# P38 ALPHA MAPK REGULATES FOXO1 AND HEPATIC GLUCOSE PRODUCTION IN HEALTH AND DISEASE

A Dissertation

by

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## ABSTRACT

Glucagon governs hepatic glucose production (HGP) by triggering glycogenolysis and gluconeogenesis. The p38α MAPK (p38α) is activated by glucagon to promote hepatic glucose production (HGP), but underlying mechanisms have not been well elucidated. In this study, we showed that p38α is activated by glucagon through the cAMP–EPAC pathway in primary hepatocytes in control of Foxo1-S273 phosphorylation. In both hepatocytes and mice, inhibition of p38α blocked Foxo1-S273 phosphorylation, decreased Foxo1 protein level, and impaired glucagon- and fasting-induced HGP. However, the effect of p38α inhibition was abolished by Foxo1-deficiency or Foxo1-S273A or D mutation. Moreover, the p38α-regulated Foxo1 pathway plays an indispensable role in stimulating HGP in inflammatory hepatocytes and insulin resistant mice. This study reveals a novel mechanism of p38α by which it regulates Foxo1-S273 phosphorylation to mediate the action of glucagon on glucose homeostasis.

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### Contributors

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## NOMENCLATURE

| | |
|---|---|
| HGP | Hepatic Glucose Production |
| PKC | Protein Kinase C |
| PKA | Protein Kinase A |
| HNF3α | Hepatocyte Nuclear Factor 3α |
| GLP1/2 | Glucagon-like Peptide 1/2 |
| GLUT1/2 | Glucose Transporter 1/2 |
| GIP | Gastric Inhibitory Polypeptide |
| GCGR | Glucagon Receptor |
| CREB | cAMP Responsive Element Binding Protein |
| EPAC | Exchange Protein Directly Activated by Camp |
| PGC-1α | Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1- α |
| PCK | Phosphoenolpyruvate Carboxykinase |
| G6PC | Glucose-6-Phosphatase |
| CaMKII | Calcium/Calmodulin-Dependent Protein Kinase II |
| CBP | CREB Binding Protein |
| CRTC2 | CREB Regulated Transcription Co-activator 2 |
| FoxOs | Forkhead Box Class Os |
| C/EBP | CCAAT/Enhancer-Binding Protein |
| RORs | Retinoid-Related Orphan Receptors |
| SIRT1 | Sirtuin 1 |

| | |
|---|---|
| PRMT1 | Protein Arginine Methyltransferase 1 |
| CREBH | cAMP Response Element-Binding Prote |
| PDC | Pyruvate Dehydrogenase Complex |
| PDK | Pyruvate Dehydrogenase Kinase |
| FBPase-1 | Fructose-2,6-Bisphosphatase |
| IRS | Insulin Receptor Substrate |
| IR | Insulin Receptor |
| MAPK | Mitogen-Activated Protein Kinase |
| ERK | Extracellular-Signal-Regulated Kinase |
| JNK | JUN N-Terminal Kinase |

**TABLE OF CONTENTS**

Page

ABSTRACT ..... ii

ACKNOWLEDGEMENTS ..... iii

CONTRIBUTORS AND FUNDING SOURCES ..... iv

NOMENCLATURE ..... v

TABLE OF CONTENTS ..... vii

LIST OF FIGURES ..... ix

CHAPTER I INTRODUCTION ..... 1

- The Discovery of Glucagon ..... 1
- Transcriptional and Posttranslational Regulation of Proglucagon ..... 2
- Regulation of Glucagon Secretion ..... 5
- Hepatic Glucagon Signaling ..... 6
- Regulation of Gluconeogenesis ..... 8
  - Regulation of Gluconeogenesis by Multiple Transcription Factors and Co-regulators ..... 9
  - Substrates Availability Controls Gluconeogenesis ..... 12
  - Hormonal Regulation in Gluconeogenesis ..... 13
- Targeting Glucagon Signaling in The Treatment of Type 2 Diabetes ..... 15
- The Role of FoxO Proteins in Regulation of Glucose and Lipid Metabolism ..... 19
- The Effect of MAPK p38 on Glucose Homeostasis ..... 23
- Specific Aims of This Thesis ..... 25

CHAPTER II PHYSIOLOGICAL RELEVANCE OF P38 TO REGULATION OF HGP ..... 26

- Introduction ..... 26
- Methods ..... 28
- Results ..... 31
  - P38α Promotes Glucagon-Induced HGP in Both Hepatocytes and Mice ..... 31
  - P38α-Mediated HGP Depends on Foxo1 ..... 33
- Summary ..... 39

CHAPTER III THE ROLE OF INSULIN SIGNALING PATHWAY IN P38 REGULATION OF HGP ..... 40

Introduction ..... 40
Methods ..... 42
Results ..... 44
Summary ..... 47

CHAPTER IV MECHANISTIC INSIGHTS: P38-FOXO1 INTERACTION IN REGULATION OF HGP ..... 48

Introduction ..... 48
Methods ..... 49
Results ..... 54
P38 Increases Foxo1 Protein Stability ..... 54
P38α Promotes Foxo1 Protein Stability via Foxo1-S273 Phosphorylation ..... 56
P38α Phosphorylates Foxo1-S273 Directly and Indirectly to Enhance Glucagon Signaling ..... 58
Glucagon Activates P38 Through cAMP-EPAC Pathway ..... 62
Foxo1-S273 Mutation Blocks the Effect of P38α on HGP in Primary Hepatocytes ..... 64
Foxo1-S273 Mutation Impairs P38α-Mediated HGP in vivo ..... 68
Summary ..... 76

CHAPTER V P38 → FOXO1 SIGNALING IN INSULIN RESISTANCE AND INFLAMMATION ..... 78

Introduction ..... 78
Methods ..... 79
Results ..... 82
Summary ..... 87

CHAPTER VI FINAL SUMMARY ..... 88

REFERENCES ..... 95

APPENDIX TABLES ..... 130

## LIST OF FIGURES

Page

Figure 1 p38α promotes hepatic glucose production in response to glucagon and fasting ..... 32

Figure 2 p38α-mediated HGP depends on Foxo1 ..... 34

Figure 3 The activity of Foxo1 and p38 in response to glucagon and fasting ..... 38

Figure 4 p38-mediated HGP is independent of insulin signaling ..... 46

Figure 5 p38α increases Foxo1 nuclear translocation and protein stability ..... 55

Figure 6 p38α stimulates Foxo1-S273 phosphorylation to increase Foxo1 activity ..... 57

Figure 7 p38α and PKA synergistically increase Foxo1 activity and promote HGP ..... 59

Figure 8 p38α directly phosphorylates Foxo1 at S273 ..... 61

Figure 9 cAMP-EPAC pathway mediates glucagon-induced p38 activation ..... 63

Figure 10 The activation of p38 by glucagon is independent of CaMKII ..... 65

Figure 11 p38α-induced HGP requires Foxo1-S273 phosphorylation in hepatocytes ..... 67

Figure 12 p38α overexpression-induced HGP is attenuated by Foxo1-S273 mutation ..... 69

Figure 13 p38α promotes Foxo1 nuclear translocation via Foxo1-S273 phosphorylation ..... 70

Figure 14 *Foxo1*$^{S273D}$ mice show resistance to SB203580-suppressed HGP ..... 72

Figure 15 Foxo1-S273D mutation blocks p38α-induced HGP in vivo ..... 74

Figure 16 Foxo1-S273A mutation abolishes p38α-induced HGP in vivo ..... 75

Figure 17 Diagram of mechanism by which p38α regulates glucagon-induced hepatic glucose production ..... 77

Figure 18 p38α→ Foxo1 signaling regulates HGP in the inflammatory state ..... 83

Figure 19 p38α→ Foxo1 signaling contributes to glucose dysregulation in insulin resistant mice ..... 86

Figure 20 Diagram of the mechanisms by which p38α regulates glucagon-induced hepatic glucose production ..... 94

# CHAPTER I

# INTRODUCTION

## The Discovery of Glucagon

Glucagon is a peptide hormone comprised of 29 amino acids and was firstly discovered as a hyperglycemic factor in the insulin extracts secreted from the pancreas (1). In 1889, Oskar Minkowski and Josef von Mering found that pancreas removal in dogs, rather than ligation of the pancreatic duct, led to diabetes, suggesting that pancreas is an important organ in maintaining glucose homeostasis and some factors secreted from pancreas regulate blood glucose (2). Subsequently, other scientists proved that pancreatic extracts successfully lower blood glucose in diabetic dog (3). In 1921, Frederick G. Banting firstly isolated the secretions from the islet cells and tout them as a potential treatment for diabetes (4). Interestingly, pancreatic extracts and insulin preparations led to a brief increase in blood glucose prior to the heralded decrease in blood glucose (5). Although the hyperglycemic effect of pancreatic extracts was ignored as an unimportant impurity, Kimball and Murlin discovered a pancreatic fraction that elevated blood glucose when injected into rabbits and dogs. This finding indicates that pancreas generates a specific substance that counteracts the hypoglycemic effect of insulin. They named this factor “the glucose agonist”, that is “glucagon” (1). Furthermore, with the efforts of Burger, Kramer, and Brandt in 1929, glucagon was demonstrated to mediate hyperglycemic effect through gluconeogenesis in liver and was reclassified as “the hyperglycemic-

glycogenolytic (H-G) factor". However, glucagon was not purified and crystallized until 1950s. Earl Sutherland successfully purified H-G factor (glucagon) from pancreas and proved its ability to increase blood glucose (6). His finding also indicates that glucagon may originate from the α-cells. Specific impairment of α-cells in pancreas using cobaltous chloride and synthalin A led to decrease in the content of glucagon in pancreas and increase in hypoglycemic effect of insulin, which further confirms that glucagon is produced in the α-cells (7). In 1953, Staub Sinn and Behren announced the preparation of crystalline glucagon and then Bromer et al. determined the amino acid sequence of glucagon, which paves the way for understanding the biological activity of glucagon at molecular level (8-10). Due to this, glucagon antibodies were produced and radioimmunoassay was established to detect glucagon (11). Finally, the discovery of glucagon makes it possible to treat severe hyperglycemia in clinic (12).

## Transcriptional and Posttranslational Regulation of Proglucagon

Glucagon is generated from proglucagon genes. The proglucagon gene is located on the long arm of human chromosome 2 and consisted of 6 exons and 5 introns, which encodes a precursor containing glucagon and two additional glucagon-like peptides (13). Pancreas, intestine, and brain (brain stem and hypothalamus) exhibit the expression of proglucagon gene, with structurally identical single mRNA transcript (14; 15). Pancreatic proglucagon gene expression is increased by fasting and hypoglycemia and inhibited by insulin. Several factors play a role in regulation of proglucagon gene expression in the

pancreas, including 1) protein kinase C (PKC) signaling pathway; 2) cAMP/protein kinase A (PKA) signaling pathway; 3) membrane depolarization and calcium influx. The mouse studies have indicated that approximately 900 kb of rat proglucagon gene 5'-flanking sequence specifically drives the transcription of proglucagon gene in the pancreatic α-cells and brain neurons (16). Transcription factors, including Pax-6, Foxa1, Cdx-2/3, Isl-1, Brn4, and c-Maf, bind to proximal promoter region of proglucagon gene, mediating its expression (17). The *Pax6* homozygous mutant mice lack glucagon-producing cells, suggesting Pax6 is important for the differentiation of α-cells in mice (18). Sander et al. further showed that Pax6 mutation results in decreased glucagon production and proglucagon mRNA expression in pancreas (19). Hepatocyte nuclear factor 3α (HNF-3α/Foxa1) null mutation mice show hypoglycemia, decreased proglucagon mRNA level, and reduced plasma glucagon level, suggesting Foxa1 plays an key role in proglucagon gene expression (20). Insulin inhibits proglucagon gene expression in α-cells via insulin-responsive element in the promoter region. Mckinnon et al. found that insulin downregulates proglucagon gene expression in α-cells through nuclear exclusion of the transcription factor Foxo1 (21).

The proglucagon mRNA is encoded to a 180-amino acid precursor protein that generates different protein profiles via tissue specific posttranslational precession in the pancreas, brain, and intestine (15). In general, the 18 kDa mammalian proglucagon is composed of three homologous hormonal sequences, glucagon, glucagon-like peptide 1 (GLP1), and glucagon-like peptide 2 (GLP2), which are separated by two intervening peptides, IP-1 and IP-2, and preceded by the N-terminal domain, glicentin-related

polypeptide (GRPP). The peptides are linked by basic amino acids (Lys-Arg or Arg-Arg) that are cleavage sites identified by enzyme during the precession. The discovery of the novel family of mammalian proteases has provided new perspective into the cellular mechanism of the most prohormones, neuropeptide precursors, and various other precursor proteins within the secretory pathway (22; 23). The prohormone convertases, including PC1/3 and PC2, play an important role in regulation of proglucagon processing. Rouille et al. demonstrated that PC2 mediates the efficient procession of glicentin into glucagon and PC3 is responsible for release of GLP1 from the C-terminal domain of proglucagon (24). The pancreas α-cell mainly processes proglucagon into glucagon, IP-1, and major proglucagon fragment (MPCF) that is a 10 kDa peptide containing the GLP-1, IP-2, and GLP-2 sequences (25; 26). Proglucagon firstly generates glicentin and MPGF through the cleavage at the $Lys^{70}$-$Arg^{71}$ site. Subsequently, glicentin is cleaved at the $Lys^{31}$-$Arg^{32}$ and $Lys^{62}$-$Arg^{63}$ sites, producing GRPP, glucagon, and IP-1, whereas MPCF is slightly and slowly cut into GLP-1-amide through the cleavage at $Arg^{109}$-$Arg^{110}$ site (24). However, proglucagon is processed into GLP-1, IP-2, and GLP-2 in the intestinal L cells (27). The N-terminal domain of proglucagon is incompletely processed into glicentin and partially cleaved into GRPP and oxyntomodulin. Thus, glucagon level is very low in the intestine (24; 28). The explanation for this specific procession of proglucagon may be attributed to the different expression levels of hormonal convertase in the α and L cells. The pancreatic α cells show high expression levels of PC2 and low expression levels of PC3 (29; 30). In contrast, the intestinal L cells exhibit PC3 activity but not PC2 (31). In

addition to glucagon, GLP-1, and GLP-2, the physiological functions of glicentin-related peptide, intervening peptide, and major proglucagon fragment are not identified.

## Regulation of Glucagon Secretion

The mechanism of regulation of glucagon secretion in α cells is similar to that in control of insulin secretion in β cells, which is sensitive to the blood glucose concentration. Glucagon is stimulated to release from α cells when blood glucose decreases (Hypoglycemia/ Fasting condition), in contrast to which the release of insulin is initiated by the elevation of blood glucose (Hyperglycemia/Feeding condition). Therefore, vascular system in the Langerhans islets is well established to ensure rapid glucose sensing.

Glucose transporter 1 (GLUT1) and glucose transporter 2 (GLUT2) mediate the glucose uptake in the Langerhans islets. Both GLUT1 and GLUT2 are expressed in β cells, whereas α cells only show the signal of GLUT1. GLUT1 has a low $K_m$ value, indicating its high affinity to glucose, which ensures continuous and rapid uptake of glucagon to regulate secretion of insulin and glucagon in the islet of Langerhans (32; 33). The ATP-sensitive potassium ($K_{ATP}$) channels plays an important role in control insulin and glucagon secretion through translating the extracellular glucose concentration variation to alternation in membrane potential (34). In α cells, due to a higher ATP sensitivity of $K_{ATP}$ channels, the lower intracellular ATP level has a certain suppressing role in $K_{ATP}$ channels (35). Thus, under low blood glucose condition, moderate $K_{ATP}$ channels activity results in opening of $Na^+$ and $Ca^{2+}$ channels and triggers glucagon secretion via granules exocytosis.

However, high glucose level (high ATP) inhibits $K_{ATP}$ channels and depolarizes the membrane potential to a point where $Na^+$ and $Ca^{2+}$ channels are blocked. This leads to the inhibition of glucagon secretion (34).

Many factors in the circulation system regulate glucagon secretion in α cells. Insulin receptor is expressed in α cells. Thus, insulin directly targets α cells to inhibit glucagon secretion through PI3K activation (36). Gamma aminobutyric acid (GABA) secreted from β cells enhances glucose-induced glucagon release (37). In 1929, a fraction from gut extracts with glucose-lowering effect through promoting insulin secretion was identified, which was named for incretin (intestine, secretion, insulin) (38). Gastric inhibitory polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) are two major incretin hormones produced from K cells and L cells in the small intestine, respectively (38). Both GIP and GLP-1 inhibit glucagon release through stimulation of insulin secretion rather than directly targeting α cells (39-42). In humans, arginine administration elevates both insulin and glucagon levels in circulation (43; 44). Somatostatin is mainly secreted from the D cells in duodenum, the brain, and the δ cells in pancreas (45). In α cells, somatostatin suppresses glucagon secretion via stimulation of $K^+$ channels that leads to hyperpolarization and VDCC closure (46).

## Hepatic Glucagon Signaling

Glucagon transduces its cascade signaling through binding to glucagon receptor (GCGR) located at the cell surface, exerting its biological action (47). Glucagon receptor

is expressed in liver, kidney, heart, adrenal glands, and adipose tissue, as well as detected in a low amount in stomach, duodenum, and brain (48) ; this results indicate that glucagon potentially has a general effect on many tissues.

The binding of glucagon to GCGR extracellular loop renders conformational alternation of GCGR, subsequently stimulating the activity of coupled G proteins (49). At least two classes of G proteins are activated by glucagon-GCGR binding, including $G_{s\alpha}$ and $G_{q.}$ Glucagon transduces its signal cascade through cellular accumulation of two second messengers, cAMP and calcium (47). Stimulatory G protein ($G_s$) is associated with GCGR in a multimeric configuration. GCGR activation leads to a conformational change of $G_s$ protein, and subsequently GTP binds to $G_{s\alpha}$ subunit, which triggers its release from G protein complex ($G_{s\alpha}$- $G_{s\beta}$- $G_{s\gamma}$). GTP-$G_{s\alpha}$ interacts and stimulates adenylate cyclase, increases cellular cAMP levels, and subsequently triggers the initiation of signaling events, including PKA, EPAC, and CRTC2/TORC2 (50). cAMP promotes PKA catalytic and regulatory subunits dissociation through binding to PKA regulatory subunits. The released catalytic subunits become active and enter the nucleus via passive diffusion, phosphorylating cAMP responsive element binding protein (CREB) at S133 and promoting its transcriptional activity (51). Active PKA also phosphorylates Foxo1 at S273, which increases Foxo1 transcriptional activity (52). Exchange protein directly activated by cAMP (EPAC) is another family of cAMP sensor proteins. EPAC may act independently of PKA, but converge synergistically with each other in regulation of some cellular events (53). CREB and Foxo1 promote gluconeogenesis through upregulation of gluconeogenic gene expression, such as peroxisome proliferator-activated receptor

gamma coactivator 1- α (PGC-1α), phosphoenolpyruvate carboxykinase (PCK), and glucose-6-phosphatase (G6PC) (54-57). PKA activation leads to glycogen phosphorylase via regulation of phosphorylase kinases, thereby promotes glycogen breakdown (58). The activation of $G_q$ stimulates the activity of phospholipase C, leading to the production of inositol 1,4,5-trisphosphate ($IP_3$), which induces downstream signals through increased $Ca^{2+}$ release from ER (49). In addition, cAMP-PKA signaling may also play a role in stimulation of cellular $Ca^{2+}$ mobilizing (59). Calcium-induced calcium/calmodulin-dependent Ser/Thr-phosphatase calcineurin (PP3CA) and calcium/calmodulin-dependent protein kinase II (CaMKII) promote gluconeogenesis through regulating Foxo1 and CREB transcriptional activity (59; 60). Thus, PKA, PLC, and calcium signaling are major mediators in glucagon action.

