previous studies, glucose stimulated insulin secretion was measured in six independent INS-1-derived cell lines. The results showed that fold stimulation of insulin secretion (average insulin secreted under high-glucose conditions divided by average insulin secreted under low-glucose conditions) in four of the cell lines were low (lines 834/105, 834/112, 832/1, and 832/2 with average responses of 2.4-, 2.1-, 2.2-, and 3.8-fold, respectively, as glucose was raised from 1 to 20 mM), whereas two cell lines were potently glucose-responsive (lines 833/15 and 832/13 with average responses of 21- and 30-fold, respectively) (Schisler et al., 2005). By comparison, INS-1 832/13 cells appears to be the most potently responsive cell line upon high glucose stimulation.

To investigate Shp2 function in INS-1 832/13 cells, we firstly transfected the cells with Shp2 or scramble (control) siRNA by using Amaxa nucleoporation system, which was tested to be most efficient way to knockdown Shp2 gene expression compared with both FuGENE and Oligofectamine transfection methodology. Shp2 protein expression level is downregulated by 90% with nucleoporation, whereas the transfection efficiency

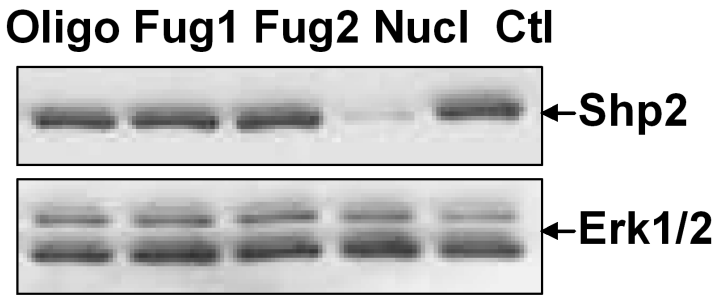


Figure 17. Immunoblot analysis of protein expression of Shp2 and Erk1/2 in protein lysates of INS-1 832/13 cells treated with siRNA against either Shp2 or Scramble (Ctl) by Oligofectamine Reagent, FuGENE 6 Transfection Reagent in two different dosage or nucleoporation.

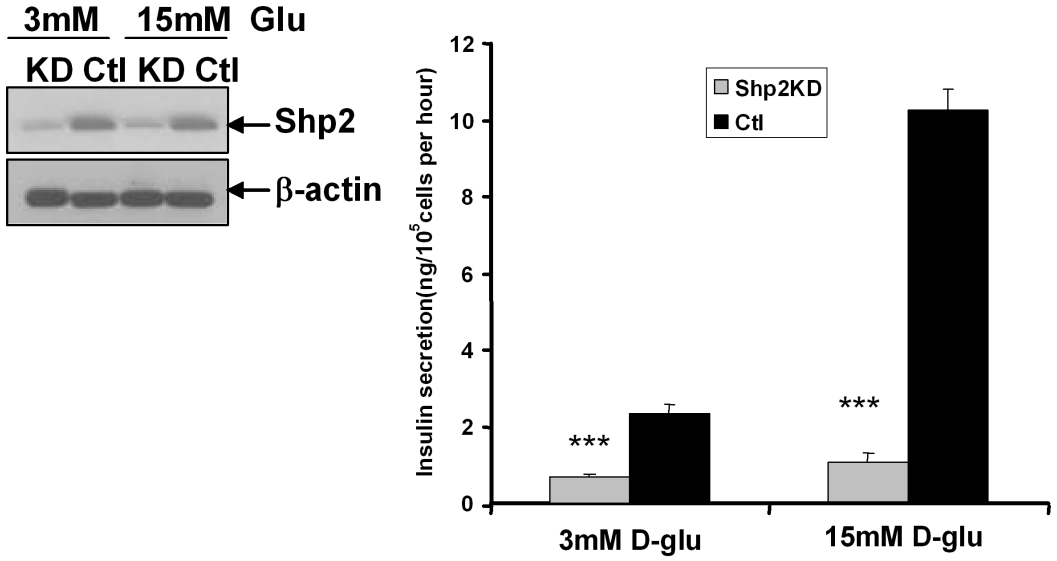


Figure 18. Immunoblot analysis of Shp2 or β-actin (loading control) from whole cell lysates from INS-1 832/13 cells treated with siRNA against either Shp2 (KD) or Scramble (Ctl). Insulin secretion in INS-1 832/13 cells following stimulation with 3 mM or 15 mM glucose media for 2 hr after a 2 hr preincubation in 3 mM glucose media. ***P < 0.001 versus controls (two-tailed Student's t-test). All data are presented as means ± s.e.m.

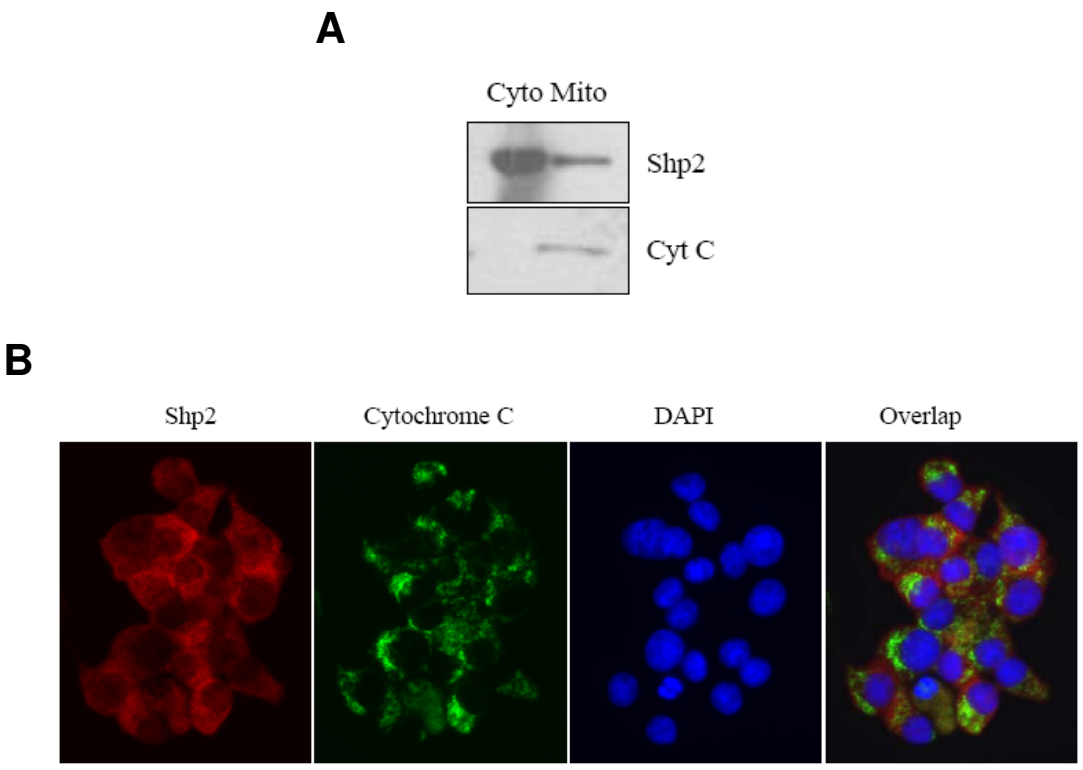


Figure 19. (A) Immunoblot of INS-1 832/13 cytosolic extraction and mitochondrial fraction with antibodies against either Shp2 or mitochondrial marker cytochrome C. (B) Immunofluorescence detection for Shp2 (red), cytochrome C (green), DAPI (blue) and overlay in INS-1 832/13 cells.

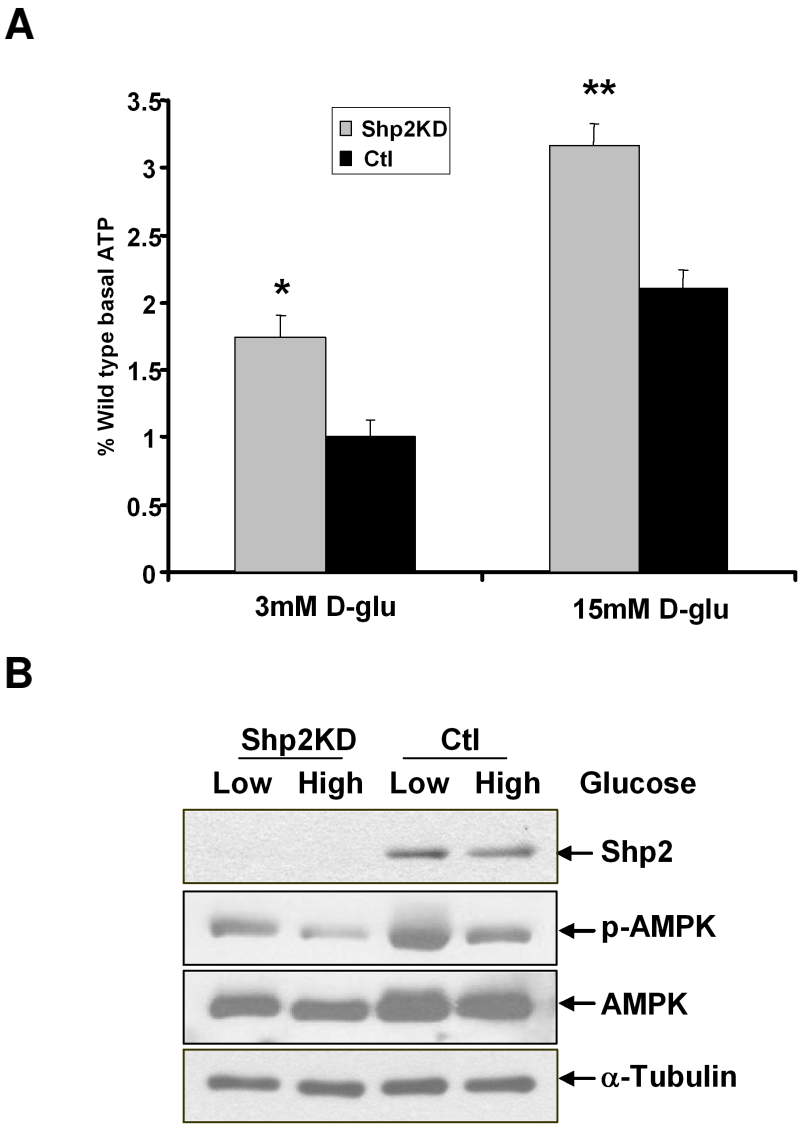


Figure 20. (A) ATP content of INS-1 832/13 cells treated with siRNA against either Shp2 (KD) or Scramble (Ctl) incubated in 3 mM or 15 mM glucose media for 2 hr post a 2 hr preincubation in 3 mM glucose media. (B) Immunoblots of AMPK, phosphothreonine 172 AMPK, Shp2 and α-Tubulin from Shp2KD and control cells with incubation in 3 mM or 15 mM glucose medium for 2 hours after a 2 hr preincubation in 3 mM glucose medium.