## Regulation of Gluconeogenesis

In mammals, gluconeogenesis takes place mainly in the liver, and to a lesser extent in renal cortex and in the epithelial cells that line the inside of the small intestine. During prolonged fasting, hepatic glycogen is depleted and hepatocytes synthesize glucose through gluconeogenesis using lactate, pyruvate, glycerol, amino acid as precursors. Some enzymatic reactions of glucogenesis are the reverse of glycolytic reactions. However, three reactions of glycolysis are essentially irreversible in vivo and cannot be used in gluconeogenesis: 1) conversion of glucose to glucose-6-phosphate by glucokinase; 2) phosphorylation of fructose-6-phophate to fructose-1,6-phosphate by

phosphofructokinase-1; 3) conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase. Conversion of pyruvate to phosphoenolpyruvate requires two exergonic reactions. Firstly, pyruvates carboxylase converts pyruvate to oxaloacetate in mitochondria. Since the mitochondrial membrane has no transporter for oxaloacetate, oxaloacetate muse be reduced to malate by mitochondrial malate dehydrogenase before export to the cytosol. Secondly, the oxaloacetate is converted to phosphoenolpyruvate via phosphoenolpyruvate carboxykinase. Conversion of fructose-1,6-biphosphate to fructose-6-phosphate is catalyzed by $Mg^{2+}$-dependent fructose-1,6-biphosphatase (FBPase-1) that promotes the essentially irreversible hydrolysis of the C-1 phosphate.

*Regulation of Gluconeogenesis by Multiple Transcription Factors and Co-regulators*

Hepatic gluconeogenesis is controlled by many transcription factors. CREB and forkhead box class Os (FoxOs) are two key transcriptional factors that directly regulate gluconeogenic genes transcription and promote the hepatic gluconeogenesis. The corresponding co-factors, such as PGC-1α, CREB binding protein (CBP/P300), and CREB regulated transcription co-activator 2 (CRTC2), contribute to gluconeogenesis through modulating CREB and FoxOs transcription activity (61).

During fasting state, glucagon secreted from pancreatic α cells activates CREB by stimulating phosphorylation at S133 via cAMP-PKA pathway. Active CREB enters into nucleus and initiate the transcription of *PCK*, *G6PC*, and *PGC-1α*. The transgenic mice with inhibition of CREB display hypoglycemia and decreased HGP (62). FoxOs (Foxo1, 3, 4) synergistically promote hepatic gluconeogenesis, among which Foxo1 has been tightly associated with hepatic gluconeogenesis (54; 56). Foxo1 is a major downstream

target of insulin, whereby insulin regulates HGP (63). Besides, Foxo1 is also regulated by glucagon-induced cAMP-PKA pathway, contributing to glucagon-induced gluconeogenesis (52). Liver specific inactivation of Foxo1 impairs both glycogenolysis and gluconeogenesis, resulting in hypoglycemia (55). PGC-1α binds and co-activates Foxo1, increasing Foxo1-induced gluconeogenic gene transcription (64). The CCAAT/enhancer-binding protein (C/EBP) family, especially C/EBPα and C/EBPβ, synergistically regulate gluconeogenesis. C/EBPα deletion decreases gluconeogenic genes expression, leading to hypoglycemia-induced neonatal death (65). C/EBPα contains different regulatory domains sensitive to contexts. Alanine mutation at the residues dephosphorylated upon fasting leads to upregulation of gluconeogenic genes and glucose intolerance (66). C/EBPβ promotes gluconeogenesis without increasing gluconeogenic genes levels and triggers fatty acid release (67). Retinoid-related orphan receptors (RORs) α, a nuclear receptor, promotes glucose production through upregulation of gluconeogenic genes expression in liver (68), whereas REV-ERB α, another nuclear receptor, suppresses gluconeogenic genes expression and glucose release through recruitment of the nuclear receptor corepressor–histone deacetylase 3 (NCoR-HDAC3) (69).

Multiple co-regulators directly or indirectly modulate the activity of CREB and Foxo1, involving the regulation of hepatic gluconeogenesis. Upon fasting, CRTC2/TORC2 is dephosphorylated and enters into nucleus, where it enhances CREB activity by forming CREB-CBP-CRTC2 complex (57; 70). Deletion of CRTC2 leads to a decrease in blood glucose and attenuation in gluconeogenesis (71). Nutrient-sensing deacetylase sirtuin 1 (SIRT1) deacetylates CRTC2, which leads to CRTC2 degradation

and reduced gluconeogenesis after prolonged fasting (72). Energy-sensing kinase AMPK inhibits the activity of CREB-CBP-CRTC2 complex by phosphorylation of CRTC2 and dampening its nuclear accumulation (57). In addition to CRTC2, AMPK also triggers phosphorylation of CBP at S436 via $PKC_{i/\lambda}$, disrupting CREB-CBP-CRTC2 complex and suppressing hepatic gluconeogenesis (73). Foxo1 activity is regulated by CBP-catalyzed acetylation (74). The mice with constitutively deacetylated Foxo1 have an increased fasting blood glucose and elevated hepatic glucose output (75). Cryptochrome 1 (CRY1) is a member of circadian clock genes and reported to regulate hepatic gluconeogenesis. CRY1, induced by insulin-SREBP1c pathway, promotes Foxo1 degradation to inhibit hepatic gluconeogenesis (76). CRY1 also suppresses hepatic glucose output via inhibiting GRs and uncoupling GCGR from $G_{\alpha}$ protein (77; 78). Protein arginine methyltransferase 1 (PRMT1), as a co-activator of Foxo1, catalyzes arginine methylation of Foxo1 at 248 and 250, thereby promoting nuclear translocation and enhancing glucose output (79; 80). Transcriptional coactivator PGC-1α, induced by fasting, upregulates gluconeogenic genes levels and glucose output through cooperation with hepatic nuclear factor-4α (HNF-4α) (81). SIRT1 induces hepatic glucose output by deacetylation of PGC-1α (82). cAMP response element-binding protein H (CREBH) is an endoplasmic reticulum (ER)-bound transcription factor and is involved in hepatic gluconeogenesis induction via binding to CRTC2 (83). ER stress-induced unfolded protein response (UPR) regulates hepatic gluconeogenesis. Three UPR are mediated through three major factors: protein kinase-like endoplasmic reticulum kinase (PERK)/eIF2α; protein kinases inositol-requiring enzyme 1 (IRE1)/XBP1; and transcription factor ATF6 (84). PERK/eIF2α mediates ER stress-

induced hepatic glucose production through upregulating C/EBP levels (85). However, IRE1/XBP1 and ATF6 inhibit hepatic gluconeogenesis through promoting Foxo1 degradation and disrupting CREB-CRTC2 interaction, respectively (86; 87).

*Substrates Availability Controls Gluconeogenesis*

In addition to the activity of gluconeogenic enzymes, gluconeogenesis rate is regulated by substrates availability. Pyruvate, the product of glycolysis, has two fates; it can be converted to lactate that is utilized to generate glucose or pyruvate is catabolized by pyruvate dehydrogenase complex (PDC) into Acetyl-CoA that enters TCA cycle for ATP production. Pyruvate dehydrogenase kinases (PDKs) phosphorylates and inactivates PDC, which is abrogated by pyruvate dehydrogenase phosphatase (88). In fasted and diabetic mice, PDK2 and PDK4 show higher expression levels in liver, muscle, and heart tissues (88). PDK4 deficiency leads to hypoglycemia in mice due to limited supply of pyruvate for gluconeogenesis induced by high PDC activity (89). The productions of lipolysis, glycerol and nonesterified fatty acids (NEFAs), also contribute to gluconeogenesis. Glycerol release from lipolysis promotes gluconeogenesis in “substrate push” mechanism (90). NEFAs are not direct substrate of gluconeogenesis, but it can be oxidized into Acetyl-CoA during β-oxidation. Acetyl-CoA is an allosteric activator of pyruvate carboxylase, which increases hepatic gluconeogenesis (91; 92). During long-term fasting, protein degradation and amino acids release are involved in gluconeogenesis by the supply of substrates. Therefore, the available substrates play a key role in regulation of gluconeogenesis.

*Hormonal Regulation in Gluconeogenesis*

Glucagon plays a dominant role in stimulating gluconeogenesis. Catecholamines also promote hepatic gluconeogenesis through cAMP-PKA signaling (93). In addition to the regulation of gluconeogenic genes expression, glucagon and catecholamines inhibit pyruvate kinase in liver and phosphorylate the fructose-2,6-bisphosphatase 1 (FBPase-1) at S36, thereby favoring hepatic gluconeogenesis. FBPase-1, activated by phosphorylation, decreases cellular concentration of fructose-2,6-bisphosphate that is an allosteric inhibitor of fructose-2,6-bisphosphatase 1 (FBPase-1)- a gluconeogenic enzyme (94; 95). Asprosin is an adipose-derived peptide hormone and stimulates hepatic glucose production (HGP) through hepatic cAMP-PKA signaling (96). Growth hormone (GH) and glucocorticoids (GCs) are important in normal growth, development, and whole body energy homeostasis (97; 98). GH signaling cascade is transduced by GH receptor (GHR), leading to activation of RAS/RAF/ERK and JAK/STAT pathway (99; 100). GH increases expression levels of PCK and G6PC through activation of JAK/STAT pathway to promote hepatic gluconeogenesis (101). Besides, GH induces PDK4 expression level through STAT5 and subsequently suppresses the oxidation of pyruvate by PDC, whereby contributing to gluconeogenesis in liver (102). The gluconeogenic role of GH is attenuated by bile acids and fibroblast growth factor (FGF) 21. The suppressive action of bile acids on GH is mediated by activation of farnesoid X receptor (FXR) that inhibits STAT5-induced PCK and PDK4 expression through stimulating SHP, a transcription repressor (101-103). FGF21 is a hormone induced by fasting through peroxisome proliferator-activated receptor α (PPARα). FGF21 inhibits GH-induced STAT5 and blocks GH-mediated

hepatic gluconeogenesis, leading to GH resistance (104). Glucocorticoids function through binding to glucocorticoids receptor (GR), a member of nuclear receptor family. Upon glucocorticoids binding, GR enters into nucleus where it initiates the gluconeogenic genes transcription (105). GR abrogation in liver decreases gluconeogenic genes expression and reduces blood glucose level during fasted condition, protecting against hyperglycemia in both STZ treated and *db/db* mice (106; 107). Yin Yang 1 (YY1) is a critical transcription factor in regulation of cell proliferation, differentiation, and hepatic gluconeogenesis. YY1 expression is induced in liver from fasted and insulin resistant mice. YY1-mediated hepatic gluconeogenesis relies on the modulation of GR expression (108). Live X receptor (LXR) is also a nuclear receptor. Upon oxysterols, LXR is activated and competes to bind to GR binding sites to inhibit gluconeogenic genes expression and suppress hepatic gluconeogenesis (109; 110).

Insulin controls blood glucose, stimulates glucose flux, and inhibits hepatic gluconeogenesis (111). Insulin action is mediated through insulin receptor (IR). Insulin binding to IR α subunit suppresses its inhibition on tyrosine kinase activity of IR β subunit, which induces tyrosine phosphorylation of insulin receptor substrates (IRS). IRS acts as docking site for Src homology-2 (SH2) domain contained proteins, such as PI3K, regulating their activities. Thus, insulin binding to IR activates PI3K/Akt pathway through tyrosine phosphorylation of IRS (112). Liver *IRS1* and *IRS2* double knockout mice exhibit hyperglycemia, hyperinsulinemia, and increased hepatic gluconeogenesis, contributing to the development of type 2 diabetes (113-115). Insulin stimulates Akt phosphorylation at S473 through mTORC2, which subsequently Foxo1 phosphorylation at S24, 256, and 319,

promoting nuclear exportation and protein degradation and suppressing hepatic gluconeogenesis (63; 116). Foxo3 and Foxo4 also contribute to hepatic gluconeogenesis, which are inhibited by insulin-induced PI3K/Akt pathway (54-56). Besides, insulin-induced Akt phosphorylates and inhibits PGC-1α, whereby abolishing PGC-1α-mediated gluconeogenic genes expression (117). Insulin abrogates TORC2 activity through activation of Ser/Thr kinase SIK2 to block gluconeogenesis in liver (118). Insulin also dismantles CREB-CBP-CRTC2 complex through PKCλ-induced CBP phosphorylation at S436, thus attenuating glucagon-induced hepatic gluconeogenesis (70; 73).

## Targeting Glucagon Signaling in The Treatment of Type 2 Diabetes

Glucagon and insulin reciprocally control glucose homeostasis and maintain a fasting blood glucose in a range of 70 to 90 mg/dL in healthy humans (119). Excessive blood glucagon level and defective insulin action and secretion result in hyperglycemia in type 2 diabetes patients (120). In the presence of insulin resistance, β cells secrete excessive insulin to maintain normal glucose homeostasis. The advanced β cells failure leads to hyperglycemia, a hallmark of diabetes (121). Thus, insulin resistance in muscle, adipose, and liver antedates hyperglycemia for several years and exhibit stably throughout the course of type 2 diabetes. Hyperglucagonemia persists in both type 1 and 2 diabetes (122; 123). The increased HGP initiates during the late period of diabetes progression (124). Several factors are attributed to the elevated HGP in diabetic patients: 1) impaired glucagon/insulin ratio; 2) increased gluconeogenic substrates (glycerol and amino acids)

supply; 3) increased hepatic lipid accumulation; 4) disrupted insulin signaling; 5) vagal regulation from hypothalamus (125). Aberrantly high glucagon stimulate glycogenolysis and gluconeogenesis, contributing to hyperglycemia, in which gluconeogenesis plays a major role (126; 127). The potential mechanisms of hyperglucagonemia in diabetes include: 1) intraislet insulin secretion defect; 2) insulin resistance in α cells; 3) insensitive of α cells to hyperglycemia (128). Thus, targeting glucagon action is a promising therapy for diabetes.

Indeed, many studies have provided evidence that intervention of glucagon signaling protects against diabetes development. Glucagon receptor deficiency leads to normal blood glucose in streptozotocin-induced type 1 diabetes mice (129; 130). In diet-induced type 2 diabetes, blocking glucagon action by glucagon receptor abrogation protects against the occurrence of hyperglycemia (131). The antagonists of glucagon receptor have been tested for the application to type 2 diabetes treatment. Glucagon receptor antibody antagonizes glucagon action, lowers blood glucose, and improves glucose tolerance in both normal and *ob/ob* mice (132). Glucagon receptor antagonists, including LY2409021 and PF-06291874, lowered $HbA_{1c}$ and glucose level with a low risk of hypoglycemia in patients with type 2 diabetes (133; 134). However, glucagon receptor antagonism leads to increases in aminotransferases, hepatic fat deposition, body weight, systolic blood pressure, and total cholesterol (133; 135-137), which prevents the application of glucagon receptor antagonists to type 2 diabetes. Thus, it is necessary to further investigate glucagon signaling and determine the applicable targets.

Many medications have been used to modulate glucagon level or action to treat patients with type 2 diabetes, including dipeptidyl peptidase-4 inhibitors (DPP-4is), glucagon-like peptide 1 receptor agonist (GLP-1RAs), and metformin. DPP-4is protect incretin from degradation, such as GIP and GLP-1. Thus, the anti-diabetic effect of DPP-4is is dominantly mediated by enhancement of GIP and GLP-1 actions. GLP-1 stimulates insulin secretion and suppresses glucagon release in patients with type 2 diabetes (138; 139). Interestingly, GIP promotes glucagon secretion during hypoglycemia and euglycemia, whereas barely affects glucagon release in hyperglycemia (140; 141). Thus, DPP-4is control the availability of GIP and GLP-1 to regulate glucagon secretion, balancing the glucose homeostasis. GLP-1RAs, as incretin mimetics, controls glucose homeostasis through stimulating GLP-1 receptor signaling. Generally, GLP-1RAs increase insulin secretion, suppress glucagon concentration, and slow gastric emptying. GLP-1 mimics, including exenatide, lixisenatide, liraglutide, and dulaglutide tend to lower glucagon levels in fasting and postprandial state (142-145).

Metformin is a clinically often used drug for the treatment of T2D for decades. It belongs to the biguanide class originally found in the plant *Galega officinalis* (146). Metformin alleviates hyperglycemia in T2D primarily through suppression of HGP (147) via several mechanisms (73; 148; 149). First, metformin promotes the formation of AMPK heterotrimeric α/β/γ-complex, activating AMPK via increasing phosphorylation of AMPKα-T172 by LKB1 at a low metformin concentration (25-100 μM) (150). AMPK activation results in phosphorylation of transcriptional cofactor CREB binding protein (CBP) at S436, which triggers CBP dissociation from the CREB-TORC2 transcription

complex and reduces gluconeogenic gene expression (73). Low-dose metformin treatment also reduces endogenous glucose production through inhibiting mitochondrial glycerophosphate dehydrogenase (151). Second, it is recently shown that AMPK also activates cyclic nucleotide phosphodiesterase-4B (PDE4B), which decreases glucagon-induced cAMP accumulation (152). Third, metformin has a role in suppression of mitochondrial oxidation via inhibiting complex I of the mitochondrial electron respiratory chain (153; 154). A high concentration of metformin (>1 mM) results in a significant decrease in ATP and increase in AMP levels (155; 156). The increased AMP/ATP ratio enhances AMPK activity and elevated AMP also inhibits adenylate cyclase, thus reducing cAMP levels and suppressing PKA activity, which blocks glucagon-induced HGP (157). Depletion of ATP by metformin also contributes to suppression of hepatic gluconeogenesis (155). Finally, AMP also inhibits the activity of fructose -1,6-bisphophatase-1 (FBP1), a rate-controlling enzyme in gluconeogenesis (158). Foxo1 is an important transcription factor that can interact with CREB to promote the expression of gluconeogenic genes, such as *G6pc* and *Pck (61)*. However, Foxo1 is believed to be controlled by Akt in insulin signaling pathway that metformin barely affects, thus the Foxo1 regulation by metformin has not yet paid attention until we recently discover Foxo1 regulation by PKA in glucagon signaling (52). Other anti-diabetic drug class, such as sulfonylureas, amylin memetics, and sodium-glucose cotransporter 2 inhibitors (SGLT2is), also tend to regulate glucagon level (159). Therefore, targeting glucagon signaling is an important therapy to treat or cure type 2 diabetes.

## The Role of FoxO Proteins in Regulation of Glucose and Lipid Metabolism

The forkhead box transcription factor class O (FoxO) family plays important roles in multiple biological events, including proliferation, apoptosis, autophagy, and gluconeogenesis (160). In mammals, FoxO family contains Foxo1, FoxO3, Foxo4, and Foxo6. Foxo family members share the conserved Akt phosphorylation sites, thereby they are regulated by insulin/Akt signaling (116; 161-163). Foxo1-null mice die from a defect in vascular development during embryogenesis. However, Foxo3- and Foxo4-null mice are viable and exhibit no distinguishable phenotypes (164). Foxo6 is highly expressed in brain and shows limited signal in liver (163). However, Foxo6 still regulates hepatic gluconeogenesis in response to insulin and glucagon (165). FoxOs synergistically regulate glucose homeostasis by promoting gluconeogenesis (54; 166), among which Foxo1 dominantly regulates hepatic glucose metabolism(166).

Insulin regulates liver adaptation between fasting and feeding through cooperating with counterregulatory hormone glucagon. Foxo1 is a key player in maintaining euglycemia in both fasting and feeding conditions. During feeding state, insulin is secreted from β cells and activates PI3K/AKT pathway, which in turn stimulates phosphorylation of Foxo1 (T24, S253, and S316) and renders Foxo1 nuclear exportation and degradation (63; 167). Thus, insulin signaling inhibits Foxo1-induced gluconeogenic gene expression, including *G6pc* and *PCK*. During the adaptation to fasting, pancreatic α cells release glucagon to combat against hypoglycemia. On one hand, glucagon increases Foxo1 mRNA expression (168; 169); on the other hand, glucagon phosphorylates Foxo1 at

several sites, thus promoting Foxo1 protein stability and nuclear translocation (52; 60; 170). Hence, glucagon increases Foxo1 activity, and subsequently induces hepatic glucose production to maintain normal blood glucose level. The importance of Foxo1 in the glucose homeostasis is further revealed in multiple genetic mice models. Transgenic mice with overexpression of constitutively nuclear Foxo1 exhibit impaired glucose homeostasis (171). Hepatic Foxo1 ablation, rather Foxo3 and Foxo4, leads to a decrease in blood glucose and no significant change in lipid profile. In *db*/*db* mice, Foxo1 deletion, but not Foxo3 deletion, significantly improves glucose tolerance; this indicates the important role of Foxo1 in glucose homeostasis (166). Liver-specific insulin receptor knockout (LIRKO) mice show severe insulin resistance and glucose intolerance. Hepatic Foxo1 deletion in LIRKO mice recuses the defects in glucose homeostasis (55; 172). Insulin receptor substrate (IRS) 1 and 2 connect insulin receptor effect to activation of PI3K/Akt pathway, which triggers enormous downstream regulators. Hepatic Foxo1 disruption normalizes hepatic IRS1 and IRS2 deletion-induced insulin resistance and glucose homeostasis dysregulation (113). The mice with hepatic knockout of Akt1 and Akt2 show glucose intolerance and insulin resistance, which are normalized by concomitant Foxo1 deletion in liver (173). These mice models provide solid evidences that Foxo1 is an important player in regulation of glucose homeostasis. In addition, FoxOs also are involved in lipid metabolism. Microsomal triglyceride transfer protein (MTP) and apolipoprotein ApoC-III are two key players in VLDL production. MTP is an endoplasmic reticulum (ER)-located protein and promotes the transfer of lipids to nascent apoB. ApoC-III inhibits lipoprotein lipase (LPL) that triggers hydrolysis of triglycerides and chylomicrons. Foxo1 mediates

insulin-regulated MTP and apoC-III levels. Constitutively active Foxo1 transgenic mice have an increase in VLDL production potentially through stimulation of MTP and apoC-III levels (174; 175). However, hepatic Foxo1 ablation has no significant effect on lipid metabolism, whereas Foxo1 and Foxo3 concomitant deletion in liver leads to hepatic steatosis with increases in serum triglyceride and cholesterol levels. These effects may be attributed to upregulation of gene expression of fatty acid synthase (*FASN*) and 3-hydroxy-3-methyl-glutaryl- CoA reductase (*HMGCR*); but the underlying mechanism remain unknown (166). Furthermore, Tao *et al.* observed similar lipid phenotype in liver-specific Foxo1, Foxo3, and Foxo4 knockout mice and indicated that nicotinamide phosphoribosyltransferase (Nampt) ,as a transcriptional target of FoxOs, regulates hepatic lipid homeostasis (176). Thus, FoxOs have a redundant role in regulation of triglyceride metabolism.

Except for liver, FoxOs also exert key functions in other tissue. In pancreas, Foxo1 plays a negative role in pancreatic β-cell proliferation. The diabetic mice lacking IRS2 suffer from progressive reduction in β-cell mass and eventually develop peripheral insulin resistance. Haploinsufficiency of Foxo1 restores β-cell mass by relieving its inhibition on *Pdx1* expression, preventing diabetes in IRS2 knockout mice (177-179). Thus, activated Foxo1 in β-cell suppresses its proliferation, reduces insulin secretion and contributes to increased blood glucose. In vivo studies show that Foxo1 inactivation or overexpression affects skeletal muscle mass. Overexpression of Foxo1 impairs glucose tolerance due to a decrease in skeletal muscle mass (180). Transgenic overexpression of Foxo1 in skeletal muscle results in severe muscular atrophy through atrogin-1, myostatin, and 4E-binding

protein-1, resulting in dysregulation of glucose homeostasis (181; 182). Besides, skeletal muscle metabolism is also affected by Foxo1. Pyruvate dehydrogenase kinase-4 is upregulated by Foxo1, inactivating pyruvate dehydrogenase and shutting down glucose oxidation (183). Therefore, skeletal muscle Foxo1 controls glucose disposal and regulates glucose homeostasis. Foxo1 plays an essential role in adipocyte differentiation at the very early state of terminal adipocyte differentiation (184). However, constitutively active Foxo1 prevents differentiation of preadipocytes, while inhibition of Foxo1 restores adipocyte differentiation of fibroblasts from insulin receptor deficient mice (185). In considering the important role of adipocyte in glucose disposal, impaired adipocyte differentiation by Foxo1 in diabetic condition may also contribute to poorly glycemic control. In brain, overexpression of nuclear mutant Foxo1 in hypothalamus results in induction of Agrp, increase in food intake, obesity, and glucose intolerance in mice (186; 187). Ablation of Foxo1 in Agrp neurons reduces food intake, increases leanness, improves glucose homeostasis, and increases sensitivity to insulin and leptin, which is mediated by Gpr17 (188). Therefore, Foxo1 in neuron, especially in Agrp neurons, plays a critical role in regulation of whole body glucose homeostasis. Osteoblast Foxo1 deficiency increases β-cell proliferation, insulin secretion, and improves insulin sensitivity through upregulation of osteocalcin expression (189). Foxo1 also promotes inflammation by enhancing Tlr4-mediated signaling in mature macrophage, which contributes to glucose homeostasis dysfunction (190). Taken together, constitutively active Foxo1 in β-cell, skeletal muscle, adipocytes, neurons, osteoblast, and macrophage also contributes to dysregulation of glucose homeostasis.

## The Effect of MAPK p38 on Glucose Homeostasis

Mitogen-activated protein kinase (MAPKs) transduce signals emanating from environmental stress and receptors on cell surface to in turn stimulate downstream transcription factors and other regulators. MAPKs are composed of four subfamilies: extracellular-signal-regulated kinase (ERKs), ERK5, JUN N-terminal kinase (JNKs), and p38 kinases (191). ERK, generally, is activated by growth-promoting mitogenic stimuli. JNKs and p38 kinase are majorly stimulated by environmental stress, including osmotic shock, hypoxia, heat shock, and ultraviolet radiation, and pro-inflammatory stimuli and cytokines, including LPS, IL-1, TGFβ, and TNF (192-195). MAPK p38 has four isoforms, p38α, p38β, p38γ, and p38δ. The p38α is ubiquitously expressed, whereas p38β, γ, and δ show high expression levels in specific tissues (196; 197). P38α and β are 75% identical in amino-acid sequence. P38γ and δ show 70% identity to each other, whereas they are 60% identical to p38α (198). Furthermore, the substrates of p38γ and δ are not similar with that of p38α and β, suggesting different functions of p38 MAPK isoforms (197).

The classical activation of p38 is induced by dual phosphorylation at Thr-Gly-Tyr motif by MAPK Kinase (MKK) 3 and MKK6 (199; 200). Two other potential MKKs-independent mechanisms of p38α activation have been proposed. Studies show that transforming growth factor-β-activated protein 1 (TAB1) specifically binds to p38α, which leads to p38α autophosphorylation and activation (201). In T cells, T cell antigen receptor (TCR) stimulates p38α phosphorylation at noncanonical residue, Tyr323 through

proximal tyrosine kinases activation; this Tyr323 phosphorylation activates p38α by inducing structure conformation and phosphorylation of Thr-Gly-Tyr motif (202). MAPK p38 regulates its substrates' activity, involving the physiological events. Their substrates include transcriptional factors, including MEF2, CHOP, SAP1, PGC-1, ATF2, p53, NFATp, CDX3, and STAT-1, as well as cellular functions related factors, such as Cdc25, NHE1, and Tau (198; 199; 203; 204).

The effect of p38 on HGP has been indicated and PKA-p38-CREB is proposed as its underlying mechanism (205). However, Jing *et al.* reported that hepatic p38α knockout did not impair activation of CREB in mice. They suggest that p38α signaling suppresses AMPK activity via inhibition of transforming growth factor b-activated kinase 1 (TAK1) in a negative feedback loop (206). The p38 phosphorylates X-box binding protein 1 (Xbp1s) at T48 and S61 and promotes Xbp1s nuclear translocation, thereby enhancing HGP (207). The p38α also regulates Foxo1 activity through stimulating its phosphorylation at S284, S295, S326, S467, and S475, potentially in control of HGP (60; 170); but whether the effect of p38α on HGP is mediated through Foxo1 and its exact mechanism remain not clearly understood. The studies from Ozcan *et al.* indicate that CaMKII mediates HGP in fasting and obese condition potentially through p38 → Foxo1 pathway signaling (60), in which they summarized the signaling as: glucagon → cAMP/PKA → p38 → Foxo1. Since Longuet *et al.* reported that glucagon-induced p38 activation is not blocked by PKA inhibition by H89 (208), it is controversial that PKA activates p38. In addition, p38 is involved in glucagon-induced fatty acid oxidation and

obese-induced insulin resistance (208; 209). Therefore, p38 plays an important role in glucose and lipid metabolism in both heath and diseases conditions.

## Specific Aims of This Thesis

Glucagon governs hepatic glucose production (HGP) by triggering glycogenolysis and gluconeogenesis; but the underlying mechanisms, especially under pathological condition, remain not well understood. MAPK p38 is activated by dual phosphorylation on Thr and Tyr within the motif Thr–Gly–Tyr in response to a series of extracellular stimuli (210). Although the role of p38 in gluconeogenesis has been indicated (205; 206), the mechanisms whereby p38 is involved in glucagon action are not well understood. In vitro study showed that p38 phosphorylates Foxo1, a key gluconeogenic transcription factor (170). Thus, we hypothesize that p38 regulates glucose homeostasis in liver through Foxo1. To this end, the aims of this thesis are: i) to identify the role of Foxo1 in p38-mediated HGP; ii) to uncover the underlying mechanisms by which p38 regulates Foxo1; iii) to explore the role of p38-Foxo1 signaling in glucose homeostasis in diabetic condition.

# CHAPTER II

# PHYSIOLOGICAL RELEVANCE OF P38 TO REGULATION OF HGP

## Introduction

Glucagon is a 29 amino acids peptide secreted from pancreatic α-cell; it regulates metabolic homeostasis by coordinating with insulin. Glucagon promotes hepatic glucose production (HGP) via stimulation of glycogenolysis and gluconeogenesis, maintaining euglycemia during fasting state. However, aberrant glucagon action leads to hyperglycemia in diabetic patients and mice mainly through gluconeogenesis (126; 211; 212). Numerous transcription factors and coactivators are involved in regulation of HGP upon controlling the expression of gluconeogenic genes, glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase (*Pck1*). Transcription factors FoxOs (1, 3, and 4) synergistically promote HGP, while Foxo1 is the dominant member in control of glucose production through stimulation of gluconeogenic genes expression (54-56). In addition, other transcription factors (including CREB, HNF-4α, and C/EBP) coordinate with several coactivators (such as p300/CBP, CRTC2, SRC-1, and PGC-1α) to promote gluconeogenesis (57; 62; 65; 70; 81; 82; 213).

p38 MAPK, one of the subfamilies of mitogen-activated protein kinases (MAPKs), is activated by inflammatory cytokines, involved in regulation of inflammatory responses (214). MAPK p38 has four isoforms, p38α, p38β, p38γ, and p38δ. The p38α is ubiquitously expressed, whereas p38β, γ, and δ show high expression levels in specific

tissues (196; 197). MAPK p38 is activated by dual phosphorylation on Thr and Tyr within the motif Thr–Gly–Tyr in response to a series of extracellular stimuli (210). Although p38 has been indicated to be involved in glucagon action, the exact roles of p38 isoforms and underlying mechanisms remain not well understood. In this study, we aim to investigate the role of p38 isoforms in glucagon-induced HGP and the role of Foxo1 in p38-mediated glucagon action.

## Methods

### Mice

The *Foxo1*$^{LivKO}$ mice were generated as previously described (215). All animal experiments were performed according to procedures approved by the Texas A&M University Institutional Animal Care and Use committee. Mice are housed at 22-24 C° and maintained on a 12 h light/12 h dark cycle with a standard chow diet. The male mice at the age of 8-12 weeks were used in all the experiments.

### Primary Mouse Hepatocytes Isolation and Culturing

Primary mouse hepatocytes were isolated from 8-12 weeks old male mice. Livers were perfused with 10 ml of HBSS with 50 mM EGTA and 1 M glucose, followed by perfusion with 15 ml HBSS supplemented with 1 M $CaCl_2$, 1 M glucose, and type II collagenase. The cells were shaken from liver and then screened through 70 μm filter and centrifuged in Percoll medium (GE Healthcare). Viable cells were seeded into low glucose DMEM Medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S).

### Pyruvate Tolerance Test

Recombinant adenovirus (1 x $10^{11}$ pfu/mice) was delivered into mice by retro orbital injection, and pyruvate tolerance test was performed 1 week after delivery of adenovirus. The mice were fasted for 16 h. After determining the basal blood glucose level, mice received 2 g/kg of body weight pyruvate sodium through i.p. injection. Blood glucose level were measured after 15, 30, 60, 90, and 120 min. The administration of SB203580

was performed, as previously described (205). Briefly, the mice were given two shots of SB203580 (10 mg/kg of body weight) via i.p. injection. The first shot was given at the beginning of the fasting, the second shot given 14 h later. After 2 h, basal blood glucose levels were measured. Afterwards, mice were i.p. injected with 2 g/kg of body weight pyruvate sodium and blood glucose levels were monitored after 15, 30, 60, 90, and 120 min.

**Quantitative Real-Time PCR**

Total RNAs were extracted with TRIzol reagent (Invitrogen Life Technologies). The cDNAs were synthesized using iScript$^{TM}$ Reverse Transcription Supermix (Bio-Rad). Quantitative real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The primers are listed in APPENDIX Table 1.

**Glucose Production in Primary Hepatocytes**

Glucose production assay was performed as previously described (216). Briefly, mouse primary hepatocytes were isolated. After attachment, hepatocytes were cultured in KRB buffer containing sodium pyruvate, sodium lactate, and BSA, and then pretreated with 10μM SB203580 for 30 min, followed by 100 nM glucagon treatment for 3 h. Finally, 200 μl medium was collected and glucose content was measured using glucose assay kit (Thermo Fisher). For transfection and adenovirus-induced overexpression experiments, attached hepatocytes were transfected with 150 nM siRNA (per well in 6-well plate) for 12 h or treated with 100 MOI adenovirus for 12 h. Afterwards, hepatocytes were cultured in KRB medium with pretreatment of 10μM SB203580 for 30 min and following 100 nM

glucagon treatment for 3 h. Cell medium was collected and glucose concentration was monitored.

**Statistical Analysis**

All results are presented as mean ± SEM. P values were calculated using the Student-t test for the comparison of difference between two groups. Significance among multiple groups was tested using one-way and two-way ANOVA followed by Tukey's multiple comparison test in GraphPad Prism. $P < 0.05$ was considered statistically significant.