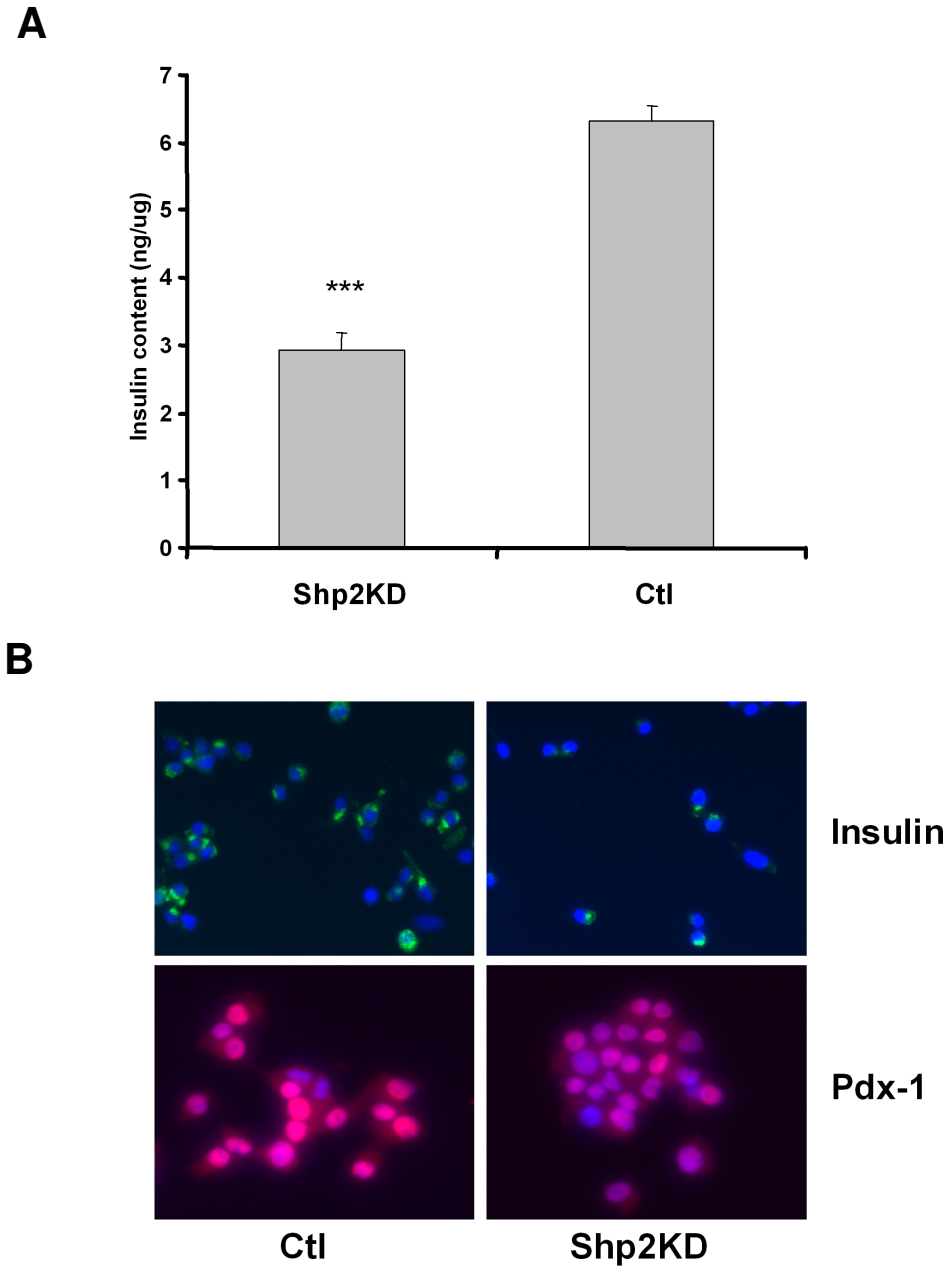


Figure 21. (A) Insulin content in Shp2 knockdown and control INS-1 832/13 cells. (B) Immunofluorescence detection for insulin and Pdx1 in Shp2KD and control INS-1 832/13 cells.

was very low by using other transfection reagents (Figure 17).

Seventy-two hours after siRNA treatment, cells were washed with PBS, and incubated in media containing 3 mM glucose for 2 hr to normalize insulin release. After the incubation, we treated the cells with either 3 mM (low) or 15 mM (high) glucose media for another 2 hr. After the 2 hr treatment, we collected the cell culture media to analyze the insulin secretion. In both basal (low) and glucose stimulated (high) status, insulin secretion in Shp2 knockdown cells is significantly lower than control cells ($P < 0.001$) (Figure 18). Upon high glucose challenge, the average insulin secretion is significantly elevated in control cells compared with low glucose stimulation. In contrast, there is no significant enhancement of insulin secretion under high-glucose conditions versus insulin secreted under low-glucose conditions. This experimental result indicates that Shp2 plays an essential role in glucose sensing and insulin secretion events in pancreatic β cells.

3.2 Elevated cellular ATP content in INS-1 832/13 cells by Shp2 gene knockdown.

Glucose metabolism through glycolysis in the cytosol and then through the tricarboxylic acid (TCA) cycle in mitochondria is pivotal to elevate glucose-induced insulin secretion from pancreatic β cells through generation of metabolic signals such as adenosine triphosphate (ATP) or increase of ATP to adenosine diphosphate (ADP) ratio (Maechler and Wollheim, 2001). Accordingly, decreased cellular ATP content or ATP to ADP ratio directly leads to defective glucose stimulated insulin secretion. We postulated that impaired insulin secretion induced by Shp2 gene silencing could be resulted from altered mitochondrial ATP generation and cellular ATP content. Previous studies

reported that knockdown of PTPMT1 (PTP localized to the Mitochondrion 1), a member of the dual-specific protein tyrosine phosphatase localized in mitochondria, can significantly elevate both ATP production and insulin secretion of pancreatic β cells (Pagliarini et al., 2005). Prior experimental data showed that Shp2 is distributed in rat brain mitochondrial as well as other Cytosolic Compartment (Salvi et al., 2004), suggesting that Shp2 might play a functional role in regulation of mitochondrial ATP generation.

To testify this postulation, we firstly analyze the subcellular location of Shp2. To this end, we isolated mitochondrial and cytosolic fractions from INS-1 832/13 cells by using mitochondrial fractionation kit. Immunoblot analysis of mitochondrial and cytosolic fraction revealed that around 10% of Shp2 protein from INS-1 832/13 cells is localized in mitochondrial (Figure 19A). Moreover, immunofluorescence staining explored a staining pattern that Shp2 is overlapped with the mitochondrial marker, Cytochrome C, suggesting that Shp2 is present in mitochondrial of INS-1 832/13 cells (Figure 19B).

We further assessed Shp2 endogenous function in mitochondrial by measuring its effect on cellular ATP levels. We transfected Shp2 or scramble siRNA into the INS-1 832/13 cell line by nucleoporation. After 3 days of transfection, the cells were rinsed with PBS and then pretreated for 2 hours in basal glucose (3 mM) standard assay buffer (SAB). After pretreatment, we replaced the media with basal or high (15mM) glucose SAB for another 2 hours, then cells were lysed and the ATP content was measured. Surprisingly, cellular ATP levels in Shp2KD cells are significantly elevated compared with control cells under both basal and high glucose condition (Figure 20A).

We further evaluated the phosphorylation level of AMP- activated protein kinase (AMPK), which is phosphorylated and activated when energy sources are low. Immunoblot analysis showed that AMPK activation levels are attenuated in Shp2 knockdown cells as compared with control cells in both basal and high glucose status (Figure 20B), which is consistent with an elevated ATP content by Shp2 gene interruption. Collectively, both ATP content and AMPK phosphorylation measurement demonstrated that Shp2 plays a negative role in ATP generation and energy homeostasis. This result manifested a previously unreported role of Shp2 in the regulation of one of the cell's most essential metabolic parameters: cellular ATP content. Together with the newly discovered function of PTPMT1 in monitoring mitochondrial ATP generation, these findings suggest that mitochondrion is an important organelle of signaling events mediated by reversible phosphorylation. By coincidence or not, both Shp2 and PTPMT1 negatively regulate cellular ATP content of INS-1 832/13 cell line, nonetheless the knockdown of Shp2 or PTPMT1 exerts opposite impact on insulin secretion. The distinct effect of these two phosphatases on output insulin secretion profiles may be due to different distribution patterns between Shp2 and PTPMT1: PTPMT1 resides exclusively in mitochondrial, whereas Shp-2 localizes throughout the whole of cytoplasm. Therefore, the effect of PTPMT to insulin secretion is merely determined by its regulation of mitochondrial ATP generation, nevertheless the impact of Shp2 on insulin secretion is a sum of its multiple roles in signaling events and energy metabolism of the entire cytoplasm.

3.3 Marked decrease of insulin content and gene transcription in β cells by Shp2 knockdown

The decreased insulin release is apparently at odds to the increased ATP generation in Shp2 knockdown cells. We subsequently analyzed insulin content by acid-ethanol extraction of insulin from Shp2 knockdown and control cells. We detected significantly decreased insulin content in Shp2 knockdown cells ($P < 0.001$) (Figure 21A). Moreover, immunofluorescence staining confirmed the decreased insulin protein expression level in Shp2 knockdown cells (Figure 21B). Quantitative RT-PCR results also showed a marked decrease of Ins-1 and Ins-2 mRNA levels by Shp2 gene silencing ($P < 0.001$) (Figure 22). Therefore, *in vitro* experimental results are consistent with the reduced insulin content and gene transcription in knockout islets shown by *in vivo* studies. It is evident that Shp2 plays a dominant role in promoting insulin biosynthesis and insulin storage, which overrides it negative role in controlling cellular ATP levels and consequently plays a positive role in regulating β-cell insulin secretion.

3.4 Altered β-cell specific gene profile by Shp2 gene silencing

To unveil the mechanism underlying the impaired insulin secretion and reduced insulin content, we measured the levels of mRNA encoding Glut2, Pdx1, Beta2/ NeuroD, glucokinase (Gck), MafA, NFATc1 and the hepatocyte nuclear factor (Hnf) family of transcriptional factors in Shp2 knockdown and control INS-1 832/13 cells by real-time reverse transcriptase polymerase chain reaction (RT–PCR). We detected significantly decreased mRNA levels for Glut2, Pdx-1, Gck, NFATc1, Hnf1α and HNF4α in Shp2 knockdown cells, which is consistent with the attenuated insulin synthesis and secretion

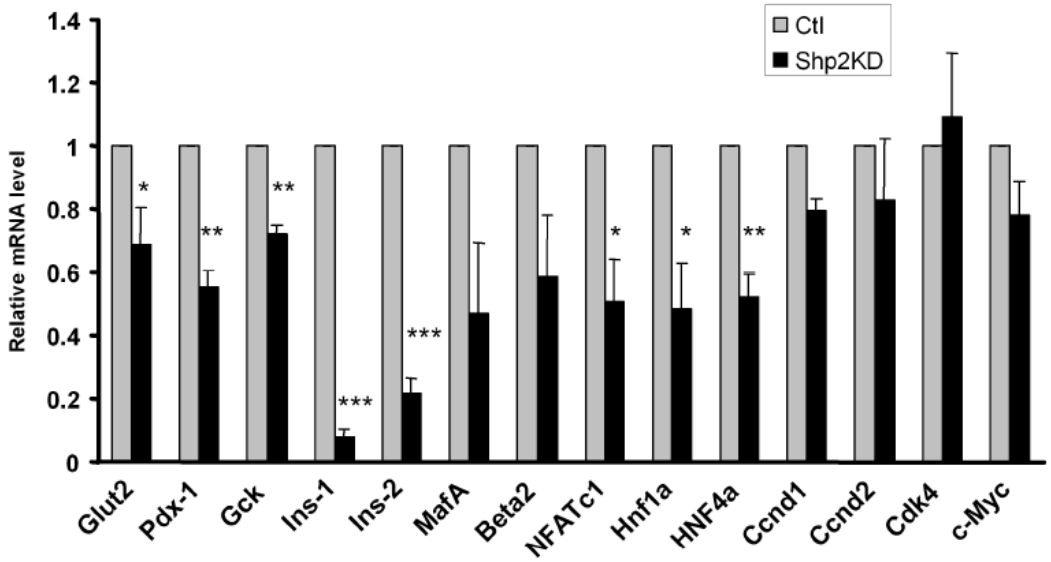


Figure 22. Quantitative real time RT-PCR analysis of expression of characterized β-cell gene products and cell-cycle regulators in control and Shp2 knockdown INS-1 832/13 cells. mRNA levels for each gene were normalized to *cph* mRNA.

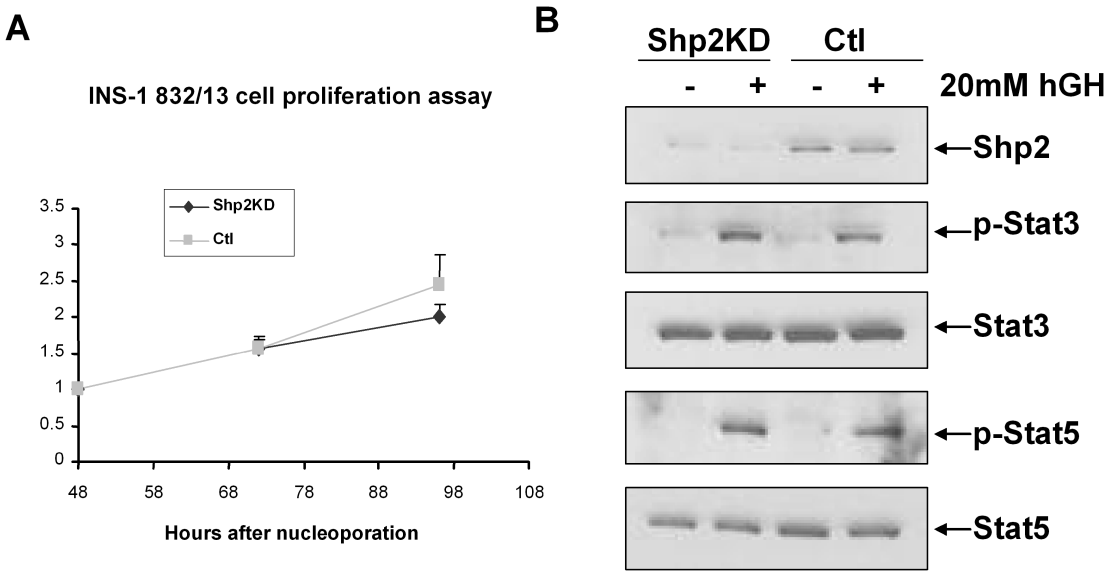
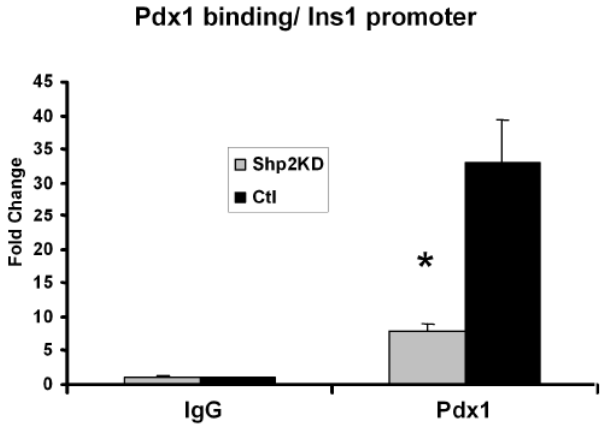


Figure 23. (A) CyQuant cell proliferation analysis of INS-1 832/13 cells treated with siRNA against either PTPMT1 or Scramble (Ctl) at different indicated time points. (B) Western blot of expression of Shp2, p-Stat3, Stat3, p-Stat5 and Stat5 in Shp2 knockdown and control cells treated with or without 20mM human growth hormone.

A



B

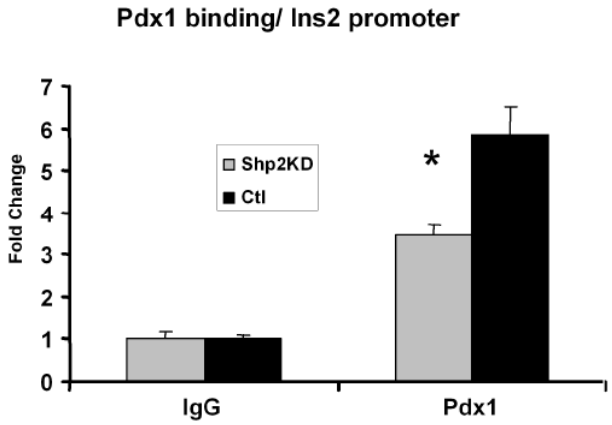


Figure 24. Anti-Pdx-1 ChIP assay performed on Shp2 knockdown and control INS-1 832/13 cells and subsequently analyzed by quantitative PCR to detect the association of Pdx1 with elements in promoters of Ins-1 (A) and Ins-2 (B) gene.

(Figure 22). This result reveals an important role for Shp2 in the modulation of several β-cell specific factors. Numerous experimental data documented that mutations or changing expression pattern of these transcription factors disrupt the glucose sensing and insulin secretion of pancreatic β cells by targeting downstream genes, most notably insulin gene. This result provided us an important clue to dissect the Shp2 function in pancreatic β cells.

In agreement with the quantitative real-time RT-PCR results, immunofluorescence staining and western blot analysis also displayed deceased protein expression levels of Pdx-1 in Shp2 knockdown cells (Figure 21B and Figure 26). Previous studies showed that Pdx-1 plays important roles in glucose-sensing, insulin synthesis and insulin exocytosis by regulating the expression of several downstream β-cell genes, including insulin, glucokinase, GLUT2 and islet amyloid polypeptide (Boyer et al., 2006). Therefore, the decreased insulin content and impaired glucose stimulated insulin secretion may result from the reduced Pdx-1 expression in Shp2 knockdown INS-1 832/13 cells.

3.5 Conserved β-cell proliferation after Shp2 gene interruption

We subsequently measured mRNA levels of several β-cell-cycle regulatory genes, including cyclin D1 (ccnd1), cyclin D2, cyclin-dependent kinase 4 (Cdk4) and c-myc. The mRNA levels of these β-cell-cycle regulators are unaltered in Shp2 knockdown cells compared with control (Figure 22).

This result is consistent with the conserved cell proliferation in Shp2 knockdown cells, which was quantitated by CyQUANT Cell Proliferation Assay (Figure 23A). It has recently become clear that signal transducer and activator of transcription (STATs) can promote pancreatic β cell proliferation after being activated by growth hormone, prolactin, cytokines and placental lactogen. Constitutively activated Stat3 was revealed to promote rat pancreatic β-cell proliferation by inducing cyclin D1 transcription. Another member of STAT family, Stat5, was proved to promote gene transcription of cyclin D2 and subsequently elevate proliferation of INS-1 832/13 cells. Herein, we analyzed the phosphorylation of Stat3 and Stat5. In basal status, the phosphorylation of Stat3 and Stat5 is barely detectable. Upon human growth hormone (20μM) treatment, there is markedly upregulated activation of Stat3 and Stat5 in both Shp2 knockdown and control cells. The phosphoryaltion levels of Stat3 and Stat5 in Shp2 knockdown cells are indistinct from control (Figure 23B), which is consistent with the normal β-cell proliferation in Shp2 knockdown cells.

3.6 Strikingly reduced Pdx-1 binding affinity to Ins1/Ins2 promoters by Shp2 gene knockdown

The modulation of insulin biosynthesis by glucose is dependent on its stimulatory effect on insulin gene transcription in the long run. Transcription of the insulin gene is mediated by transcription factors association with multiple *cis*-acting elements within the insulin promoter located with the region up to 350 bp from the start site. Previous studies revealed that Pdx-1 can directly bind to A3 and A1 elements in insulin promoter and regulate insulin promoter activity by forming a complex with other transcriptional

coactivators (Mosley et al., 2004). To determine whether interrupting Shp2 gene transcription in pancreatic β cells would affect the direct association of Pdx-1 with its target genes, Ins-1 and Ins-2, chromatin immunoprecipitation (ChIP) assay was carried out by using antibodies specific for Pdx-1 in Shp2 knockdown and control cells.