## Results

### *P38α Promotes Glucagon-Induced HGP in Both Hepatocytes and Mice*

To test the role of p38 in HGP upon glucagon treatment, wild type (WT) mice with inhibition of p38 using an inhibitor, SB203580 via intraperitoneal (i.p.) injection were assessed (205). Control mice received 10% DMSO. WT mice with the administration of SB203580 had a 10% decrease in fasting blood glucose and a 17% reduction in HGP during intraperitoneal pyruvate tolerance test (ipPTT) (Figures 1A-C). In WT hepatocytes, glucagon increased HGP by 39% and SB203580 treatment attenuated glucagon effect by 23% (Figure 1D). The p38 family has four members: α, β, γ, and δ. As previously reported (217), p38α is the dominant isoform in mouse hepatocytes. p38β, γ, and δ showed relatively low expression levels in mouse primary hepatocytes (Figure 1E). Although the lower expression level of p38γ and δ in liver, they play roles in reprogramming liver metabolism and regulating hepatic cell cycle progression (218; 219). In considering SB203580 targets both p38α and β, as well as the potential roles of p38γ and δ in liver, we tested the individual effect of p38 isoforms on glucagon-induced HGP in WT hepatocytes. Glucagon increased HGP by 37%, and RNAi-mediated p38α knockdown abrogated HGP induction by glucagon. However, knockdown of other p38 isoforms (p38β, γ, or δ) barely affected glucagon-induced HGP (Figure 1F). Further, we analyzed WT mice with disruption of p38α by injection with adeno-associated virus 8 (AAV8)-p38α small hairpin RNA (shRNA). Control mice received AAV8-expressing scramble shRNA. Fasting blood

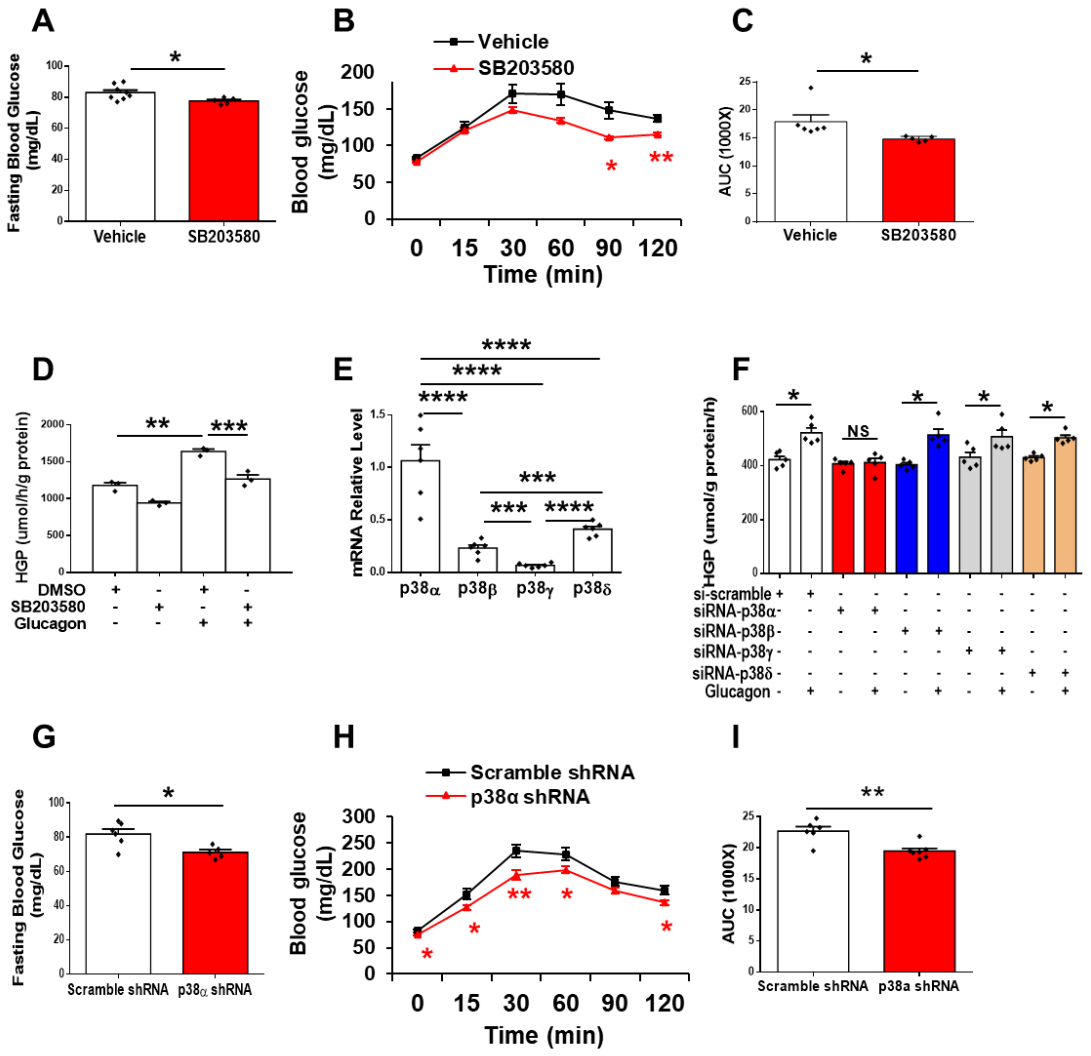



**Figure 1 p38α promotes hepatic glucose production in response to glucagon and fasting**
(A) Fasting blood glucose level in WT mice treated with SB203580, n=5-8. (B) Pyruvate tolerance test in WT mice treated with SB203580, *P<0.05, **P<0.01 versus Vehicle, n=5-6. (C) AUC of pyruvate tolerance test in (B). (D) Hepatocytes were isolated from WT mice, then pretreated with 10 μM SB203580 followed by 100 nM glucagon treatment. Glucose output in cells was measured, n=3. (E) mRNA expression levels of p38 isoforms in mouse primary hepatocytes, n=6. (F) p38 isoforms siRNAs were transfected into mouse hepatocytes for 12 h. Then cells were treated with 100 nM glucagon for 3 h. Glucose content was measured using glucose kit, n=5. (G) Fasting blood glucose level in WT mice treated with AAV8-p38α shRNA, n=5-6. (H) Pyruvate tolerance test in WT mice treated with AAV8-p38α shRNA, *P<0.05, **P<0.01 versus Scramble, n=5-6. (I) AUC of pyruvate tolerance test in (H). All data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

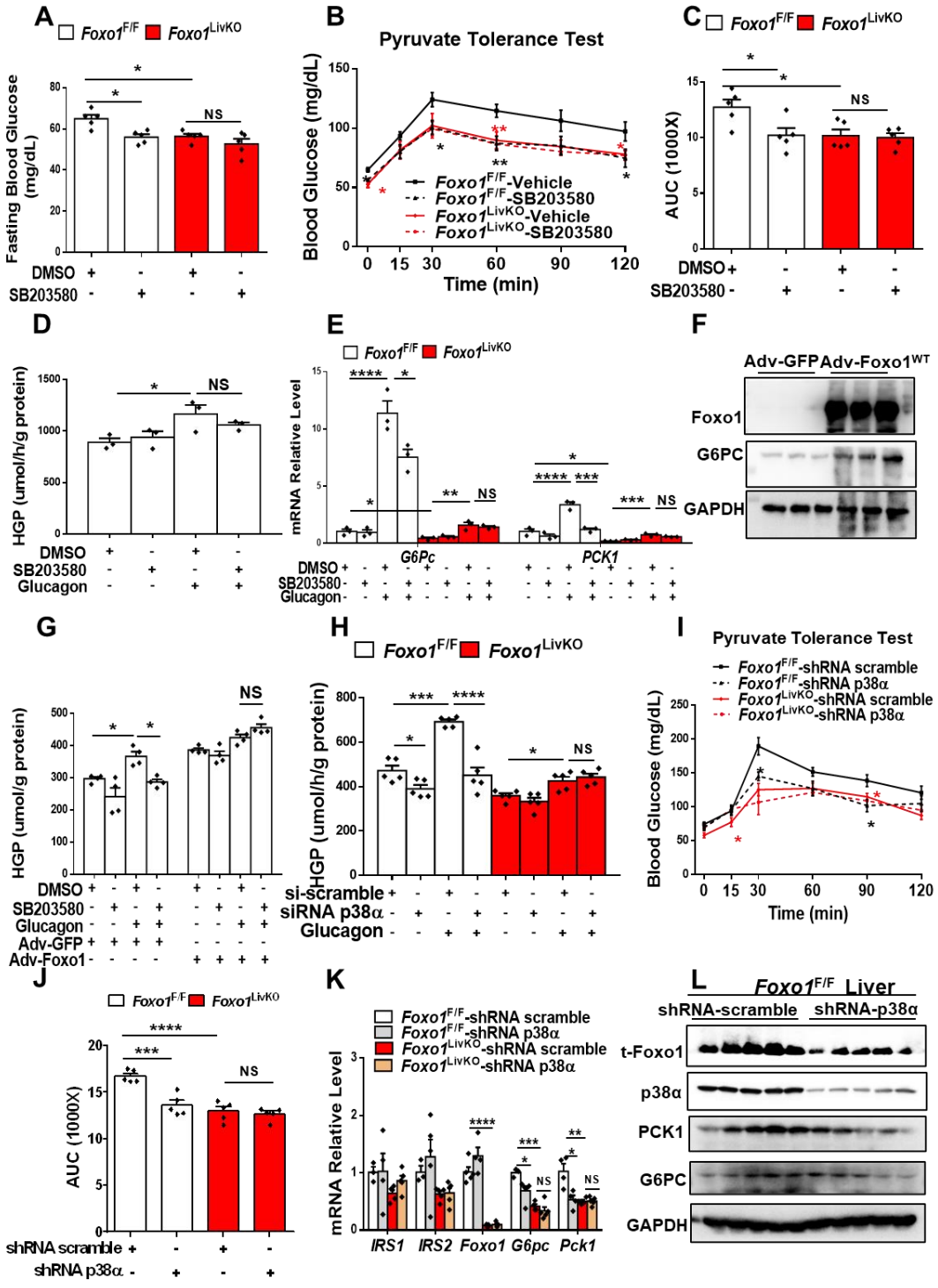
glucose level was significantly reduced by p38α disruption. Hepatic gluconeogenesis, assessed by ipPTT, revealed a 14% decrease in glucose output in *Foxo1*$^{F/F}$ mice with p38α ablation. These results indicate that p38α, but not other p38 isoforms, regulates glucagon-induced HGP (Figures 1G-I).

*P38α-Mediated HGP Depends on Foxo1*

Foxo1 is an important transcription factor that promotes HGP in response to glucagon (52; 55). To determine whether p38 regulates glucagon-induced HGP via Foxo1, Floxed *Foxo1* (*Foxo1*$^{F/F}$) and liver Foxo1 knockout (*Foxo1*$^{LivKO}$; albumin promoter-CRE recombinase) mice with inhibition of p38 using an inhibitor, SB203580 via intraperitoneal (i.p.) injection were assessed (205). Control mice received 10% DMSO. *Foxo1*$^{F/F}$ mice with the administration of SB203580 had a 13% decrease in fasting blood glucose (Vehicle: 63.6 ± 2.6 versus SB203580: 55.5 ± 1.5 mg/dL) and a 23% reduction in HGP during intraperitoneal pyruvate tolerance test (ipPTT). *Foxo1*$^{LivKO}$ mice showed an 11% decrease in fasting blood glucose and a 20% reduction in HGP, as compared to that in *Foxo1*$^{F/F}$ mice. p38 inhibition by SB203580 did not further markedly suppress both fasting blood glucose (Vehicle: 56.4 ± 1.2 versus SB203580: 52.7 ± 2.5 mg/dL) and HGP in *Foxo1*$^{LivKO}$ mice (Figures 2A-C). In addition, Glucagon led to a significant increase in HGP in primary hepatocytes from *Foxo1*$^{LivKO}$ mice, whereas inhibition of p38 had no further suppressive effect on glucagon-induced HGP in *Foxo1*$^{LivKO}$ hepatocytes (Figure 2D). Consistently, *G6pc* and *Pck1* mRNA expression levels were significantly stimulated in *Foxo1*$^{F/F}$ hepatocytes upon glucagon treatment. Inhibition of p38 by SB203580 blocked

**Figure 2 p38α-mediated HGP depends on Foxo1**

(A) Blood glucose in 16 h fasted *Foxo1*$^{F/F}$ and *Foxo1*$^{LivKO}$ mice injected with or without 10 mg/kg SB203580, n=5. (B) Pyruvate tolerance test in *Foxo1*$^{F/F}$ and *Foxo1*$^{LivKO}$ mice administered with 10 mg/kg SB203580 via i.p. injection. *****P<0.05; ******P<0.01 versus *Foxo1*$^{F/F}$-Vehicle, n=5. (C) AUC of pyruvate tolerance test in (B), n=5. (D) *Foxo1*$^{LivKO}$ hepatocytes were pretreated with 10 μM SB203580 for 30 min, then treated with 100 nM glucagon for 3 h. Glucose content was measured using glucose kit, n=3. (E) Hepatocytes were pretreated with 10 μM SB203580 for 30 min, then treated with 100 nM glucagon for 3 h. Total RNA was extracted, and gluconeogenic gene mRNA levels were measured using Q-PCR, n=3. (F) *Foxo1*$^{WT}$ was overexpressed in mouse Hepatocytes. Foxo1 and G6PC protein levels were detected. (G) Foxo1 was overexpressed in hepatocytes using adenovirus for 12 h. 10 μM SB203580 was used to treat cells for 30 min, followed by 100 nM glucagon treatment for 3 h. Glucose content in cells were measured, n=4. (H) Hepatocytes isolated from *Foxo1*$^{F/F}$ and *Foxo1*$^{LivKO}$ mice were transfected with p38α siRNA for 12 h, and then treated with 100 nM glucagon for 3 h. Glucose content was measured using glucose kit, n=5. (I) Pyruvate tolerance tests in *Foxo1*$^{F/F}$ and *Foxo1*$^{Livko}$ mice. *****P<0.05 versus Foxo1F/F-shRNA scramble, n=5. (J) AUC of pyruvate tolerance tests in (I), n=5. (K) mRNA levels of gluconeogenic genes in the livers of *Foxo1*$^{F/F}$ and *Foxo1*$^{Livko}$ mice infected with AAV8-shRNA-p38α, n=3-5. (L) The protein levels in livers from *Foxo1*$^{F/F}$ mice infected with AAV8-shRNA-p38α. All data are presented as mean ± SEM. *****P<0.05, ******P<0.01, *******P<0.001, ********P<0.0001


A
Foxo1F/F Foxo1LivKO
Fasting Blood Glucose (mg/dL)
NS
DMSO
SB203580
B
Pyruvate Tolerance Test
Blood Glucose (mg/dL)
Foxo1F/F-Vehicle
Foxo1F/F-SB203580
Foxo1LivKO-Vehicle
Foxo1LivKO-SB203580
Time (min)
C
Foxo1F/F Foxo1LivKO
AUC (1000X)
NS
DMSO
SB203580
D
HGP (umol/h/g protein)
NS
DMSO
SB203580
Glucagon
E
Foxo1F/F Foxo1LivKO
mRNA Relative Level
G6Pc
PCK1
NS
DMSO
SB203580
Glucagon
F
Adv-GFP
Adv-Foxo1WT
Foxo1
G6PC
GAPDH
G
HGP (umol/g protein)
NS
DMSO
SB203580
Glucagon
Adv-GFP
Adv-Foxo1
H
Foxo1F/F Foxo1LivKO
HGP (umol/h/g protein)
NS
si-scramble
siRNA p38α
Glucagon
I
Pyruvate Tolerance Test
Foxo1F/F-shRNA scramble
Foxo1F/F-shRNA p38α
Foxo1LivKO-shRNA scramble
Foxo1LivKO-shRNA p38α
Blood Glucose (mg/dL)
Time (min)
J
Foxo1F/F Foxo1LivKO
AUC (1000X)
NS
shRNA scramble
shRNA p38α
K
Foxo1F/F-shRNA scramble
Foxo1F/F-shRNA p38α
Foxo1LivKO-shRNA scramble
Foxo1LivKO-shRNA p38α
mRNA Relative Level
NS
IRS1
IRS2
Foxo1
G6pc
Pck1
L
Foxo1F/F Liver
shRNA-scramble
shRNA-p38α
t-Foxo1
p38α
PCK1
G6PC
GAPDH

glucagon-induced *G6pc* and *Pck1* expression. Glucagon also markedly increased *G6pc* and *Pck1* mRNA expression levels in *Foxo1*$^{LivKO}$ hepatocytes, whereas SB203580 treated *Foxo1*$^{LivKO}$ hepatocytes exhibited no dramatic decreases in *G6pc* and *Pck1* expression in response to glucagon (Figure 2E). Transduction with adenoviral Foxo1 into WT hepatocytes led to significant increases in Foxo1 and G6PC proteins (Figure 2F). Consistently, glucagon increased HGP by 23% in GFP overexpressed hepatocytes, which is attenuated by SB203580; however, glucagon treatment resulted a nonsignificant increase in HGP and p38 inhibition by SB203580 barely affected glucagon-induced HGP in Foxo1 overexpressed hepatocytes (Figure 2G).

To test whether Foxo1 is involved in p38α-mediated HGP, we knocked down p38α in *Foxo1*$^{Livko}$ hepatocytes using siRNA. Consistent with SB203580, Foxo1 deficiency blocked the effect of p38α on glucagon-induced HGP (Figure 2H). Further, we analyzed *Foxo1*$^{F/F}$ and *Foxo1*$^{LivKO}$ mice with disruption of p38α by injection with adeno-associated virus 8 (AAV8)-p38α small hairpin RNA (shRNA). Control mice received AAV8-expressing scramble shRNA. Hepatic gluconeogenesis, assessed by ipPTT, revealed a 19% decrease in glucose output in *Foxo1*$^{F/F}$ mice with p38α ablation. As compared with that in *Foxo1*$^{F/F}$ mice, *Foxo1*$^{LivKO}$ mice had a 20% decrease in HGP. Importantly, p38α disruption did not further decrease HGP in *Foxo1*$^{LivKO}$ mice (Figures 2I and 2J). Gene expression analysis revealed that knockdown of p38α did not affect hepatic mRNA levels of *IRS1*, *IRS2*, and *Foxo1* in either *Foxo1*$^{F/F}$ or *Foxo1*$^{LivKO}$ mice. However, p38α ablation downregulated mRNA levels of *G6pc* and *Pck1* in *Foxo1*$^{F/F}$ livers, but not in *Foxo1*$^{LivKO}$ livers. Foxo1 deficiency led to significant decreases in *G6pc* and *Pck1* expression (Figure

2K). AVV8-shRNA-mediated p38α knockdown decreased t-Foxo1, PCK1, G6PC, and p38α proteins in *Foxo1*$^{F/F}$ mice liver (Figure 2L).

Finally, we detected the protein abundance of Foxo1 and p38 phosphorylation at T180/Y182 (pp38-T180/Y182) in response to glucagon. Glucagon treatment significantly stimulated pp38-T180/Y182 – which is a measure of its activation state – with a corresponding increase in Foxo1 protein in WT primary hepatocytes (Figures 3A and 3B). We also compared the expression levels of pp38-T180/Y182 and Foxo1 during the transition from a fed to fasted state in WT mice. In accordance with results in hepatocytes, pp38-T180/Y182, Foxo1, G6PC, and PCK1 levels were simultaneously increased upon fasting (Figures 3C and 3D). Taken together, the effect of p38α on HGP is mediated by Foxo1.

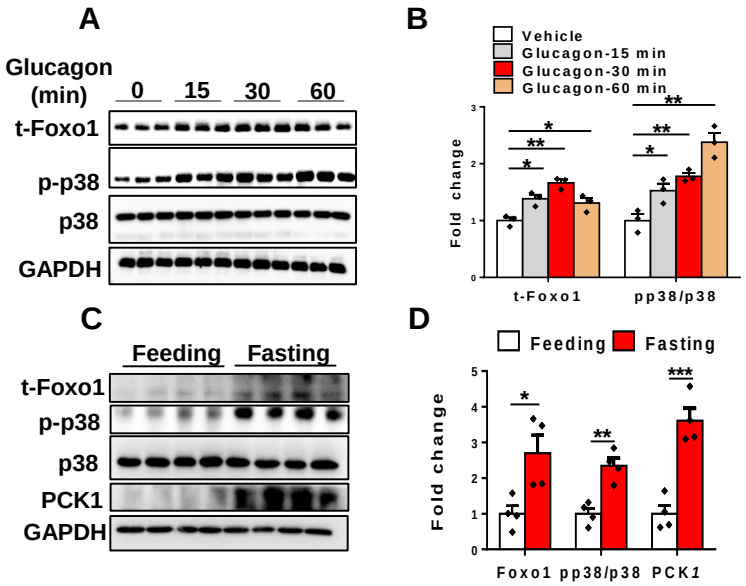



**Figure 3 The activity of Foxo1 and p38 in response to glucagon and fasting**

(A) Mouse primary hepatocytes were treated with 100 nM glucagon for 15, 30, and 60 min, respectively. Foxo1 and p38 phosphorylation levels were analyzed using Western blotting; (B) Quantification of (A), n=3; (C) Protein abundance of gluconeogenic genes in the livers from 16 h fasted and random-fed WT mice. (D) Quantification of (C), n=4. All data are presented as mean ± SEM. *****P<0.05, ******P<0.01, *******P<0.001.

## Summary

The results in this chapter indicate that p38α plays a major role in regulation of glucagon-induced hepatic glucose production, whereas other isoforms, p38β, γ, or δ, barely affect glucagon action on hepatic glucose production. Further study in hepatic Foxo1 ablation mice suggests that Foxo1 is required for the regulation of p38α in hepatic glucose production.