ChIP analysis showed direct interaction of Pdx-1 with *cis*-regulatory elements in Ins1 and Ins2 promoter region. Quantitative real-time polymerase chain reaction (Q–PCR) revealed that the binding affinity of Pdx-1 with elements within the Ins-1 and Ins-2 promoters was significantly lower in Shp2 knockdown cells in comparison with control cells ($P < 0.05$) (Figure 24A, 24B). Therefore, Shp2 can regulate both Pdx-1 gene expression level and functional activity of Pdx-1. This experimental data provides unique evidence that a cytoplasmic protein tyrosine phosphatase, Shp2, can control the binding affinity of certain transcription factor to its target genes through a series of intermediate signaling molecules. The transactivating activity of Pdx-1 is controlled by multiple factors, such as phosphorylation and nuclear localization. *In vitro* studies showed that Pdx-1 can be phosphorylated by MAPK family members, including p38, Erk1/2 and c-Jun N-terminal kinase (Jnk) (Macfarlane et al., 1997). Erk2 shares the same two phosphorylation sites on Pdx-1 with p38. Glucose is reported to promote Pdx-1 nuclear localization and enhance its transcriptional activation capacity. The glucose-stimulated Pdx-1 functional activity is suppressed by Erk1/2 inhibitor, whereas it can not be restricted by p38 activity blocker. One the basis of positive regulation on Erk1/2 activation by Shp2 in fibroblasts and several other cell types as previously reported, we postulate that Shp2 gene silencing might reduce Erk1/2 phosphorylation, thereby repressing Pdx-1 transactivating activity.

3.7 Analysis of signaling cascades medicated by Shp2 during glucose stimulated insulin secretion

3.7.1 Altered IRS2/PI3K/Akt/Foxo1 activation in pancreatic β cells by Shp2 gene silencing

Although it is evident that Shp2 can regulate insulin production and glucose stimulated insulin secretion both in *vivo* and *in vitro*, the molecular basis underlying its regulation of pancreatic β-cell function remains largely unknown. To decipher the molecular mechanism of how Shp2 regulate insulin biosynthesis, we accessed IRS2/PI3K/Akt/Foxo1 and MAPK pathways in INS-1 832/13 cells, which were reported to be mediated by Shp2 signaling in other cells types and be essential for pancreatic β-cell function. Previous studies reported that targeted deletion of the N-terminal SH2 domain of SHP-2 severely impaired PDGF- and IGF-induced Akt phosphorylation as well as EGF-induced Erk1/2 activation in mouse fibroblast cells (Wu et al., 2001), indicating a positive role of Shp2 in regulating these two signaling pathways.

To assess Shp2 function in IRS2/PI3K/Akt/Foxo1 activation, we firstly examine whether Shp2 interacts with two important upstream molecules of this pathway: IRS2 and PI3K regulatory subunit P85. We detected an association of Shp2 with IRS2 protein in INS-1 832/13 cells by immunoprecipitation and immunoblot. The interaction was markedly enhanced with high glucose exposure in comparison with low glucose exposure (Figure 25). This result is consistent with the previous finding that tyrosine phosphorylated motifs in the IRS proteins bind to the SH2 domains of multiple adaptor proteins, including Shp2. Insulin stimulation was shown to promote IRS2 association

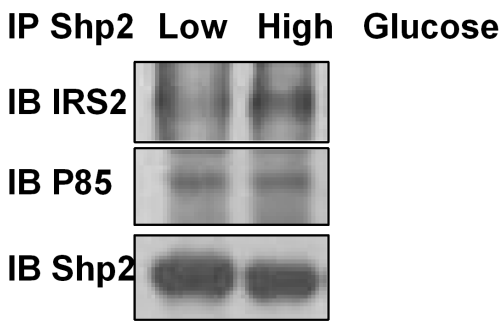


Figure 25. Immunoprecipitation and immunoblot analysis of association of Shp2 with IRS2 and P85.

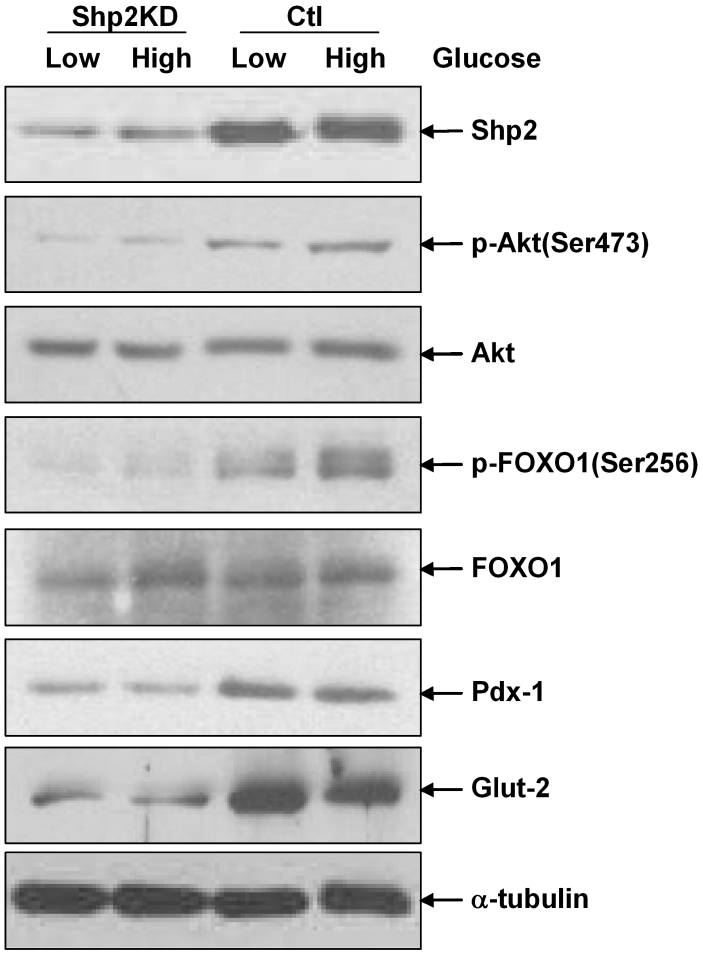


Figure 26. Western blot of expression of Shp2, p-Akt(Ser473), Akt, p-FOXO1(Ser256), FOXO1, Pdx1, Glut2 and α-tubulin in Shp2 knockdown and control cells with incubation in 3 mM or 15 mM glucose medium for 2 hours following a 2 hr preincubation in 3 mM glucose medium.

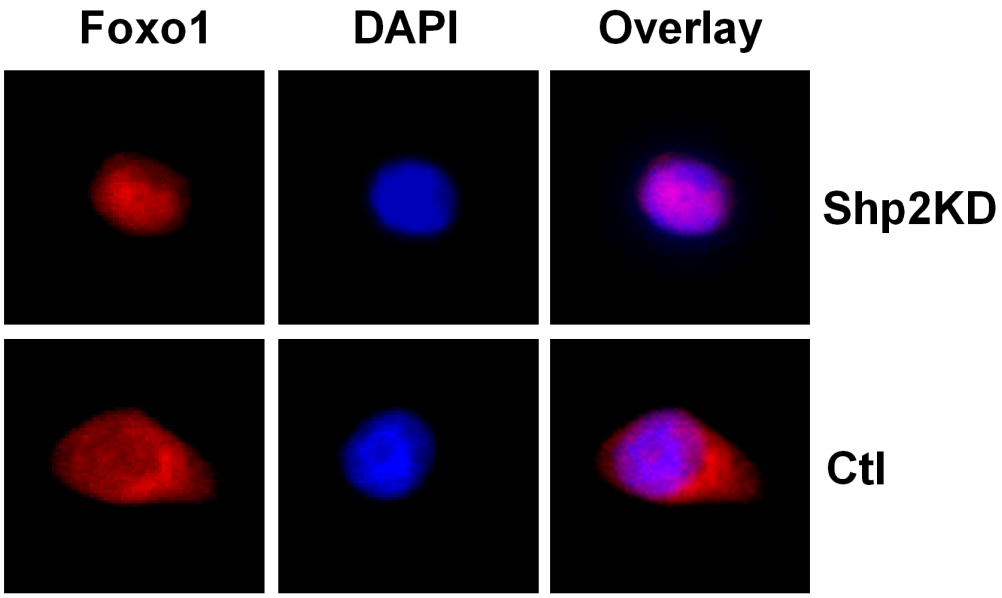


Figure 27. Immunofluorescence detection of FOXO1 (red), DAPI (blue) and overlay in Shp2KD and control cells.

with Shp2 in muscle of DHEA-treated rats (Campbell et al., 2004). Moreover, we observed an interaction between Shp2 and P85 regulatory subunit of PI3K (Figure 25). This result implicated that Shp2 may facilitate IRS2/P85 interaction by forming a complex with them.

We subsequently investigate whether gene silencing of Shp2 affects phosphorylation of Akt and Foxo1. To this end, we introduced Shp2 or scramble siRNA into INS-1 832/13 cells by nucleoporation. After transfection for 72 hours, cells were preincubated for 2 hours in basal glucose (3 mM) media and then treated with basal or high (15mM) glucose media for another 1 hour. Then cells were harvested and lysed for immunoblot analysis. As expected, we detected markedly reduced levels of Akt (Ser473) and Foxo1 (Ser253) in Shp2 knockdown cells compared with control under both basal (3mM) and high glucose (15mM) condition (Figure 26). With high glucose stimulation, the phosphorylation level of Akt (Ser473) and Foxo1 (Ser253) is promoted in control cells, whereas in contrast, there is barely detectable activation of these two molecules in Shp2 knockdown cells. Under the same condition, total Akt and Foxo1 protein levels showed no change (Figure 26). Moreover, glucose stimulated Akt phosphorylation is significantly diminished in pancreatic extraction of $Shp2^{panc-/-}$ mice compared with control, whereas there is no distinction of Akt activation level in knockout liver extraction as compared with control (Figure 29). These data indicated that Shp2 is required for glucose mediated activation of Akt and Foxo1 in INS-1 832/13 cells. Previous studies of transgenic mice expressing a kinase-dead mutant of Akt in β cells exhibited defective glucose handling capability as a result of reduced insulin secretion and increased susceptibility to high fat diet-induced diabetes (Bernal-Mizrachi et al.,

2004). Conversely, transgenic mice with β-cell constitutively active Akt overexpression displayed improved glucose tolerance, hyperinsulinemia and resistance to streptozotocin-induced diabetes due to increased β-cell size and proliferation (Bernal-Mizrachi et al., 2001). On the other hand, glucose promotes Foxo1 activity in β cells through autocrine/paracrine effect of secreted insulin on its own receptor (Martinez et al., 2006). Activation of FOXO1 is implicated to regulate β-cell growth and function through linking insulin signaling to Pdx1 expression (Kitamura et al., 2002). Taken together, reduced activation of Akt and Foxo1 by Shp2 gene silencing/deletion may account for attenuated Pdx-1 expression, defective insulin secretion and glucose intolerance.

3.7.2 Disruption of insulin signaling by interrupting Shp2 gene inhibits Foxo1 nuclear exclusion upon high glucose stimulation

The Foxo family members of transcriptional factors are known to be negatively regulated by Akt activation in β-cell and other cell types (Nakae et al., 2002). Activation of Foxo1 by Akt leads to suppression of its transcriptional activity and nuclear exclusion to cytoplasm. To determine whether decreased Foxo1 phosphorylation in Shp2 knockdown cells affects its nuclear/cytoplasmic localization pattern, we treated cells with high glucose (25mM) media for 1 hour after starvation and then examine the Foxo1 distribution. Consistent with the western blot results, we detected cytoplasmic translocation of Foxo1 upon high glucose stimulation in control cells, whereas disruption of insulin signaling by Shp2 gene silencing prevented nuclear exclusion of Foxo1 (Figure 28). Previous studies showed that constitutively nuclear localization of Foxo1 mutant represses Pdx1 expression, therefore exclusively nuclear localization of Foxo1 in Shp2

knockdown cells leads to repression of Pdx1 and consequently defective insulin gene transcription.

3.7.3 Suppression of Erk1/2 activation by Shp2 gene silencing

In parallel with the Akt/Foxo1 pathway, activated Erk1/2 kinases have been shown to transactivate *Pdx1* and *insulin* gene expression in pancreatic β-cells (Lawrence et al., 2008). We examined the effect of *Shp2* gene silencing on Erk1/2 activation and detected markedly lower phospho-Erk1/2 levels under high glucose condition in Shp2 knockdown INS-1 832/13 cells, as compared to control (Figure 29B). These results are accordant with the well-documented perception that Shp2 promotes full activation of Erk1/2 in response to various stimuli. Erk1/2 activation was reported to positively regulate insulin transcription and phosphorylate Pdx-1, thereby increasing PDX-1 transactivating activity (Khoo et al., 2003). Therefore, the attenuation of serine phosphorylation of Erk1/2 by interrupting Shp2 gene may lead to the decreased binding affinity of Pdx-1 to Ins-1 and Ins-2 promoters.

3.7.4 Association of Shp2 with Sprouty 1 in pancreatic β cells

The next question we asked was how Shp2 promotes Erk activation in β-cells. Prior studies showed that Shp2 promotes receptor tyrosine kinase signaling by inactivating a feedback inhibitor Sprouty 1 in cultured *Drosophila* cells and developing eye (Jarvis et al., 2006; Mason et al., 2006). Based on these findings, we postulated that Shp2 might regulate Erk1/2 activation by dephosphorylating Sprouty 1 protein in INS-1 832/13 cells. To testify this hypothesis, we determined whether Shp2 associate with

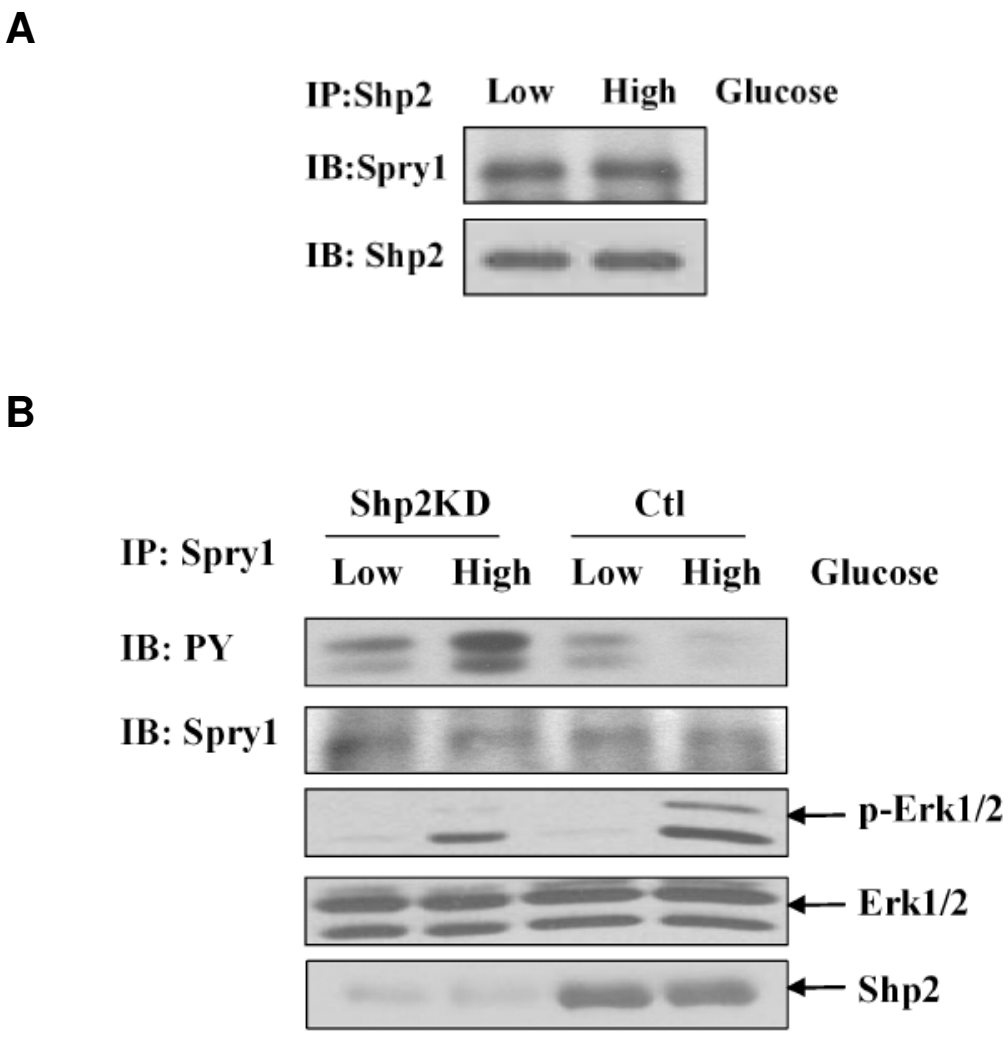


Figure 28. (A) Immunoprecipitation and immunoblot analysis of association of Shp2 with Sprouty 1 under both low (3mM) and high (15mM) glucose conditions. (B) Immunoprecipitation and immunoblot analysis of tyrosine phosphorylation of Sprouty 1 in Shp2 knockdown and control cells in low (3mM) or high (15mM) glucose status. Immunoblot analysis of Shp2, p-Erk and Erk in Shp2 knockdown and control cells in low (3mM) or high (15mM) glucose status.

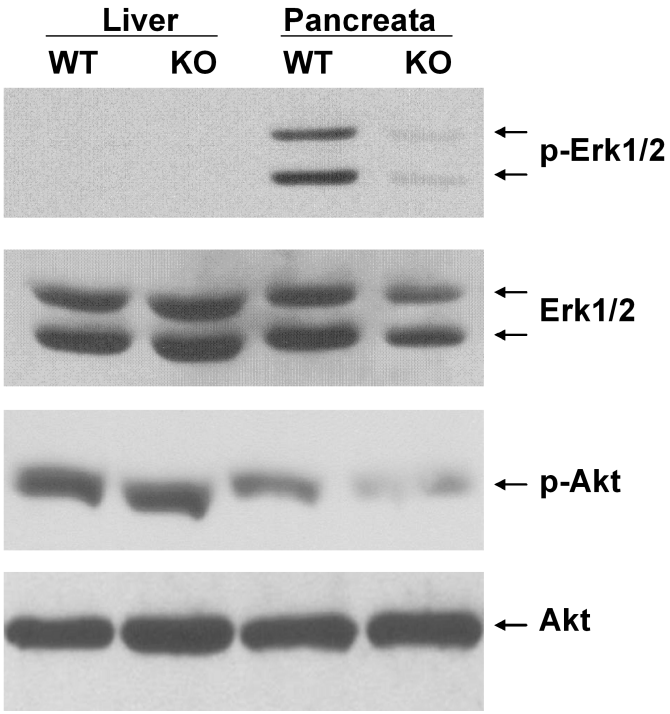


Figure 29. Erk and Akt activation is reduced in pancreatic protein extraction from *Shp2*$^{Panc-/-}$ mice compared with controls (WT) after glucose IP injection.

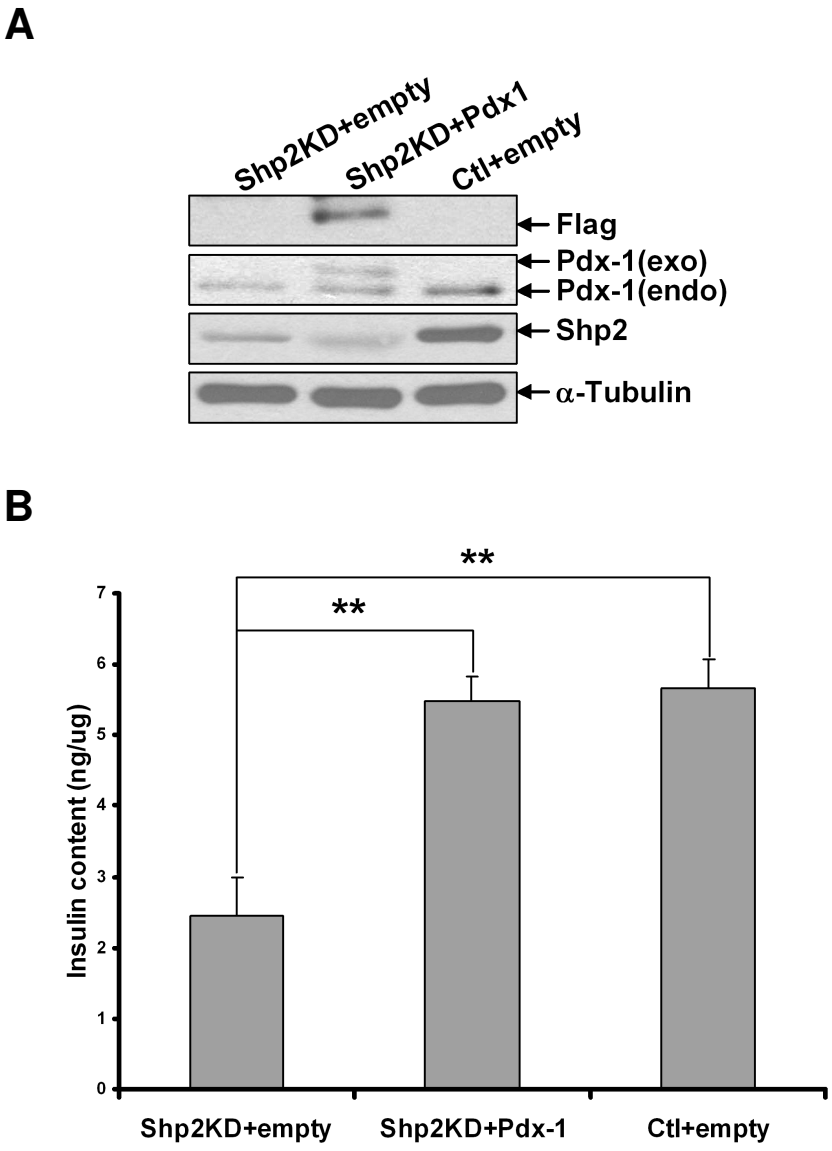


Figure 30. (A) Immunoblot analysis of Flag, Pdx-1, Shp2 and α-Tubulin of protein extraction from these three groups of INS-1 832/13 cells with 72 hr transfection of Shp2-siRNA+empty vector, Shp2-siRNA+Pdx1 cDNA, and scramble-siRNA+empty vector. (B) Insulin content of these three groups of INS-1 832/13 cells.