# CHAPTER III

# THE ROLE OF INSULIN SIGNALING PATHWAY IN P38 REGULATION OF HGP

## Introduction

Glucose homeostasis is maintained by the balance between glucose production mainly in liver or absorption during feeding and glucose disposal in skeletal muscle and adipose tissue. These processes are tightly regulated by insulin and counterregulatory hormones. Insulin, secreted by pancreatic β cells during nutrient uptake period, promotes glucose uptake in skeletal muscle and adipose tissue, as well as suppresses HGP (220). The suppressive effect of insulin on HGP is mediated by insulin-induced Foxo1 inactivation. Insulin binds to insulin receptor and activates its intrinsic tyrosine-kinase activity; this results in insulin receptor substrate (IRS) phosphorylation at tyrosine residues, acting as anchoring sites for regulatory subunits of PI3K kinase (221). This catalyzes the generation of phosphatidyl 3,4,5-phohate, which activates PDK1 and Akt. In turn, Akt phosphorylates Foxo1 at T24, S256, and S319, inducing Foxo1 nuclear exportation and degradation, whereby inhibiting Foxo1-induced gluconeogenesis (63; 116). In addition, insulin phosphorylates CBP at S436 via PDK1-$PKC_{i/\lambda}$; this disassociates CBP-CREB-TORC and suppresses gluconeogenesis (70).

We previously found that p38α impairs insulin signaling through promoting the degradation of insulin receptor substrates (IRS) (222). Ozcan et al. showed that p38α is

one of the casual factors that renders hepatic insulin resistance (209). Since we showed that p38 mediates HGP through Foxo1, to determine whether the p38 effect is mediated through impairing insulin signaling. In this study, we detected the role of p38 in HGP in liver-specific IRS1 and IRS2 knockout (DKO) mice.

## Methods

### Mice

The *IRS1/IRS2*$^{LivKO}$ (DKO), *IRS1/IRS2/Foxo1*$^{LivKO}$ (TKO) (113), and *Foxo1*$^{253A}$ (223) mice were generated as previously described. All animal experiments were performed according to procedures approved by the Texas A&M University Institutional Animal Care and Use committee. Mice are housed at 22-24 C° and maintained on a 12 h light/12 h dark cycle with a standard chow diet. The male mice at the age of 8-12 weeks were used in all the experiments.

### Primary Muse Hepatocytes Isolation and Culturing

Primary mouse hepatocytes were isolated from 8-12 weeks old DKO and *Foxo1*$^{253A}$ male mice. Livers were perfused with 10 ml of HBSS with 50 mM EGTA and 1 M glucose, followed by perfusion with 15 ml HBSS supplemented with 1 M $CaCl_2$, 1 M glucose, and type II collagenase. The cells were shaken from liver and then screened through 70 μm filter and centrifuged in Percoll medium (GE Healthcare). Viable cell were seeded into low glucose DMEM Medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S).

### Pyruvate Tolerance Test

The administration of SB203580 was performed, as previously described (205). Briefly, the mice were given two shots of SB203580 (10 mg/kg of body weight) via i.p. injection. The first shot was given at the beginning of the fasting, the second shot given 14 h later. After 2 h, basal blood glucose levels were measured. Afterwards, mice were i.p.

injected with 2 g/kg of body weight pyruvate sodium and blood glucose levels were monitored after 15, 30, 90, and 120 min.

**Glucose Production in Primary Hepatocytes**

Glucose production assay was performed as previously described (216). Briefly, mouse primary hepatocytes were isolated. After attachment, hepatocytes were cultured in KRB buffer containing sodium pyruvate, sodium lactate, and BSA, and then pretreated with 10μM SB203580 for 30 min, followed by 100 nM glucagon treatment for 3 h. Finally, 200 μl medium was collected and glucose content was measured using glucose assay kit (Thermo Fisher).

**Statistical Analysis**

All results are presented as mean ± SEM. P values were calculated using the Student-t test for the comparison of difference between two groups. Significance among multiple groups was tested using one-way and two-way ANOVA followed by Tukey's multiple comparison test in GraphPad Prism. $P < 0.05$ was considered statistically significant.

## Results

Insulin/Akt triggers Foxo1 degradation and nuclear exclusion via phosphorylation of T24, S256, and S319 (63; 116). Ozcan *et al.* showed that p38α is a causal factor that instigates hepatic insulin resistance (209). We previously found that p38α impairs insulin signaling by promoting the degradation of insulin receptor substrates (222). To determine whether the effect of p38 on HGP relies on insulin-induced Foxo1 degradation and nuclear exclusion, we assessed the role of p38 in HGP in hepatic IRS1 and IRS2 of double knockout (DKO) mice using albumin promoter driving CRE recombinase. As previously reported (113), The DKO mice showed about 2-fold increases in blood glucose at the random-fed state (*IRS1*/*IRS2*$^{F/F}$: 143.6 ± 6.5 versus DKO: 339.2 ± 56.0 mg/dL, P<0.01) and the overnight fasting state (*IRS1*/*IRS2*$^{F/F}$: 66.2 ± 5.6 versus DKO:125.3 ± 14.5 mg/dL, P<0.01). However, deletion of hepatic *Foxo1* gene in DKO mice (TKO) largely rescued both feeding and fasting blood glucose to the normal levels (TKO: Feeding: 147.3 ± 5.6; Fasting: 78.6 ± 4.8 mg/dL; Figure 4A). SB203580 injection resulted in a 16% and 29% decrease in fasting blood glucose level in control *IRS1*/*IRS2*$^{F/F}$ and DKO mice, respectively (*IRS1*/*IRS2*$^{F/F}$ mice: vehicle: 96.5 ± 1.7 versus SB203580: 80.8 ± 4.7 mg/dL, and DKO mice: vehicle: 152.2 ± 17.6 versus SB203580: 107.4 ± 9.2 mg/dL). However, SB203580 administration had no significant effect on blood glucose in TKO mice (Vehicle: 79.1 ± 7.8 versus SB203580: 89.0 ± 12.7 mg/dL; Figure 4B). The control and DKO mice injected with SB203580 had a 24% and 32% reduction in HGP during ipPTT, respectively (Figures 4C and 4D). The effect of SB203580 on HGP was abolished in TKO

mice (Figures 4C and 4D). In DKO hepatocytes, *IRS1* and *IRS2* deficiency resulted in a 26% elevation in HGP at basal level, compared to *IRS1*/*IRS2*$^{F/F}$ hepatocytes. Glucagon treatment increased HGP by 42% and 40% in *IRS1*/*IRS2*$^{F/F}$ and DKO cells, respectively (Figure 4E). However, SB203580 treatment resulted in a 26% and 31% decrease in glucagon-induced HGP in both *IRS1*/*IRS2*$^{F/F}$ and DKO hepatocytes, respectively (Figure 4E).

Given that IRS1 and IRS2 are major insulin signaling components in control of intracellular activation of phosphoinositide 3-kinase (PI3K) and downstream target of Akt (224), we used wortmannin, the PI3K inhibitor to further assess the role of PI3K in suppression of HGP in hepatocytes. Wortmannin treatment did not block the suppression of HGP by SB203580 in glucagon-treated WT hepatocytes (Figure 4F), suggesting that PI3K is not required for p38 action on HGP. Foxo1-S253 is a key phosphorylation site mediating the effect of insulin and PI3K/Akt on suppression of Foxo1 activity and gluconeogenic genes expression (63). We previously showed that global *Foxo1*$^{S253A}$ mutation mice displayed mild increases in feeding blood glucose and insulin levels (223). To further detect whether the Foxo1-S253 phosphorylation is involved in p38-mediated HGP, we detected HGP in *Foxo1*$^{S253A}$ hepatocytes treated with SB203580. Glucagon treatment increased HGP by 52% and 30% in WT and *Foxo1*$^{S253A}$ hepatocytes, respectively. Remarkably, SB203580 treatment attenuated the glucagon effect in both WT and *Foxo1*$^{S253A}$ hepatocytes (Figure 4G). Glucagon also increased Foxo1 protein and SB203580 treatment markedly attenuated the glucagon-induced Foxo1 protein level in *Foxo1*$^{S253A}$ hepatocytes (Figure 4H).

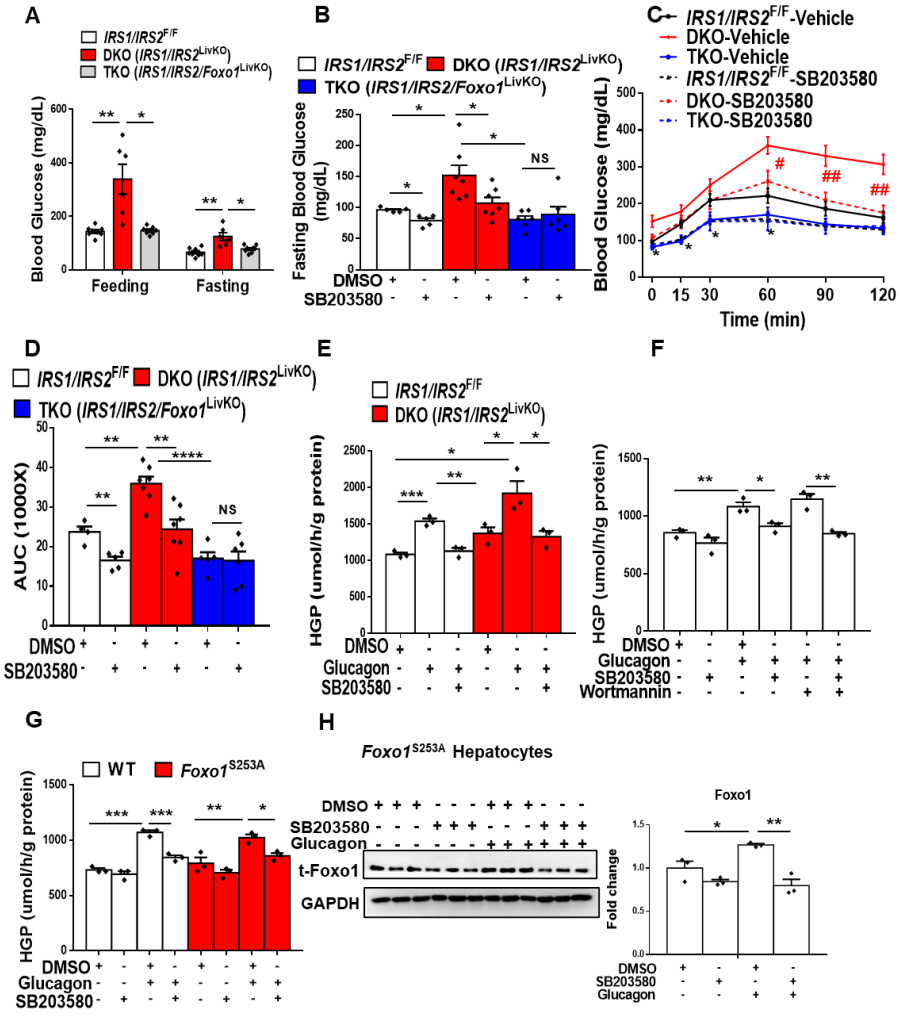



**Figure 4 p38-mediated HGP is independent of insulin signaling**

**(**A) Blood glucose in *IRS1/IRS2*$^{F/F}$, DKO, and TKO mice under 16 h fasted and random-fed state, n=6-7. (B) Blood glucose levels in *IRS1/IRS2*$^{F/F}$, DKO, and TKO mice treated with SB203580 under 16 h fasted condition, n=5-7. (C) Pyruvate tolerance tests in *IRS1/IRS2*$^{F/F}$, DKO, and TKO mice treated with SB203580. *P<0.05 versus *IRS1/IRS2*$^{F/F}$-Vehicle; #P<0.05 versus DKO-Vehicle, n=4-7. (D) AUC of pyruvate tolerance test in (C), n=4-7. (E) Hepatocytes isolated from *IRS1/IRS2*$^{F/F}$ and DKO mice were pretreated with 10 μM SB203580 for 30 min and then treated with 100 nM glucagon for 3 h. Glucose content was measured by glucose kit, n=3. (F) WT hepatocytes were pretreated with 200 nM wortmannin and 10 μM SB203580 for 30 min, followed by 100 nM glucagon for 3 h. Glucose content was measured by glucose kit, n=3. (G) Hepatocytes were isolated from WT and *Foxo1*$^{S253A}$ mice, then pretreated with 10 μM SB203580 for 30 min and treated with 100 nM glucagon for 3 h. Glucose content was measured by glucose kit, n=3. (H) S253A hepatocytes were pretreated with 10 μM SB203580 for 30 min and treated with 100 nM glucagon for 1 h. Foxo1 level was analyzed by Western blotting, n=3. All data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

## Summary

The results in this chapter show that suppression of p38 decreases fasting blood glucose and glucose output during pyruvate tolerance test in DKO mice, whereas p38 function on glucose production is abolished in TKO mice. Furthermore, p38 inhibition blocks glucagon-induced HGP in both DKO and *Foxo1*$^{S253A}$ hepatocytes. Thus, these results indicate hat insulin signaling is not necessary for the regulation of p38 in hepatic glucose production. However, Foxo1 is a key regulator in p38 action on glucagon-induced hepatic glucose production.

# CHAPTER IV

# MECHANISTIC INSIGHTS: P38-FOXO1 INTERACTION IN REGULATION OF HGP

## Introduction

Foxo1 is a major player in response to insulin action and regulates hepatic glucose production through upregulation of PCK1 and G6PC. Insulin action results in phosphorylation of Foxo1 at T24, S253, and S316 through AKT, leading to its degradation, nuclear exportation, and inactivation (63). Foxo1 also has several non-AKT phosphorylation sites by multiple protein kinases, including S246, S284, S295, S326, and T553 (60; 170). Recently, we have identified a new phosphorylation site of Foxo1 by PKA at S273 and Foxo1-S273 phosphorylation increases Foxo1 protein stability and nuclear translocation (52). In addition to phosphorylation modification, the activity of Foxo1 is modulated by deacetylation and glycosylation (225-227).

p38 is a mitogen-activated protein kinase (MAPK) that induces a wide variety of biological effects in response to a broad range of stimuli (228). It has been indicated that p38 regulate glucagon-induced HGP (205). Here, our data show that p38α-mediated HGP relies on Foxo1, whereas its underlying mechanism is not clearly understood. Therefore, in this study we will further investigate the molecular mechanism by which p38α regulates Foxo1 activity and glucagon activates p38 in hepatocytes.

## Methods

### Mice

*Foxo1*$^{S273D}$ and *Foxo1*$^{S273A}$ (52) mice were generated as previously described. All animal experiments were performed according to procedures approved by the Texas A&M University Institutional Animal Care and Use committee. Mice are housed at 22-24 C° and maintained on a 12 h light/12 h dark cycle with a standard chow diet. The male mice at the age of 8-12 weeks were used in all the experiments.

### Primary Mouse Hepatocytes Isolation and Culturing

Primary mouse hepatocytes were isolated from 8-12 weeks old male mice. Livers were perfused with 10 ml of HBSS with 50 mM EGTA and 1 M glucose, followed by perfusion with 15 ml HBSS supplemented with 1 M $CaCl_2$, 1 M glucose, and type II collagenase. The cells were shaken from liver and then screened through 70 μm filter and centrifuged in Percoll medium (GE Healthcare). Viable cells were seeded into low glucose DMEM Medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S).

### Pyruvate Tolerance Test

Recombinant adenovirus (1 x $10^{11}$ pfu/mice) was delivered into mice by retro orbital injection, and pyruvate tolerance test was performed 1 week after delivery of adenovirus. The mice were fasted for 16 h. After determining the basal blood glucose level, mice received 2 g/kg of body weight pyruvate sodium through i.p. injection. Blood glucose level were measured after 15, 30, 60, 90, and 120 min. The administration of SB203580

was performed, as previously described (205). Briefly, the mice were given two shots of SB203580 (10 mg/kg of body weight) via i.p. injection. The first shot was given at the beginning of the fasting, the second shot given 14 h later. After 2 h, basal blood glucose levels were measured. Afterwards, mice were i.p. injected with 2 g/kg of body weight pyruvate sodium and blood glucose levels were monitored after 15, 30, and 90 min.

**Glucose Tolerance Test**

Recombinant adenovirus ($1 \times 10^{11}$ pfu/mice) was delivered into mice by retro orbital injection, and glucose tolerance test was performed 2 weeks after delivery of adenovirus. Mice were fasted for 16 h and then injected with 2 g/kg body weight glucose. Blood glucose levels were measured after 15, 30, 60, 90, and 120 min.

**Insulin Tolerance Test**

Recombinant adenovirus ($1 \times 10^{11}$ pfu/mice) was delivered into mice by retro orbital injection, and insulin tolerance test was performed 3 weeks after delivery of adenovirus. Mice were fasted for 4 h, and then injected with 1 U/kg body weight insulin via i.p. injection. Blood glucose level was monitored after 15, 30, 45, and 60 min.

**Glucose Production in Primary hepatocytes**

Glucose production assay was performed as previously described (216). Briefly, mouse primary hepatocytes were isolated. After attachment, hepatocytes were cultured in KRB buffer containing sodium pyruvate, sodium lactate, and BSA, and then pretreated with 10μM SB203580, 10μ H89, or 10μM ESI-09 for 30 min, followed by 100 nM glucagon treatment for 3 h. Finally, 200 μl medium was collected and glucose content was measured using glucose assay kit (Thermo Fisher). For transfection and adenovirus-

induced overexpression experiments, attached hepatocytes were transfected with 150 nM siRNA (per well in 6-well plate) for 12 h or treated with 100 MOI adenovirus for 12 h. Afterwards, hepatocytes were cultured in KRB medium with pretreatment of 10μM SB203580 for 30 min and following 100 nM glucagon treatment for 3 h. Cell medium was collected and glucose concentration was monitored.

**In Vitro Kinase Assay and Liquid Chromatography-Mass Spectrometry**

To determine whether p38α directly phosphorylates Foxo1 at S273, we performed in vitro kinase assay. Recombinant Foxo1-GST fusion protein, 20 μg (Millipore) was incubated with 1 μg active p38α (Millipore) for 30 min at 30 °C, in 50 μl reaction buffer (25 mmol/L Tris-HCl, 1 mmol/L EGTA, 0.5 mmol/L EDTA, 0.5 mmol/L β-mercaptoethanol, and 3 mmol/L magnesium acetate) with or without 0.1 mmol/L ATP. The phosphorylation of Foxo1 at S273 was analyzed by Western blotting using pFoxo1-S273 antibody. The interesting protein bands were also incised from the SDS-PAGE gel and subjected to dimethyl formamide (DMF)-assisted trypsin digestion. The digested peptides were analyzed by liquid chromatograph-mass spectrometry (LC-MS), as previously described (52). The raw spectrum data were analyzed and MASCOT-compatible mfg files were generated through DataAnalysis software. MASCOT software was used for peptide searching and analysis.

**cAMP Assay**

Mouse primary hepatocytes were isolated and transfected with siRNA-p38α for 24 h, followed by 100 nM glucagon treatment for 1 h. Cellular cAMP was measured using cAMP ELISA kit (Cayman Chemicals).