Sprouty 1 protein by co-immunoprecipitation and immunoblot analysis. Physical association of Shp2 with Sprouty 1 was detected in both low and high glucose conditions (Figure 29A).

3.7.5 Negative regulation of Sprouty 1 phosphorylation and activation by Shp2

We subsequently assess the effect of Shp2 on activation of Sprouty 1 by analyzing tyrosine phosphorylation levels of Sprouty1 upon glucose stimulation in Shp2 knockdown cells and control. As shown in Figure 30B, tyrosine phosphorylation of Sprouty 1 was significantly higher under high glucose condition in Shp2 knockdown cells compared with control, suggesting that Shp2 negatively regulates activation of Sprouty 1. Taken together, Shp2 can directly dephosphorylate and inactivate Sprouty 1 protein and relieve its inhibition on MAPK signaling activation, thereby promoting Erk1/2 phosphorylation in INS-1 832/13 cells (Figure 29B). Our results identify Sprouy 1 protein as a substrate of Shp2 in pancreatic β cells, and provide important evidence to support the conclusion that Shp2 generates a double-negative regulatory circuit in which Shp2 elevates signaling output profile by inactivating a feedback inhibitor.

Collectively, Shp2 deletion leads to altered activation of IRS2/PI3K/Akt/Foxo1 and Sprouty1/Erk1/2 pathways, which cooperatively suppress Pdx-1 expression level and its transactivating activity, thereby decreasing insulin gene transcription and biosynthesis in pancreatic β cells. These findings reveal a novel and crucial role of Shp2 in regulating insulin signaling events via its enzymatic activity as well as adaptor protein function.

3.8 Restoration of decreased insulin content in Shp2 knockdown cells by Pdx-1 cDNA transfection

According to our experimental data, Pdx-1 plays an important role in integrating different signaling pathways modulated by Shp2 into the regulation of insulin gene transcription and production in pancreatic β cells. To test the hypothesis that reduced Pdx1 expression and activity is responsible for suppression of insulin biosynthesis in Shp2-deficient cells, we performed experiment to examine whether restoration of Pdx1 gene expression can retrieve the reduced insulin content in Shp2 knockdown cells. Three groups of INS-1 832/13 cells were transfected with Shp2-siRNA+empty vector, Shp2-siRNA+Pdx-1 cDNA, and scramble-siRNA+empty vector. After 72 hours of transfection, we detected that the reduced insulin content by Shp2 gene silencing can be enhanced to control level by transfection of Pdx-1 cDNA (Figure 30). Western blot analysis revealed that Shp2 knockdown lowered endogenous Pdx-1 protein expression levels by approximately 50%, which can be restored by transfection of Pdx-1 cDNA tagged with Flag (Figure 30). This result suggests that Pdx-1 is an essential downstream target of Shp2 in control of insulin biosynthesis in pancreatic β cells.

IV. Discussion

Studies in this dissertation have demonstrated that protein tyrosine phosphatase Shp2 is necessary for pancreatic β cell function and explored a novel regulatory role for Shp2 in insulin secretion and glucose homeostasis. Our results revealed that Shp2 can modulate insulin signaling in β cells and therefore control Pdx-1 gene expression and transactivational activity, which are required in adult humans and animals for normal glucose sensing and insulin gene transcription. In this chapter, I will discuss the implication of the experimental data acquired from both *in vivo* pancreatic Shp2 knockout mouse model and *in vitro* studies of Shp2 knockdown INS-1 832/13 cells.

1. Shp2 plays an essential role in acute-phase insulin secretion and glucose tolerance.

Full understanding of molecular signaling mechanisms underlying control of insulin biosynthesis in β-cells is a pre-requisite for unveiling and correcting β-cell dysfunction in type 2 diabetes. Accumulating experimental data suggest that multiple pathways are involved in physiological control of insulin production and secretion from β cells. This model of action predicts the requirement of one or more signaling molecules acting to fine-tune and coordinate a variety of signals in β cells, although little is known in this regard. In this study, we have generated and characterized conditional knockout mice in which the *Shp2/PTPN11* gene is selectively deleted in the pancreas, leading to revelation of a critical role of Shp2 as a signal regulator/coordinator in β cells.

A distinct phenotype of $Shp2^{panc-/-}$ mice is the marked reduction of first-phase insulin secretion *in vivo* and age-related progressively impaired glucose tolerance. In

patients with defective glucose tolerance or in the early stages of type 2 diabetes, loss of acute phase insulin release is almost constantly detected despite the enhancement of second-phase secretion (Unger, 1991). The stimulus coupling insulin secretion pathway begins with glucose uptake through Glut2 transporter, and then glucose is phosphorylated by glucokinase and converted to pyruvate by glycolysis. Pyruvate subsequently goes into mitochondrial and undergoes TCA cycle to generate ATP, which leads to rise in the cellular ATP: ADP ratio. The increase of the ratio causes closure of ATP-sensitive K^+ channels and depolarization of plasma membrane. This opens voltage-gated Ca^{2+} channels and triggers calcium influx, which induces exocytosis of insulin-containing granules (Maechler and Wollheim, 2001).

Loss of the rapid first-phase insulin secretion could be resulted from abnormality of glucose entry, glucose metabolism, availability of readily releasable insulin granules, exocytosis of insulin-containing granules and alterations in intracellular glucose/lipid balance within β cells. In addition, reduced β-cell mass may contribute to the absence of acute-phase insulin secretion. Deletion of Shp2 in pancreas resulted in no aberrant change in islet morphology or β-cell mass in chow-diet fed mice, whereas in contrast, islet insulin content was markedly reduced in $Shp2^{panc-/-}$ mice. These results suggest that the loss of first-phase insulin secretion in knockout mice is specifically due to defect in β-cell function instead of alteration in β-cell replication/neogenesis. Besides *in vivo* studies, *in vitro* study consistently showed that knockdown of Shp2 in INS-1 832/13 cells decreases insulin secretion in both basal and high glucose condition without affecting β-cell

proliferation, indicating that the defective insulin secretion was resulted from unique role of Shp2 in β-cell function.

Pancreatic Shp2 gene knockout results in age-dependently inability to handle a glucose challenge, indicating that appropriate levels of Shp2 expression in pancreatic β cells are crucial in maintaining glucose metabolism. It is well recognized that intact first-phase insulin secretion is importance in maintaining normal glucose tolerance. Studies of human subjects with impaired glucose tolerance (IGT) showed multiple defects in both qualitative and quantitative measures of insulin secretion (Byrne et al., 1996). Reduced early-phase insulin secretion in response to oral glucose was detected in subjects with normal glucose tolerance who are relatives of type 2 diabetic patients, therefore measure of insulin secretion has been used as the foremost predictor of future development of type 2 diabetes (Elbein et al., 1999; Lundgren et al., 1990). Impaired glucose tolerance in $Shp2^{panc-/-}$ mice points to a decline in β-cell function in the absence of Shp2, suggesting that strategies directly targeting Shp2 may improve β-cell function and glucose metabolism in type 2 diabetes.

2. The absence of Shp2 expression in β cells leads to abnormality of glucose sensing.

During glucose stimulated insulin secretion, glucose entry is the rate-limiting step governing the response to high glucose challenge and glucose utilization of pancreatic β cells. Type 2 diabetes has been related with polymorphisms in the glucose-sensing machinery (Ashcroft and Rorsman, 2004). A variety of monogenic forms of diabetes (MODYs) are directly connected with disruptive glucose signal transduction machinery

and consequently loss of first-phase insulin secretion of the β-cell (Porter and Barrett, 2005). The classical view of glucose sensing machinery in β cells includes a high capacity, low affinity glucose transporter-2 (Glut2), which balances the intra- and extracellular glucose signal and the high K_M glucokinase (Gck), which phosphorylates glucose to glucose-6-phospate at a rate that is very sensitive to the change of circulating glucose levels. Reduced expression level of Glut2 is a progressive factor for both type 1 and type 2 diabetes. On the other hand, glucokinase plays a dominant role in glucose sensing by exerting high control strength over the rate of glycolysis (Sweet and Matschinsky, 1997). Our studies of $Shp2^{panc-/-}$ mice showed that the expression levels of both Glut2 and glucokinase in pancreatic islets are significantly reduced in the absence of Shp2, which is consistent with *in vitro* studies by utilizing Shp2 gene silencing methodology. The impaired expression of glucose sensors by Shp2 gene deletion directly links to loss of first-phase insulin secretion and consequent glucose intolerance. The mechanism underlying the decreased expression levels of Glut2 and glucokinase is relevant to the altered transcriptional regulation of Pdx-1 in the absence of Shp2. This result suggests that appropriate expression level of Shp2 is crucial to glucose signaling machinery in pancreatic β cells.

3. Shp2 is essential for insulin gene transcription and biosynthesis in β cells.

The pancreatic β cell synthesizes and secretes appropriate amounts of insulin to maintain blood glucose levels within a narrow physiological range. The regulation of insulin production in pancreatic β cells is mediated by nutrient and hormones. Metabolic

regulation of insulin gene expression enables β cells to retain sufficient stores of intracellular insulin to sustain the secretory demand. Among these regulators, glucose and fatty acids are the most important nutrients modulating insulin gene expression in both physiological and pathological status (Prentki and Nolan, 2006). In the short term, elevated circulating glucose levels following food intake regulates insulin biosynthesis mainly at the level of translation by rapidly replenishing insulin stores and increasing insulin exocytosis. In the long term, β-cell adaption in response to increased metabolic demand such as the insulin resistance associated with obesity and aging is dependent on the transcriptional and translational regulation of insulin biosynthesis. More specifically, glucose can enhance the association of Pdx-1 with the A3 element of insulin promoter and its functional activity. Pdx-1, together with MafA and B2, act in a synergistic manner to induce insulin gene transcription. Moreover, phosphorylation and transcriptional activity of Pdx-1 are regulated by several kinases, including p38 mitogen-activated protein kinase, phosphatidylinositol-3 kinase, and extracellular signal-regulated kinases (Macfarlane et al., 1997).

Our experimental data revealed that insulin gene transcription and protein expression levels in pancreatic β cells are markedly downregulated by reducing Shp2 gene expression both *in vivo* and *in vitro*. The mechanistic studies revealed that the altered insulin gene transcription and biosynthesis are mostly resulted from the decreased expression level and transactivating activity of Pdx-1 in Shp2 deficient β cells. As mentioned before, Pdx-1 modulates insulin gene transcription in a synergistic way with other transcriptional factors and plays a dominant role, therefore reduced Pdx-1 gene transcription level exerts a profound impact on insulin gene transcription. Notably, forced

expression of exogenous Pdx1 in Shp2 knockdown INS-1 832/13 cells improved insulin production. Therefore, Pdx-1 is the main downstream target of Shp2 in control of insulin biosynthesis. We could propose to improve insulin production by promoting Shp2 activity as well as expression level in pancreatic β cells.

In addition, quantitative real-time RT-PCR results showed that gene transcription levels of NFATc1, HNF1α and HNF4α are markedly reduced by Shp2 gene silencing. Recent studies demonstrate that NFATc1 controls the expression of Ins1, Ins2 and all six genes mutated in hereditary forms of monogenic type 2 diabetes (MODY). Heterozygous mutations in gene encoding HNF-1α and HNF-4α leads to MODY3 and MODY1, respectively. Therefore, the altered gene transcription of NFATc1, HNF1a and HNF4a might partially lead to defects of insulin biosynthesis in the absence of Shp2. Moreover, recent studies reported that deletion of Shp2 in primary myoblasts during the differentiation impaired NFAT transcriptional activity and myotube multinucleation (Mara Fornaro, et al. 2006 JCB), which is consistent with our observation of decreased NFAT gene transcription by Shp2 gene silencing in pancreatic β cells. The underlying molecular basis of Shp2 modulation of gene transcription of NFATc1, HNF1α and HNF4α in β cells remains to be identified in the future work.

4. Shp2 modulates mitochondrial ATP generation in pancreatic β cells.

After glucose entry into β-cell, glucose is phosphorylated by glucokinase and undergo glycolysis and then generate ATP in mitochondrial. Glycolysis produces small amounts of ATP, but the majority (around 98%) of ATP is generated through the

mitochondrial oxidation of pyruvate, a glycolytic product of glucose (Maechler and Wollheim, 2001). In the mitochondria, pyruvate is dehydrogenated and carboxylated to assure anaplerosis (provision of carbons) to the TCA cycle. ATP is generated through the electron transfer from the TCA cycle to the respiratory chain by NADH and FADH2, and then ATP is transported from mitochondrial to cytoplasm. The ratio of ATP:ADP is subsequently elevated, which leads to the closure of ATP-sensitive K+ channels (K_{ATP}) and depolarization of the plasma membrane. The depolarization opens the voltage-gated Ca^{2+} channels and induces exocytosis of insulin-containing granules. Recent studies showed that reversible phosphorylation is involved in the regulation of mitochondrial ATP generation through the TCA cycle and oxidative phosphorylation. Disruption of PTPMT1 (*PTP* localized to the *Mit*ochondrion *1*), a member of dual-specific protein tyrosine phosphatase (DS-PTP) family, leads to marked increase of cellular ATP content and insulin secretion under both basal- and glucose-stimulated conditions in INS-1 832/13 cells.

Shp2 was the first protein tyrosine phosphatase shown to be present in mitochondria by analysis of extraction from purified rat brain mitochondria (Salvi et al., 2004), which implicates a potential role of Shp2 in regulation of mitochondrial ATP generation. Our experimental data revealed that approximate 10% of Shp2 protein is distributed in the mitochondrial compartment in INS-1 832/13 cells. Consistent with the negative regulation of mitochondrial ATP generation by PTPMT1, Shp2 gene silencing in pancreatic β cells also induced significant elevation of cellular ATP content. Notably, increased of ATP generation is opposite from decrease of insulin secretion in Shp2 knockdown cells, nevertheless these results implicated multiple functional roles of

different aspects played by Shp2 in pancreatic β cells. The gap between decreased insulin secretion and increased cellular ATP content by Shp2 knockdown is derived from impaired insulin storage and supply. On the one hand Shp2 positively regulate insulin biosynthesis by controlling insulin gene transcription, but on the other hand Shp2 negatively regulating mitochondrial ATP production through undefined mechanism. More importantly, the positive effect of Shp2 for insulin production overrides its negative role for ATP generation in the entire secretion event, therefore the output insulin secretion turns out to be positively regulated by Shp2.

The underlying molecular basis of Shp2 regulation of mitochondrial ATP generation remained to be elucidated. The further study may provide new insight into reversible tyrosine phosphorylation in the regulation of mitochondrial TCA cycle and oxidative phosphorylation.

5. Interrupting Shp2 gene leads to disrupted gene profile of several β-cell transcription factors

Numerous studies have documented that optimal expression of key transcription factors are necessary for normal pancreatic development and maintaining β-cell function. Genetic mutations of these transcription factors are directly link to pathogenesis of type 2 diabetes. Our studies revealed that gene profile of several β-cell specific genes, including Ins-1, Ins-2, Glut2, Pdx-1, glucokinase, NFATc1, Hnf1α and Hnf4α, was disrupted in pancreatic β cells by Shp2 gene silencing. It is well-known that mutations in Pdx-1, glucokinase, Hnf4α or Hnf1α can lead to monogenic forms of type 2 diabetes known as

maturity onset of the young (MODY) (O'Rahilly et al., 2005). Reduced expression of these genes is consistent with the attenuated insulin production and secretion in Shp2 knockout and knockdown β cells.

As mentioned before, Pdx-1 is an essential regulator in both islet development and β-cell function. It is required for pancreatic development during the initial stage of gut formation. A single nucleotide deletion of gene encoding human Pdx-1 leads to pancreas agenesis (Stoffers et al., 1997). In adult animals, Pdx-1 modulates the gene transcription of many pancreas-specific genes such as insulin, glucokinase, Glut2, IAPP and somatostatin. Disruption of Pdx-1 in pancreatic β cells attenuates insulin secretion and leads to age-related β-cell loss, which causes impaired glucose tolerance and maturity onset diabetes (Ahlgren et al., 1998). Pdx-1 heterozygote mouse model and non-diabetic humans with a mutation in one Pdx-1 allele display glucose intolerance and defective glucose-stimulated insulin secretion, whereas retain normal pancreatic islet morphology and pancreatic islet insulin content (Ahlgren et al., 1996; Brissova et al., 2002). The physiological consequences of Pdx-1 heterozygote mice share some common characteristic features with the phenotype of *Shp2*$^{panc-/-}$ mice, nonetheless insulin content and insulin gene transcription of *Shp2*$^{panc-/-}$ mice are markedly reduced. In *Shp2*$^{panc-/-}$ mice, Pdx-1 gene expression in islets was decreased by approximate 40%, which may lead to the similarity of phenotype exhibited by Pdx-1 heterozygote mice and Shp2 knockout mice. However, deletion of Shp2 in pancreas also results in attenuated gene expression of other transcription factors as well as reduced Pdx-1 transactivating activity, therefore insulin biosynthesis is significantly downregulated in Shp2$^{panc-/-}$ mice but not in Pdx-1 heterozygote mice. This result implicates that disrupting Shp2 gene in pancreatic β cells

may enhance susceptibility to diabetes. On the other hand, strategies specifically promoting Shp2 expression/activity in β-cell may improve β-cell function in type 2 diabetes.

6. Shp2 is a coordinator/regulator of Akt/FoxO1 and Erk pathways.

In exploring the Shp2-modulated signaling cascades upstream of Pdx1 for control of *insulin* gene expression, we have found that glucose-stimulated Akt/FoxO1 and Erk pathways are altered in Shp2-deficient cells. In previous studies (Feng, 1999; Kuhne et al., 1993; Neel et al., 2003), we and others have detected physical association of Shp2 with IRS1 and other members of the IRS family, and indeed these adaptor/scaffolding proteins share two tyrosyl residues at the C-terminal tail as Shp2-docking sites. In this study, we detected physical association of Shp2 with IRS2 and PI3K in INS-1 832/13 cells. Several reports suggest that Shp2 may regulate the PI3K pathway negatively or positively depending on cellular context or stimulators (Zhang et al., 2002). High level of glucose stimulation can enhance IRS2/Shp2 association, suggesting that Shp2 may regulate glucose-stimulated activation of the IRS2/PI3K pathway. Indeed, impaired Akt activation was detected in Shp2 knockdown INS-1 cells, leading to reduced phosphorylation of FoxO1 and increased nuclear accumulation of FoxO1. FoxO1 acts as a repressor of Pdx1 promoter, and phosphorylation of FoxO1 by Akt leads to nuclear exclusion to the cytoplasm and suppression of its transcription repressor activity. Thus, reduced Akt activity leads to nuclear accumulation of FoxO1 and suppression of *Pdx1* expression in Shp2-deficient cells. Consistent to our observation reported here, transgenic animals with

expression of a kinase-dead mutant of Akt in pancreatic β cells displayed impaired glucose tolerance and defective insulin secretion (Bernal-Mizrachi et al., 2004).