**MAP Kinase Activity Assay**

MAPK kinase activity assay was performed using MAP Kinase/Erk Assay Kit (Sigma). Briefly, mouse primary hepatocytes were isolated and pretreated with 10 μM H89 or ESI-09 for 30 min, followed by 100 nM glucagon treatment for 1 h. Phosphorylated p38 was purified using immunoprecipitation and added into the reaction buffer (10 μl $Mg^{2+}$/ATP cocktail and 10 μl ADBI), followed by the addition of 10 μl MAP Kinase substrate cocktail. After incubation for 30 min at 30 °C in a shaking incubator, phosphorylation of MBP was detected by immunoblotting.

**PKA Activity Assay**

PKA activity was measured using PKA (Protein Kinase A) Colorimetric Activity Kit (Thermo fisher). Briefly, primary hepatocytes were isolated and transfected with p38α-siRNA for 24 h, then treated with 100 nM glucagon for 1 h. We prepared the cell lysate and analyzed the PKA activity according to manufacturer's instructions.

**Co-immunoprecipitation**

Primary hepatocytes were isolated and cultured in the 10 cm dish with DMEM medium supplemented with 10% FBS. Hepatocytes proteins were collected in 1 ml TNE buffer (Sigma). Antibodies were added into hepatocytes lysis (1:50) and incubated overnight at 4 °C. We then transferred 100 ul protein A beads (Thermo fisher) into the protein-antibody complex and incubated at room temperature for 3 h. We washed the beads and denatured samples. The protein-protein interaction was analyzed using Western blotting.

**Quantitative Real-Time PCR**

Total RNAs were extracted with TRIzol reagent (Invitrogen Life Technologies). The cDNAs were synthesized using iScript$^{TM}$ Reverse Transcription Supermix (Bio-Rad). Quantitative real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The primers are listed in APPENDIX Table 1.

**Immunoblotting, Immunofluorescence and Immunoprecipitation**

For immunoprecipitation, antibodies were added into the cell lysis (1:50) and incubated overnight at 4 °C. Protein A beads (Thermo fisher) were added into the protein-antibody complex and incubated at room temperature for 3 h. The protein-protein interaction was analyzed using Western blotting. For immunofluorescence, Cells were fixed using 4% paraformaldehyde for 10 min and permeabilized for 10 min using 0.2% Triton X-100. After blocking for 30 min in 1% BSA, cells were incubated overnight in Foxo1 antibody (1:100) at 4 ºC. Cells were incubated with secondary antibody (1:500) in the dark for 1 h at room temperature. Cells were then incubated with DAPI for 1 min and mounted coverslip with a drop of mounting medium. Antibodies information is listed in APENDIX Table 2.

**Statistical Analysis**

All results are presented as mean ± SEM. P values were calculated using the Student-t test for the comparison of difference between two groups. Significance among multiple groups was tested using one-way and two-way ANOVA followed by Tukey's multiple comparison test in GraphPad Prism. $P < 0.05$ was considered statistically significant.

## Results

### *P38 Increases Foxo1 Protein Stability*

In view of Foxo1 as a key controller of p38-induced HGP, we examined whether p38 regulates Foxo1 expression in response to glucagon. Glucagon administration increased the signal intensity of Foxo1, leading to a marked redistribution of Foxo1 immunoreactivity from cytoplasm to nuclei in HepG2 cells. However, such an effect of glucagon was diminished by p38 inhibitor, SB203580, in HepG2 cells (Figure 5A). In WT mouse primary hepatocytes, glucagon increased Foxo1 protein level by 2.2-fold. SB203580-mediated p38 inhibition caused a decrease in Foxo1 protein abundance by 29% upon glucagon treatment (Figure 5B).

In considering that glucagon upregulates *Foxo1* mRNA expression (168; 169), we examined the effect of p38 on *Foxo1* mRNA expression in WT hepatocytes. Glucagon, as expected, elevated the mRNA expression levels of *Foxo1*, *G6pc*, and *Pck1*, whereas SB203580 treatment dampened glucagon-induced *G6pc* and *Pck1* mRNA expression rather than dampening *Foxo1* expression, suggesting that p38 regulates Foxo1 protein abundance through post-translation modification (Figure 5C). Foxo1 is phosphorylated by insulin, glucagon, or growth factor signaling cascades, thus regulating its cytoplasmic retention and cellular protein stability (52; 60; 229). We next treated WT hepatocytes with MG132, a 20S proteasome complex inhibitor. Consistent with our data above (Figure 5B), glucagon treatment resulted in a marked increase in Foxo1 protein and p38 inactivation

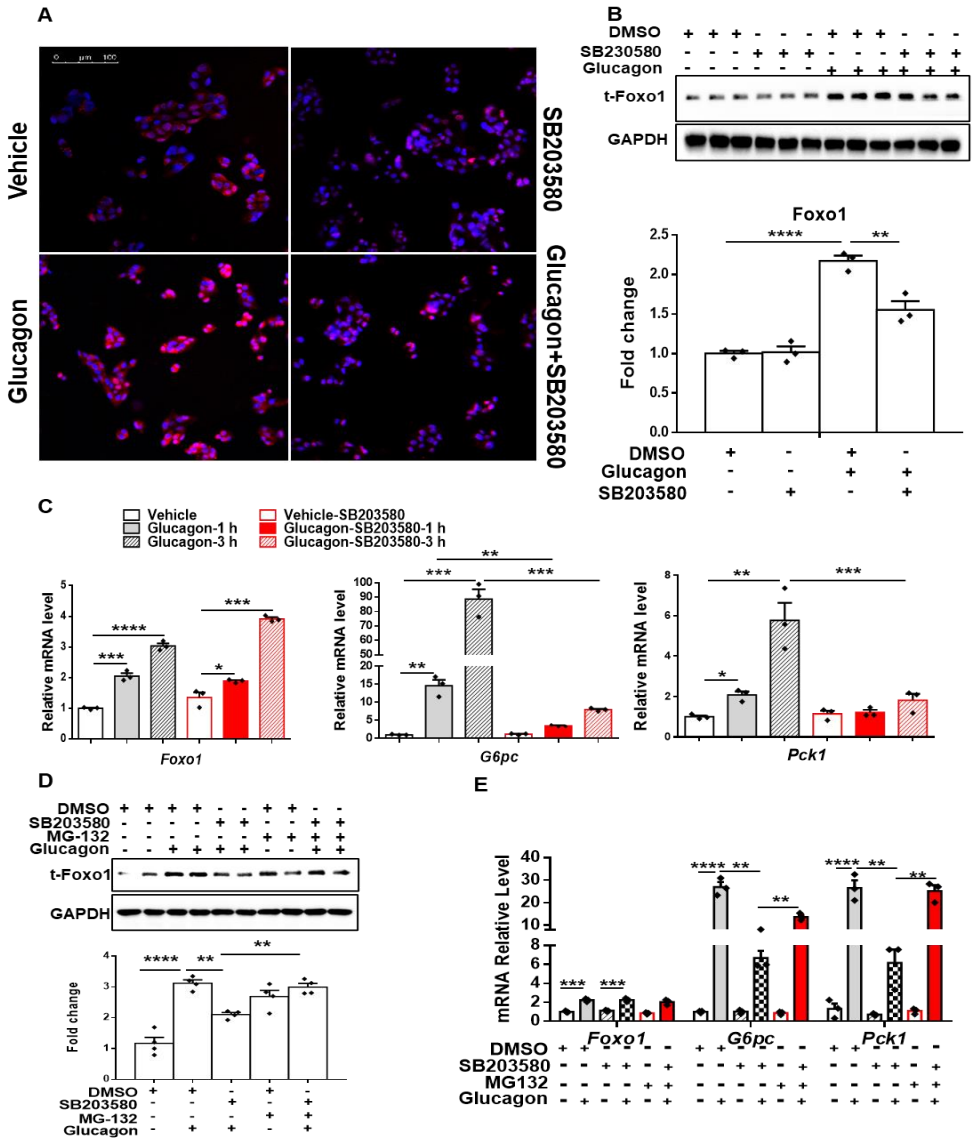



**Figure 5 p38α increases Foxo1 nuclear translocation and protein stability**

(A) HepG2 cells were pretreated with 10 μM SB203580 for 30 min, then treated with 100 nM glucagon for 1 h. Foxo1 subcellular translocation was analyzed by immunofluorescence. Red color: Foxo1; Blue color: Nucleus. (B) Hepatocytes isolated from WT mice were pretreated with 10 μM SB203580 for 30 min, then treated with 100 nM glucagon for 1 h. t-Foxo1 was detected using Western blotting, n=3. (C) Hepatocytes were pretreated with 10 μM SB203580 for 30 min, then treated with 100 nM glucagon for 1 h and 3 h, respectively. *Foxo1*, *G6pc*, and *Pck1* mRNA levels were measured by Q-PCR, n=3. (D) Hepatocytes were pretreated with 10 μM MG132 and 10 μM SB203580, then treated with 100 nM glucagon for 1 h. t-Foxo1 abundance was detected, n=4. (E) WT Hepatocytes were pretreated with 10 μM MG132 and 10 μM SB203580, then treated with 100 nM glucagon for 1 h. *Foxo1*, *G6pc*, and *Pck1* mRNA levels were measured by Q-PCR, n=3. All data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

abrogated such an effect of glucagon in hepatocytes. However, MG132 administration significantly diminished the SB230580 inhibitory effect on Foxo1 protein upon glucagon treatment (Figure 5D). Furthermore, downregulation of *G6pc* and *Pck1* mRNA expression by SB203580 was rescued by MG132 in hepatocytes treated with glucagon; on the other hand, MG132 did not affect the mRNA expression of *Foxo1* (Figure 5E). Thus, these results indicate that p38 promotes Foxo1 nuclear translocation and increases Foxo1 protein stability to involve glucagon-induced HGP.

*P38α Promotes Foxo1 Protein Stability via Foxo1-S273 Phosphorylation*

We previously reported that glucagon phosphorylates Foxo1 at S273 via PKA, increasing Foxo1 protein stability and nuclear translocation (52). Indeed, p38 also increases Foxo1 protein abundance and nuclear distribution upon glucagon treatment. We next asked whether p38α regulates Foxo1 via Foxo1-S273 phosphorylation (pFoxo1-S273), increasing Foxo1 activity and protein stability. As we previously reported (52), blockage of PKA by H89 attenuated glucagon-induced pFoxo1-S273 and total Foxo1 (t-Foxo1) protein levels. Similarly, SB203580 treatment also decreased pFoxo1-S273 and t-Foxo1 protein levels in response to glucagon in control hepatocytes (Figure 6A); this suggests that pFoxo1-S273 might also be a target of p38 in regulation of Foxo1 activity. The p38 family has four members: α, β, γ, and δ isoforms. Western-blot analysis revealed that knockdown of p38α resulted in significant decreases in pFoxo1-S273, t-Foxo1, pCREB-S133, and PCK1 levels in response to glucagon (Figure 6B). However, loss-function of p38β, γ, or δ barely affected glucagon effect (Figures 6C-E). As expected,

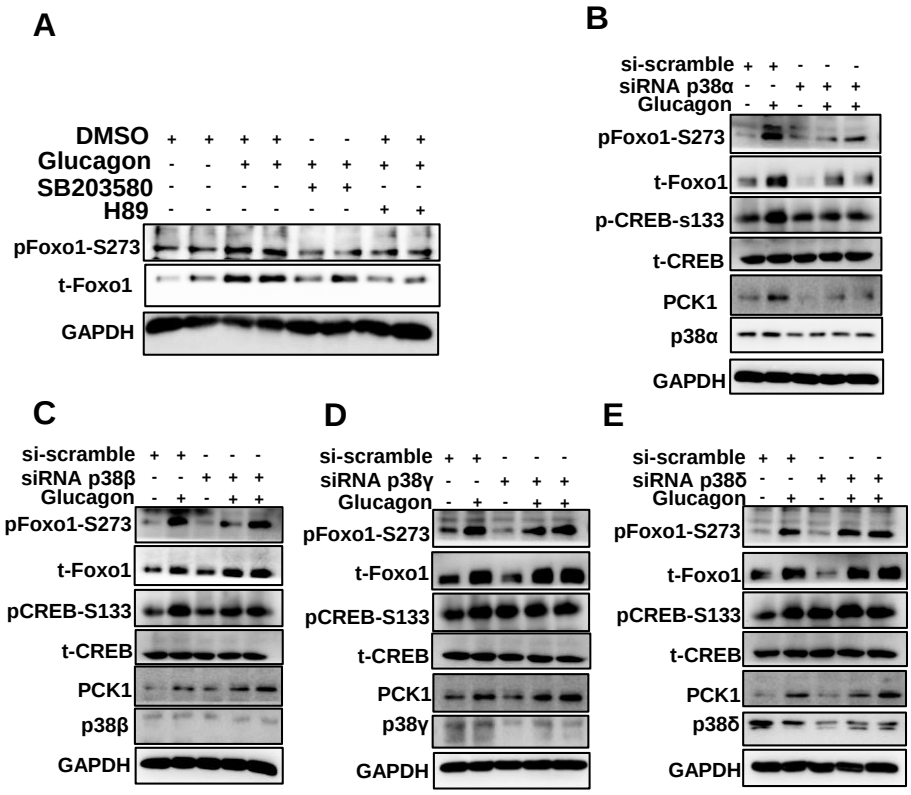



**Figure 6 p38α stimulates Foxo1-S273 phosphorylation to increase Foxo1 activity**
(A) Primary hepatocytes were pretreated with 10 μM H89 and SB203580, and then treated with 100 nM glucagon for 1 h. pFoxo1-S273, t-Foxo1 and pp38-T180/Y182 levels were detected. (B-E) Primary hepatocytes were transfected with p38 isoforms siRNAs and treated with 100 nM glucagon for 1 h. The protein levels of pFoxo1-S273, t-Foxo1, PCK1, and pCREB-S133 were measured using Western blotting.

p38α, β, γ, and δ expression levels were remarkably downregulated by their corresponding siRNAs (Figures 6B-E, bottom two blots).

*P38α Phosphorylates Foxo1-S273 Directly and Indirectly to Enhance Glucagon Signaling*

PKA regulates pFoxo1-S273 in glucagon signaling (52). We next asked whether PKA activation is required for p38 phosphorylation/activation that controls Foxo1-S273 phosphorylation in glucagon action in hepatocytes. Inhibition of PKA through H89 or siRNA attenuated glucagon-induced pFoxo1-S273 and t-Foxo1 protein levels, but did not block the phosphorylation/activation of p38 in response to glucagon in hepatocytes (Figure 7A), suggesting that PKA activation is not required for p38 activation in glucagon action in hepatocytes. We next determined whether p38α activation is required for PKA activation and Foxo1-S273 phosphorylation in glucagon signaling. We performed in vitro kinase assays and glucagon dramatically increased PKA activity by ~ 2.7-fold, whereas RNAi-mediated p38α knockdown partially blocked PKA activity by 22% upon glucagon treatment (Figure 7B). Consistently, glucagon stimulated PKA substrates' phosphorylation, indicating PKA activity, which is partially attenuated by p38α knockdown (Figure 7C).

Given that suppression of PKA largely but incompletely blocks glucagon-induced Foxo1-S273 phosphorylation, we continue to investigate other kinases that can potentially regulate Foxo1-S273 and here we test the role of p38. As expected, glucagon increased pFoxo1-S273 and t-Foxo1 protein levels, which was partially blocked by either siRNA-

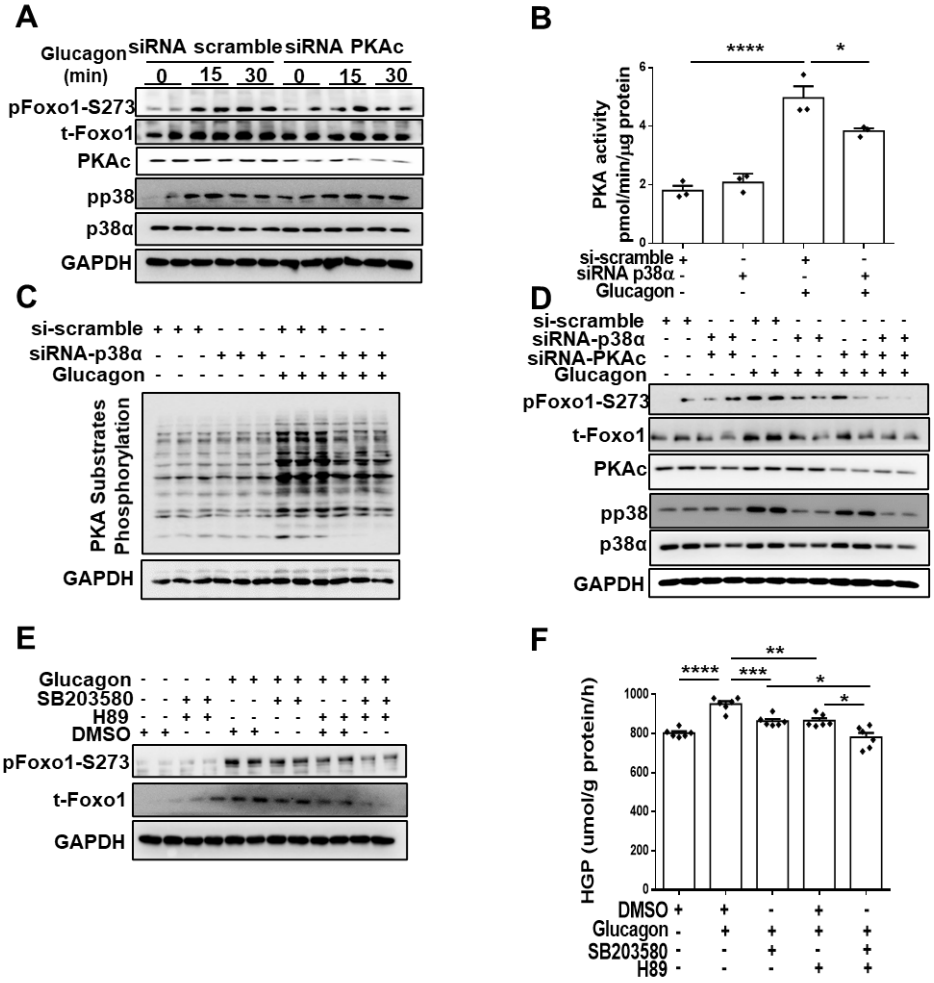



**Figure 7 p38α and PKA synergistically increase Foxo1 activity and promote HGP**

(A) Hepatocytes were transfected with siRNA-PKAc for 24 h, then treated with 100 nM glucagon for 15 min and 30 min. pFoxo1-S273, t-Foxo1, and pp38-T180/Y182 levels were analyzed. (B) Mouse primary hepatocytes were transfected with p38α-siRNA for 24 h, and then treated with 100 nM glucagon for 1 h. PKA activity was measured by PKA activity assay, n=3. (C) Mouse Hepatocytes were transfected with siRNA-p38α for 24 h, then treated with 100 nM glucagon for 1 h. Phosphorylation of PKA substrates were detected by western blotting. (D) Mouse primary hepatocytes were transfected with siRNA-p38α and siRNA-PKAc, and then treated with 100 nM glucagon for 1 h. pFoxo1-S273, t-Foxo1, and pp38-T180/Y182 levels were detected. (E) WT Hepatocytes were pretreated with 10 μM H89 and SB203580 for 30 min, followed by 100 nM glucagon treatment for 1 h. pFoxo1-S273 and t-Foxo1 levels were analyzed. (F) Mouse hepatocytes were pretreated with 10 μM H89 and SB203580 for 30 min, followed by 100 nM glucagon treatment for 3 h. Glucose content was measured by glucose kit, n=6. All data are presented as mean ± SEM. *P<0.05,**P<0.01, ***P<0.001, ****P<0.0001.

mediated inhibition of p38α or PKA in hepatocytes (Figure 7D). Importantly, suppression of both p38α and PKA had an additive effect on abolishing pFoxo1-S273 and t-Foxo1 levels in response to glucagon in hepatocytes (Figures 7D and 7E, top two blots). Moreover, glucagon-induced p38-T180/Y182 phosphorylation was not blocked by suppression of PKA (Figure 7C, bottom three blots), suggesting that Foxo1-S273 phosphorylation is controlled by both PKA and p38 independently, even though p38 activation partially regulates PKA activity in glucagon action We next performed HGP for functional analyses of the PKA and p38 in control of glucose production in vitro. The HGP assay revealed that glucagon increases HGP by 21% in hepatocytes; however, suppression of PKA by H89, a chemical PKA inhibitor, or suppression of p38 by SB203580 resulted in a 12% or 11% decrease in glucagon-induced HGP, respectively. Importantly, suppression of both PKA and p38 further reduced the glucagon-induced HGP by 19% ($p<0.05$, Figure 7F). These results suggest that both PKA and p38α regulate pFoxo1-S273 and HGP independently. There was a report suggesting that p38 phosphorylates Foxo1 directly (170), we next asked whether p38α directly phosphorylates Foxo1-S273. LC-tandem MS analysis revealed that human Foxo1 is phosphorylated by p38α at S276, even though other sites detected including S279, S287, S298, S329, T425, and T428 (Figures 8A). The co-immunoprecipitation analysis was performed, showing that p38α, rather than p38β, γ, or δ, interacts with Foxo1 (Figure 8B). We further performed in vitro kinase assay in which GST-Foxo1 protein and active p38α were incubated in the protein kinase assay buffer system. We observed recombinant human Foxo1-S276 (equivalent to mouse Foxo1-S273) is phosphorylated by p38α in vitro by

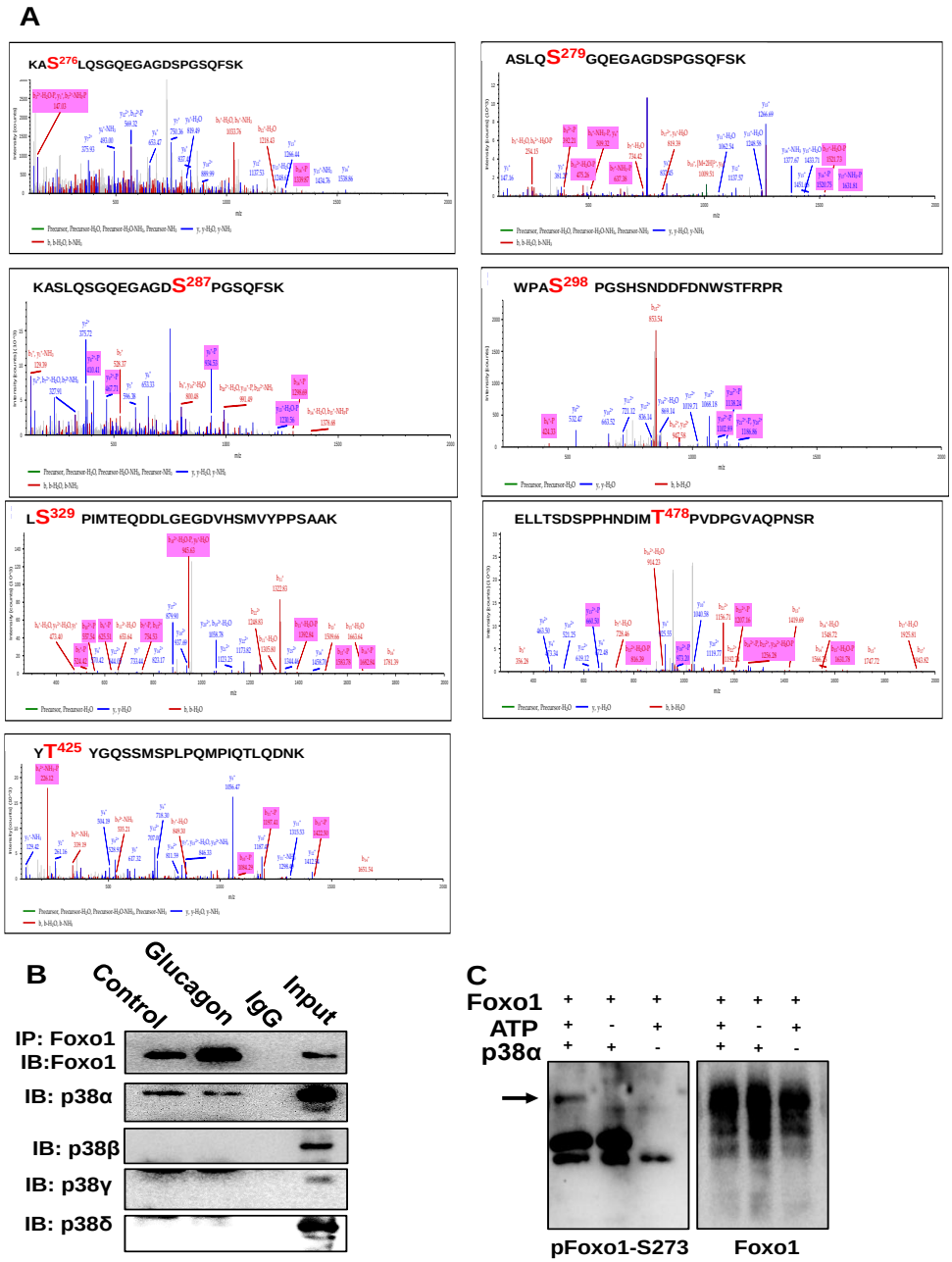



**Figure 8 p38α directly phosphorylates Foxo1 at S273**

(A) MS/MS spectra of Foxo1 peptide containing phosphorylation sties by p38α. The Foxo1-GST was incised from the SDS-PAGE gel and subjected to LC-MS/MS. (B) The interaction between p38α, β, γ, or δ and Foxo1 in hepatocytes. (C) Active p38α and GST-Foxo1 were incubated in the reaction buffer for 30 min at 30 ºC. pFoxo1-S273 was analyzed using Western blotting.

Western blot (Figure 8C). Moreover, Therefore, these data indicate that p38α stimulates Foxo1-S273 phosphorylation directly through binding to Foxo1.

*Glucagon Activates P38 Through cAMP-EPAC Pathway*

It is known that glucagon activates p38, but the underlying mechanism and functional significance remain incompletely understood. Glucagon activates adenylyl cyclase by binding to glucagon receptor (GCGR), increasing the production of cAMP to trigger signal transduction (152). Firstly, we examined the role of GCGR in activation of p38 by glucagon. The GCGR antagonist attenuated p38 phosphorylation in liver (Figure 9A). Secondly, we treated WT hepatocytes with cAMP to test whether cAMP is a key in p38 activation; the results showed that cAMP increases pFoxo1-S273, elevates t-Foxo1 protein level, and stimulates pp38-T180/Y182 (Figure 9B). Consistently, inhibition of PKA by H89 did not block the stimulation of p38α by cAMP (Figures 9C). cAMP significantly increased PKA substrates phosphorylation, whereas RNAi-mediated p38α knockdown barely attenuated cAMP-induced PKA substrates' phosphorylation (Figure 9D). RNAi-mediated p38α suppression attenuated glucagon-induced cellular cAMP level (Figure 9E), suggesting that p38α potentially elevates cellular cAMP level that enhances PKA activity.

In addition to PKA, exchange protein directly activated by cAMP (EPAC) is another family of cAMP sensor proteins (53). Inhibition of EPAC by ESI-09, a chemical inhibitor, largely blocked glucagon-induced pFoxo1-S273 and pp38-T180/Y182, as well as t-Foxo1 (Figure 9F). The MAPK kinase activity analysis showed that glucagon

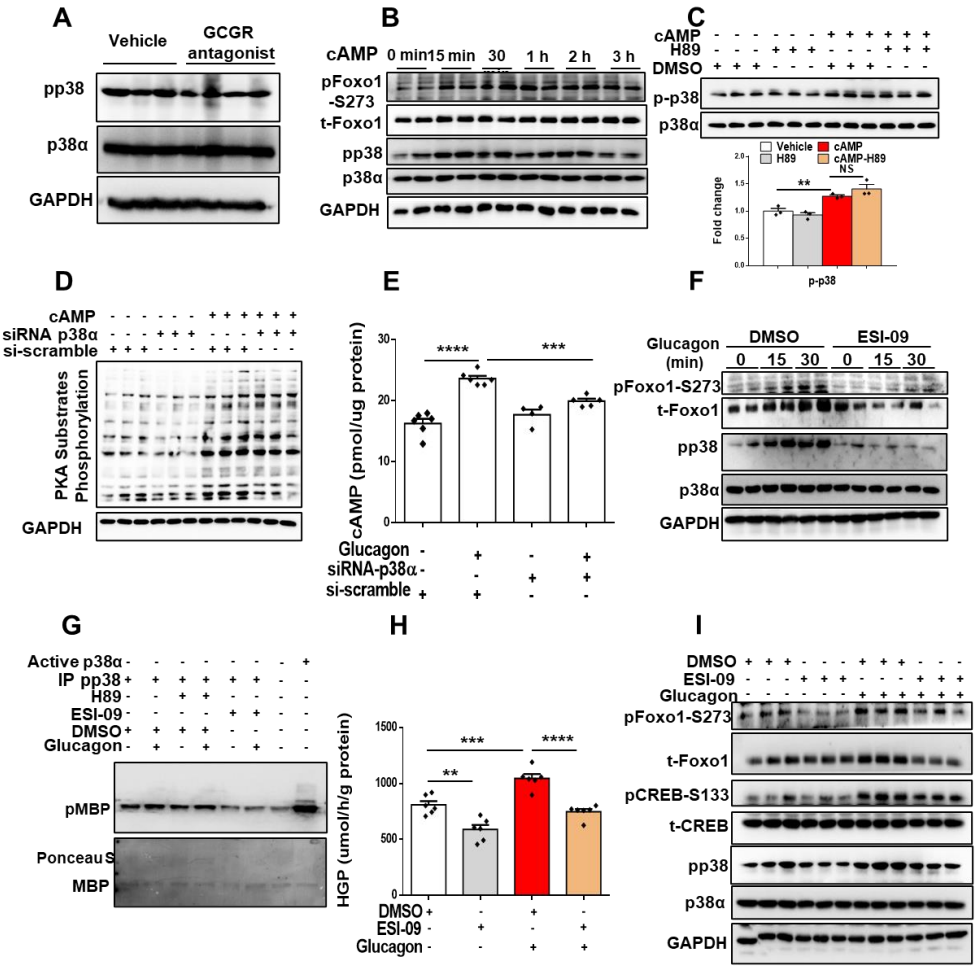



**Figure 9 cAMP-EPAC pathway mediates glucagon-induced p38 activation**

(A) WT mice were intravenously injected with glucagon receptor (GCGR) antagonist ([des-His1, Glu9]-Glucagon amide, 1mg/kg body weight). Liver samples were collected after 45 min and phosphorylation of p38 was analyze using western blotting. (B) Hepatocytes were treated with 100 nM cAMP in a time-dependent manner, as indicated. Protein levels were detected using western blotting. (C) WT hepatocytes were pretreated with 10 μM H89 for 30 min , then treated with 100 nM cAMP for 1 h. pp38-T180/Y182 level was detected, n=3. (D) Mouse Hepatocytes were transfected with siRNA-p38α for 24 h, then treated with 100 nM cAMP for 1 h. PKA substrates phosphorylation were detected. (E) Hepatocytes were transfected with siRNA-p38α for 24 h, then treated with 100 nM glucagon for 1 h. The cellular cAMP levels were measured, n=4-6. (F) Primary hepatocytes were pretreated with 10 μM ESI-09 and treated with 100 nM glucagon for 15 min and 30 min. Protein levels were analyzed by Western blotting. (G) pp38 was immunoprecipitated from hepatocytes treated with 100 nM glucagon after treatment of H89 and ESI-09. The activity of p38α was determined by MAPK kinase assay. (H) Hepatocytes were pretreated with 10 μM ESI-09 for 30 min, followed by 100 nM glucagon treatment for 3 h. Glucose content was measured by glucose kit, n=6. (I) Mouse primary hepatocytes were treated with ESI-09 for 30 min, followed by 100 nM treatment for 3 h. Protein abundances were analyzed by Western blotting. **P<0.01, ***P<0.001, ****P<0.0001.

increases p38 activity that is indicated by MBP phosphorylation. Inhibition of EPAC, rather than PKA, markedly abrogated glucagon-stimulated p38 activity (Figure 9G). Moreover, glucagon-induced HGP was significantly decreased by inhibition of EPAC (Figure 9H). Consistently, glucagon increased the levels of pFoxo1-S273, pCREB-S133, pp38-T180/Y182, as well as t-Foxo1. However, EPAC inhibition by ESI-09 treatment blocked theses effects of glucagon (Figure 9I).

Ozcan *et al.* reported that pp38-T180/Y182 is decreased in the CaMKII-deficiency liver (60). We finally answered the question of whether CaMKII plays a role in pFoxo1-S273 and pp38-T180/Y182 in response to glucagon. We found that although pp38-T180/Y182 is moderately decreased in CaMKII-suppressed hepatocytes at basal level, RNAi-mediated CaMKII suppression has no marked effect on glucagon-induced pp38-T180/Y182, pFoxo1-S273, and pCREB-S133 (Figure 10). These results demonstrate that glucagon stimulates p38 activity largely through the GCGR–cAMP–EPAC signaling pathway.

*Foxo1-S273 Mutation Blocks the Effect of P38α on HGP in Primary Hepatocytes*

To evaluate the role of Foxo1-S273 phosphorylation in p38α-mediated HGP, we generated *Foxo1*$^{S273D}$ and *Foxo1*$^{S273A}$ mice, in which Foxo1-S273 is mutated into aspartate (D) mimicking phosphorylation and alanine (A) blocking phosphorylation at Ser-273, respectively (52), and monitored HGP in primary hepatocytes isolated from these mice. In WT hepatocytes, RNAi- or SB203580-mediated p38α suppression largely abrogated glucagon-induced HGP. *Foxo1*$^{S273D}$ and *Foxo1*$^{S273A}$ hepatocytes showed impaired basal and glucagon-induced HGP. Importantly, suppression of p38α either by siRNA or

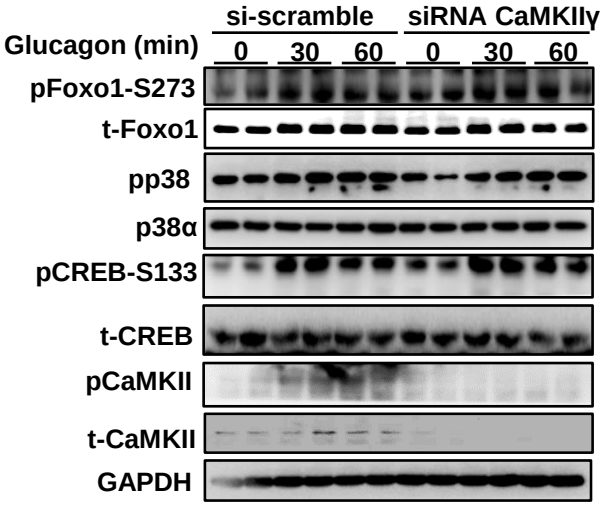



**Figure 10 The activation of p38 by glucagon is independent of CaMKII**
Mouse Hepatocytes were transfected with siRNA-CaMKIIγ for 24 h, then treated with 100 nM glucagon for 1 h. pFoxo1-S273, t-Foxo1, pp38-T180/Y182, pCREB-S133, and pCaMKII-T287 levels were detected using western blotting.

SB203580 treatment had little effect on glucagon-induced HGP in both *Foxo1*$^{S273D}$ and *Foxo1*$^{S273A}$ hepatocytes (Figures 11A-C). Protein analysis revealed that glucagon significantly increased pFoxo1-S273, t-Foxo1, and G6PC levels in WT hepatocytes, while p38α knockdown blocked the glucagon stimulatory effect (Figure 11D); however, *Foxo1*$^{S273D}$ and *Foxo1*$^{S273A}$ hepatocytes with p38α suppression had no changes in t-Foxo1 protein abundance upon glucagon treatment (Figures 11E and 11F).