In addition to reduced Akt activation, we also show that glucose-stimulated Erk1/2 activation was blunted in Shp2 knockdown INS-1 832/13 cells, suggesting a positive role of Shp2 in promoting Erk activation by glucose and insulin, in support of previous observations in other cell types (Noguchi et al., 1994). The biochemical mechanism by which Shp2 promotes the Erk pathway has not been fully understood, and apparently different mechanisms are involved depending on cell types. The biochemical data presented here suggest that Sprouty 1 is a Shp2 substrate in β-cells, co-immunoprecipitation of Shp2 and Sprouty 1 was detected in INS-1 832/13 cells, and tyrosine-phosphorylation level of Sprouty was increased in Shp2 knockdown cells. Sprouty proteins are negative feedback modulators of receptor tyrosine kinase signaling to the Erk pathway (Mason et al., 2006). This is the first demonstration of Sprouty involvement in Shp2 regulation of Erk signaling for control of insulin synthesis in β-cells. Activated Erk has been shown to phosphorylate and regulate Pdx1 activity in control of insulin gene expression in pancreatic β-cells (Lawrence et al., 2008).

In the process of analyzing Shp2 mediated singling events, we also investigated the involvement of Shp2 in other signaling pathways, which play important roles in glucose stimulated insulin secretion. Bone morphogenetic protein 4 (Bmp4) and its high-affinity receptor, Bmpr1a, was recently reported to be critical for glucose stimulated insulin secretion of pancreatic β-cell function (Goulley et al., 2007). Mice with suppressed BMPR1A signaling in β cells displayed reduced expression of essential genes

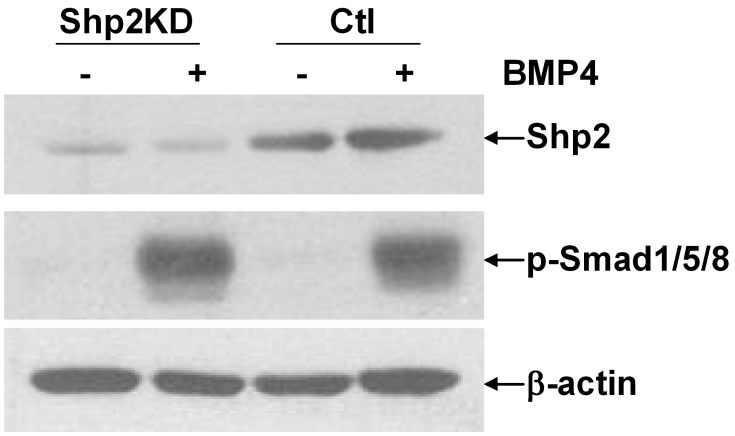


Figure 31. Immunoblot analysis of expression of Shp2, p-Smad1/5/8 and β-actin (loading control) in Shp2 knockdown and control cells treated with or without BMP4.

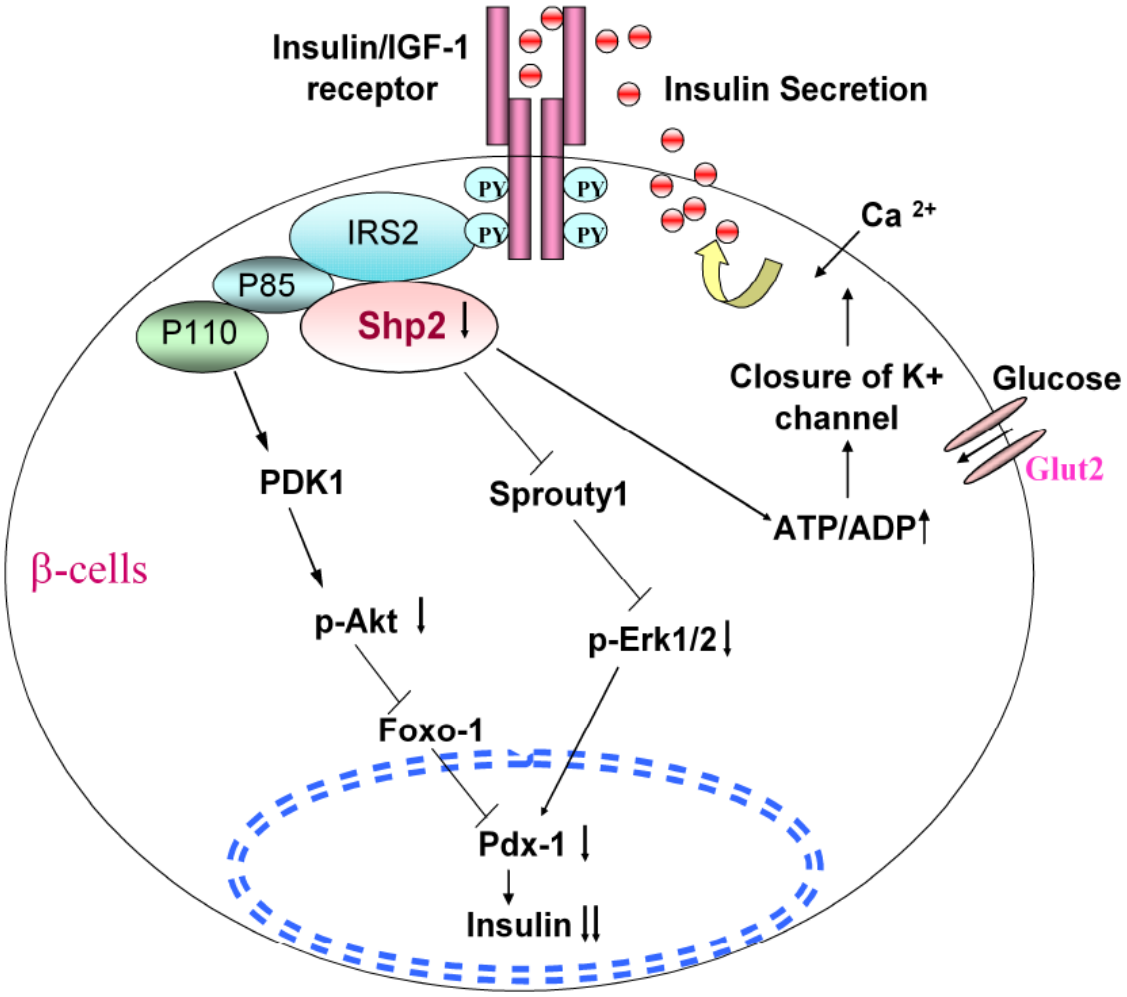


Figure 32. A model for Shp2 orchestration of signaling events controlling insulin biosynthesis in pancreatic beta cells.

involved in insulin gene expression, proinsulin processing, glucose sensing, stimulus coupling insulin secretion, incretin signaling, and insulin exocytosis and develop diabetes due to impaired insulin secretion. On the other hand, enhanced expression of Bmp4 in β cells markedly elevated GSIS and glucose handling capability. Systemic treatment of BMP4 molecules to adult mice can improve glucose disposal ability and insulin secretion in a mouse model of glucose intolerance. In our study, phosphorylation of Smad1/5/8, a downstream target of BMP4 signaling pathway, exhibited no alteration in Shp2 knockdown cells as compared with control (Figure 31). Therefore, our results excluded the possibility of Shp2 regulation of BMP4 signaling in pancreatic β cells, which implicates that the modulation of Shp2 in mediating insulin signaling is unique and specific.

Taken together, the impaired insulin synthesis in Shp2-deficient cells is due to reduced expression and activity of Pdx1, resulting from defects in PI3K/Akt/FoxO1 and Erk1/2 activation, respectively. A working model for Shp2 orchestration of signaling events controlling insulin biosynthesis and secretion in pancreatic β cells is summarized in Figure 32.

7. Future Perspective

Previous studies showed that β-cell-specific insulin receptor knockout (βIRKO) and IGF1 receptor knockout (βIGFRKO) displayed normal β-cell proliferation and development, indicating that insulin/IGF1 is not crucial for early development of islet β-cells (Kulkarni et al., 1999; Kulkarni et al., 2002). Results shown here also suggest that

Shp2 has a limited role in pancreatic development, but is a critical player in regulation of β-cell function in adults. However, these studies do not rule out the possibility that a failure of observing developmental defect is due to partial deletion of loxp-floxed target gene sequence by Pdx1-Cre in conditional gene knockout mouse models.

Further study evaluated the insulin/IGF1 receptor function in β-cell compensatory growth by feeding βIRKO and βIGFRKO with high-fat diets. βIRKO, instead of βIGFRKO, displayed defective islet growth with high-fat diet challenge, indicating that insulin signaling is critical for compensatory β-cell growth instead of early β-cell growth. Our study also indicates that deletion of Shp2 in pancreas, although not causing diabetes, may increase susceptibility to diabetes. In the absence of progressive factors, subjects with susceptible Shp2-deficient β cells exhibit glucose intolerance, impaired glucose stimulated insulin secretion and attenuated insulin biosynthesis, but still maintain glucose homeostasis and normal β-cell mass. If defective insulin production and secretion in Shp2 null subjects are in conjunction with increased metabolic demand in states such as pregnancy, obesity, ages and high-fat diets, diabetes may ensue. Thus, reduced Shp2 expression or activity, from either genetic or environmental factors, may represent a significant risk for type 2 diabetes. Herein I speculate that to challenge Shp2 knockout animals with high fat diet for 20 weeks will induce severe hyperglycemia and early onset of diabetes. To monitor Shp2 expression level/activity in pancreatic β cells in human subjects would be beneficial to diagnosis and treatment of type 2 diabetes.

On the basis of our studies of $Shp2^{panc-/-}$ animal model, we postulate that constitutively activating Shp2 in pancreatic β-cells might improve insulin secretion,

glucose handling and glucose homeostasis in diabetic mice. To testify this postulation, we can create Pdx1-Shp2^{active} mouse model and cross them with existing diabetic mouse model. To this end, we can take advantage of the existing Tet-ON system and Shp2D61A/E76A constitutively active mutant to generate pancreatic-specific and inducible Shp2^{active} animal model. This will allow us to evaluate whether activating Shp2 can improve β−cell function in diabetic model and provide direct evidence of Shp2 as a useful therapeutic target for treating type 2 diabetes.

8. Concluding remarks

In this study, we generated conditional mutant mice (Shp2$^{Panc-/-}$) with Shp2 specifically deleted in the pancreas. *Shp2$^{Panc-/-}$* mice exhibit reduced glucose-stimulated insulin secretion and progressive impairment of glucose tolerance. Shp2-deficient β-cells have attenuated insulin gene expression and reduced insulin content. Consistently, siRNA-mediated Shp2 knockdown in INS-1 832/13 cells resulted in significantly decreased insulin secretion/biosynthesis and disrupted expression profile of several β-cell specific genes. Biochemical analyses revealed defects in the Akt/FoxO1 and Erk pathways, leading to decreased Pdx1 expression/activity and insulin biosynthesis in Shp2-deficient β-cells. Together, this study identifies Shp2 as a novel coordinator of multiple signals controlling insulin gene transcription and production in β-cells.

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