We next performed p38α gain-of-function using adenovirus (Adv-p38α) in primary hepatocytes isolated from WT, *Foxo1*$^{S273D}$, and *Foxo1*$^{S273A}$ mice. In WT cells, overexpression of p38α stimulated HGP by 23%; glucagon treatment resulted in a 30% increase in HGP, which was not further enhanced by p38α overexpression (Figure 12A). Adv-p38α treatment markedly increased pFoxo1-S273, t-Foxo1, G6PC, and PCK1 protein levels, as well as slightly stimulating PKA substrates' phosphorylation; however, glucagon markedly induced PKA substrates' phosphorylation, pFoxo1-S273, t-Foxo1, G6PC, as well as PCK1 proteins in cells, which were not further enhanced by p38α overexpression (Figures 12B and C). *Foxo1*$^{S273D}$ hepatocytes exhibited a 14% induction in HGP upon glucagon challenge, whereas p38α overexpression had no further effect on HGP in the *Foxo1*$^{S273D}$ cells (Figure 12D). Protein expression analysis in *Foxo1*$^{S273D}$ cells revealed that p38α overexpression had little effect on increasing the Foxo1 protein level. Of note is that glucagon slightly increased Foxo1 level in *Foxo1*$^{S273D}$ cells (Figure 12E). In *Foxo1*$^{S273A}$ hepatocytes, glucagon resulted in a 10% elevation in HGP. Distinct to WT cells, Adv-p38α treatment had no stimulatory effect on HGP in the *Foxo1*$^{S273A}$ cells (Figure 12F). These data suggest that p38α regulates HGP in response to glucagon largely

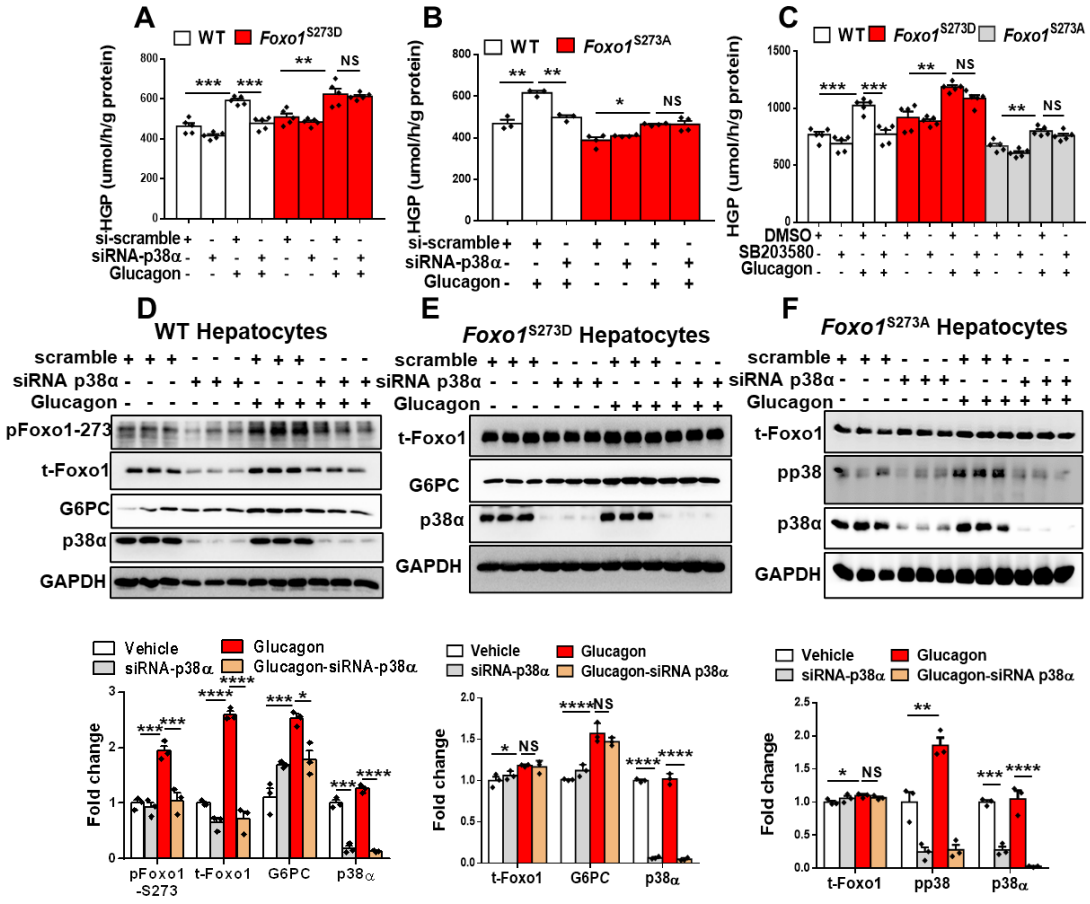



**Figure 11 p38α-induced HGP requires Foxo1-S273 phosphorylation in hepatocytes**
(A) Hepatocytes were isolated from WT and *Foxo1*$^{S273D}$ mice, and then transfected with siRNA-p38α for 12 h, followed by 100 nM glucagon treatment for 3 h. Glucose content was measured by glucose kit, n=5. (B) WT and *Foxo1*$^{S273A}$ hepatocytes were transfected with siRNA-p38α for 12 h, followed by 100 nM glucagon treatment for 3 h. Glucose content was measured by glucose kit, n=3-4. (C) Hepatocytes from WT, *Foxo1*$^{S273D}$, and *Foxo1*$^{S273A}$ mice were pretreated with 10 μM SB203580 for 30 min and treated with 100 nM glucagon for 3 h. Glucose content was measured by glucose kit, n=5. (D) p38α siRNA was transfected into Hepatocytes, followed by 100 nM glucagon treatment for 1 h. Protein levels were analyzed, n=3. (E) Hepatocytes were isolated from *Foxo1*$^{S273D}$ mice, then transfected with siRNA-p38α for 24 h, followed by 100 nM glucagon treatment for 1 h. t-Foxo1, G6PC, and p38α were analyzed, n=3. (F) Hepatocytes were isolated from *Foxo1*$^{S273A}$ mice, then transfected with siRNA-p38α for 24 h, followed by 100 nM glucagon treatment for 1 h. t-Foxo1 and p38α levels were analyzed, n=3. All data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

via Foxo1-S273 phosphorylation.

We further examined whether p38 mediates glucagon-induced Foxo1 nuclear translocation through pFoxo1-S273. Glucagon increased cytosolic and nuclear Foxo1 levels by ~ 5- and 2-fold, respectively, in WT hepatocytes; however, SB203580 treatment largely blocked the effect of glucagon on Foxo1 distribution (Figure 13A, upper three blots). In *Foxo1*$^{S273D}$ hepatocytes, glucagon slightly increased cytosolic and nuclear Foxo1 abundance, while inhibition of p38 by SB203580 did not significantly impair the Foxo1 levels in both cytosol and nucleus of *Foxo1*$^{S273D}$ cells (Figure 13A, bottom three blots). Furthermore, we overexpressed *Foxo1*$^{WT}$ and *Foxo1*$^{S273D}$ in WT primary hepatocytes using adenoviral expression system and detected exogenously expressed Foxo1 nucleus distribution. Similarly, glucagon increased Foxo1 abundance in nucleus upon glucagon treatment, and inhibition of p38 by SB203580 diminished glucagon's stimulatory effect on Foxo1 nuclear translocation in Adv-*Foxo1*$^{WT}$ expressed cells. Adv-*Foxo1*$^{S273D}$ expressed hepatocytes showed accumulated signal of Foxo1 in both cytosol and nucleus. Importantly, SB203580 treatment failed to suppress Foxo1 nuclear localization in Adv-*Foxo1*$^{S273D}$ expressed cells (Figure 13B). Taken together, these data suggest that p38-mediated Foxo1 nuclear translocation largely depends on the Foxo1-S273 phosphorylation.

*Foxo1-S273 Mutation Impairs P38α-Mediated HGP in vivo*

We further investigated physiological significance of Foxo1-S273 phosphorylation in p38α-mediated HGP by using the *Foxo1*$^{S273D}$ and *Foxo1*$^{S273A}$ mice. We injected SB203580 with a dose of 10 mg/kg body weight into mice and performed ipPTT.

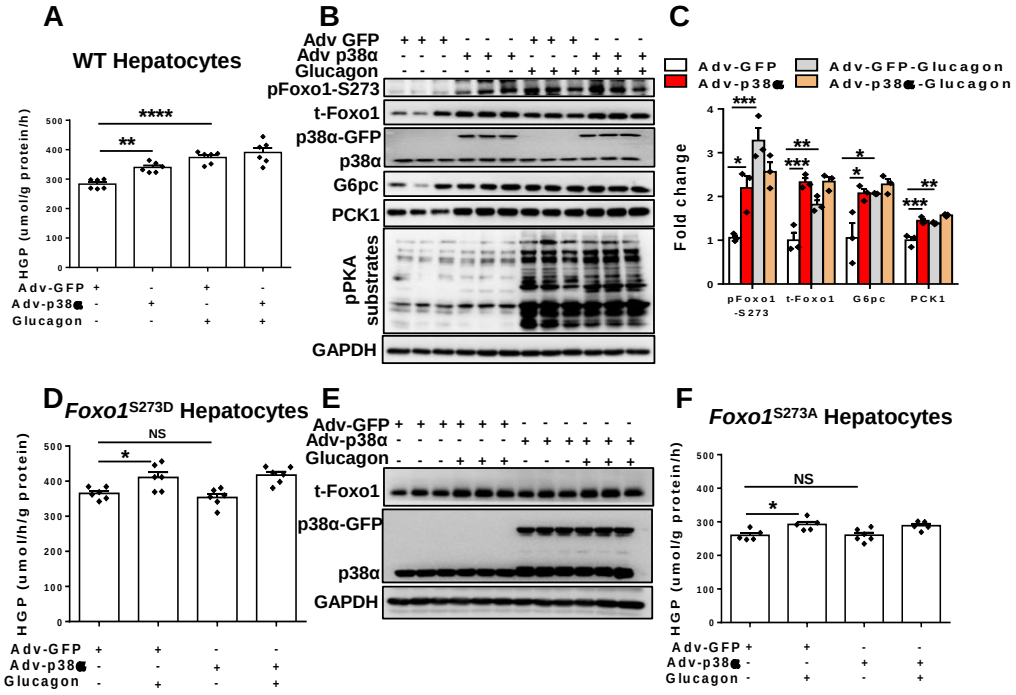



**Figure 12 p38α overexpression-induced HGP is attenuated by Foxo1-S273 mutation**
(A) WT Mouse hepatocytes were transduced with Adv-p38α for 12 h, then treated with 100 nM glucagon for 3 h. Glucose content was measured by glucose kit, n=6. (B) p38α was overexpressed in WT hepatocytes via adenovirus, followed by 100 nM glucagon treatment for 3 h. Protein levels were analyzed via Western blotting. (C) Quantification of (B), n=3. (D) *Foxo1*$^{S273D}$ hepatocytes were transduced with Adv-p38α for 12 h, then treated with 100 nM glucagon for 3 h. Glucose content was measured by glucose kit, n=6. (E) p38α was overexpressed in *Foxo1*$^{S273D}$ hepatocytes via adenovirus, followed by 100 nM glucagon treatment for 3 h. Protein levels were analyzed via western blotting. (F) *Foxo1*$^{S273A}$ hepatocytes were treated with glucagon for 3 h. Glucose production was measured by glucose kit, n=5-6. All data are presented as mean ± SEM. *****P<0.05, ******P<0.01, *******P<0.001, ********P<0.0001.

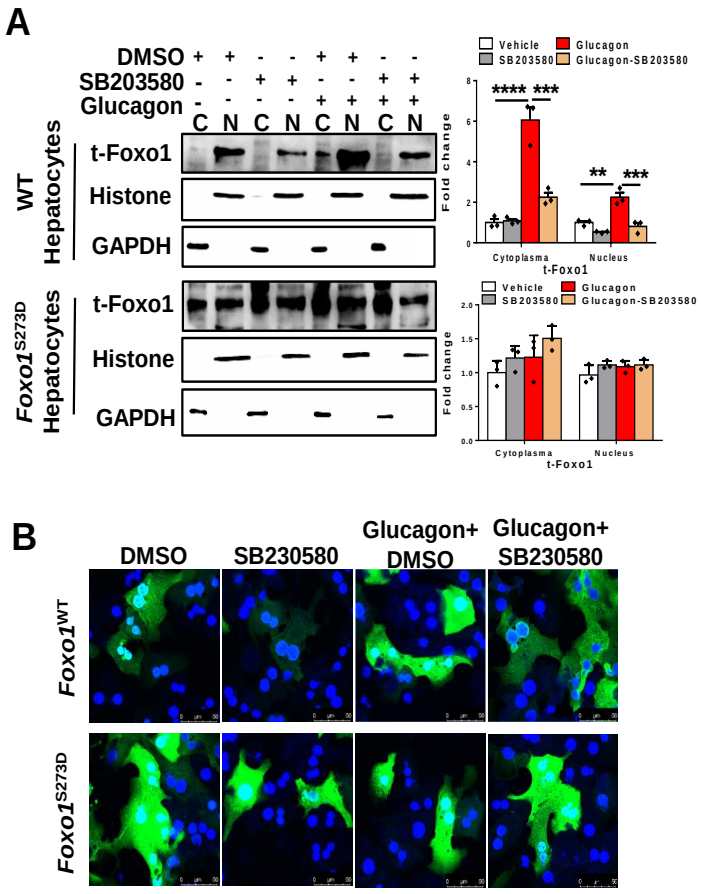



**Figure 13 p38α promotes Foxo1 nuclear translocation via Foxo1-S273 phosphorylation**
(A) Mouse hepatocytes were isolated from WT and *Foxo1*$^{S273D}$ mice, and then pretreated with 10 μM SB203580 for 30 min, followed by 100 nM glucagon treatment for 1 h. Cytoplasmic and nucleus proteins were extracted and Foxo1 protein level was analyzed by Western blotting, n=3. The data is presented as mean ± SEM. (B) WT hepatocytes were transduced with *Foxo1*$^{WT}$ and *Foxo1*$^{S273D}$ adenovirus for 12 h, and then treated with 10 μM SB203580 for 30 min, followed by 100 nM glucagon treatment for 1 h. Foxo1 was detected using immunofluorescence. All data are presented as mean ± SEM. **P<0.01, ***P<0.001, ****P<0.0001.

SB203580-treated WT mice decreased fasting blood glucose by 26% (Vehicle: 57.3 ± 2.4 versus SB203580: 42.2 ± 1.5 mg/dL, $p<0.05$) and reduced HGP by 10% in WT mice (Figures 13A-C). However, *Foxo1*$^{S273D}$ mice, as compared with WT mice, had a slight increase in fasting blood glucose and a significant elevation in HGP. Importantly, SB203580-mediated p38 inhibition failed to significantly suppress the fasting blood glucose (Vehicle: 67.3 ± 3.1 versus SB203580: 60.5 ± 1.5 mg/dL) and HGP in *Foxo1*$^{S273D}$ mice (Figures 14A-C). SB203580 administration decreased Foxo1 protein level by 60% in the liver of WT mice liver. However, *Foxo1*$^{S273D}$ liver exhibited a 35% increase in Foxo1 protein, as compared to WT mouse liver; *Foxo1*$^{S273D}$ mice also displayed resistance to SB203580-induced suppression of Foxo1 protein in liver (Figures 14D and 14E). Inhibition of p38 had no effect on *Foxo1* mRNA expression level, but *Pck1* mRNA expression level was decreased by 28% in the liver of WT mice-treated with SB203580. The effect of SB203580 on suppression of *Pck1* expression was blocked in the Foxo1-S273D liver (Figure 14F).

We next examined whether p38α suppression impairs blood glucose in *Foxo1*$^{S273D}$ mice using AAV8-shRNA expression system. Retroorbital injection of AAV8-p38α-shRNA for a week successfully reduced hepatic p38α by 80% in WT mice (Figures 15E, bottom two blots) without changing body weight and liver weight (data not shown). The AAV8-p38α-shRNA treatment resulted in a 16% reduction in fasting blood glucose in WT mice (scramble-shRNA: 76.2 ± 1.7 versus p38α-shRNA: 64.3 ± 2.3 mg/dL, $p<0.05$). The fasting blood glucose in *Foxo1*$^{S273D}$ mice was increased, as compared with WT mice, and delivery of AAV8-p38α-shRNA did not significantly impair fasting blood glucose in

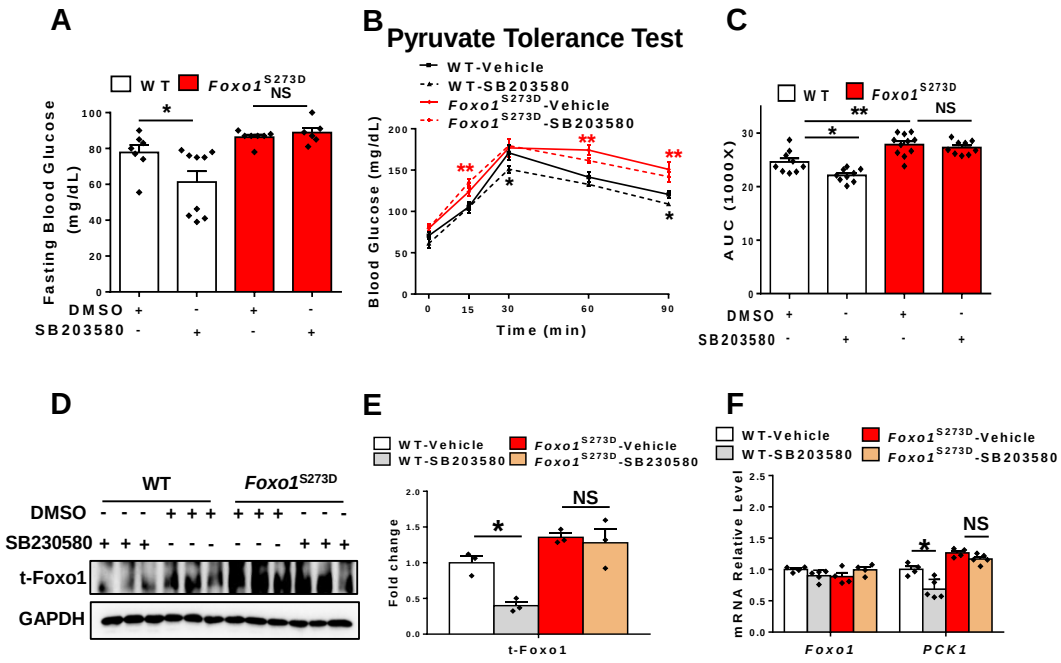



**Figure 14 *Foxo1*$^{S273D}$ mice show resistance to SB203580-suppressed HGP**
(A) Blood glucose in 16 h fasted WT and *Foxo1*$^{S273D}$ mice treated with 10 mg/kg body weight of SB203580, n=6-9. (B) WT and *Foxo1*$^{S273D}$ mice were treated with 10 mg/kg body weight of SB203580. After 16 h fasting, 2 g/kg body weight pyruvate was i.p. injected into the mice. Blood glucose levels were measured. *****P<0.05 versus WT-Vehicle, n=4. (C) AUC of pyruvate tolerance tests, n=9-11. (D) Foxo1 protein level in WT and *Foxo1*$^{S273D}$ mice treated with SB203580 under 16 h fasting condition, n=3. (E) Quantification of (D). (F) *Foxo1* and *Pck1* mRNA levels in WT and *Foxo1*$^{S273D}$ mice treated with SB203580 under 16 h fasting condition, n=4-5. All data are presented as mean ± SEM. *****P<0.05, ******P<0.01.

*Foxo1*$^{S273D}$ mice (scramble-shRNA: 92.0 ± 4.6 versus p38α-shRNA: 84.2 ± 1.4 mg/dL; Figure 15A). Furthermore, shRNA-mediated p38α suppression inhibited HGP during pyruvate tolerance tests by 14% in WT mice; however, S273D mutation blocked the inhibitory effect of p38α suppression on HGP (Figures 15B and 15C). Suppression of p38α had little effect on the mRNA levels of *IRS1*, *IRS2*, and *Foxo1* in both WT and *Foxo1*$^{S273D}$ mouse liver. However, suppression of p38α reduced *G6pc* and *Pck1* mRNA levels by 26% and 30% in WT livers, respectively; this effect of p38α suppression was blocked in *Foxo1*$^{S273D}$ mouse liver (Figure 15D). The WT mice-infected with AAV8-p38α-shRNA exhibited decreases in the level of pFoxo1-S273, t-Foxo1, and G6PC protein in the liver (Figure 15E). However, Foxo1-S273D mutation disrupted this effect of p38α suppression in the liver of mice (Figure 15E). p38α disruption had no significant effect on glucose tolerance and insulin sensitivity in both WT and *Foxo1*$^{S273D}$ mice under physiological condition (Figures 15F-I).

Finally, we used *Foxo1*$^{S273A}$ mice to evaluate the effect of p38 on HGP. In WT mice, inhibition of p38 by SB203580 decreased the fasting blood glucose by 16% (Vehicle: 80.6 ± 2.5 versus SB203580: 67.8 ± 0.59 mg/dL) and attenuated HGP by 17%. However, the alanine mutation in Foxo1-S273 blocked the inhibitory role of SB230580 in both fasting blood glucose level (Vehicle: 61.8 ± 3.1 versus SB203580: 56.0 ± 3.8 mg/dL, $p<0.05$) and HGP (Figures 16 A-C). Therefore, these results indicate that Foxo1-S273 phosphorylation plays a key role in p38α-mediated glucose homeostasis and HGP *in vivo*.

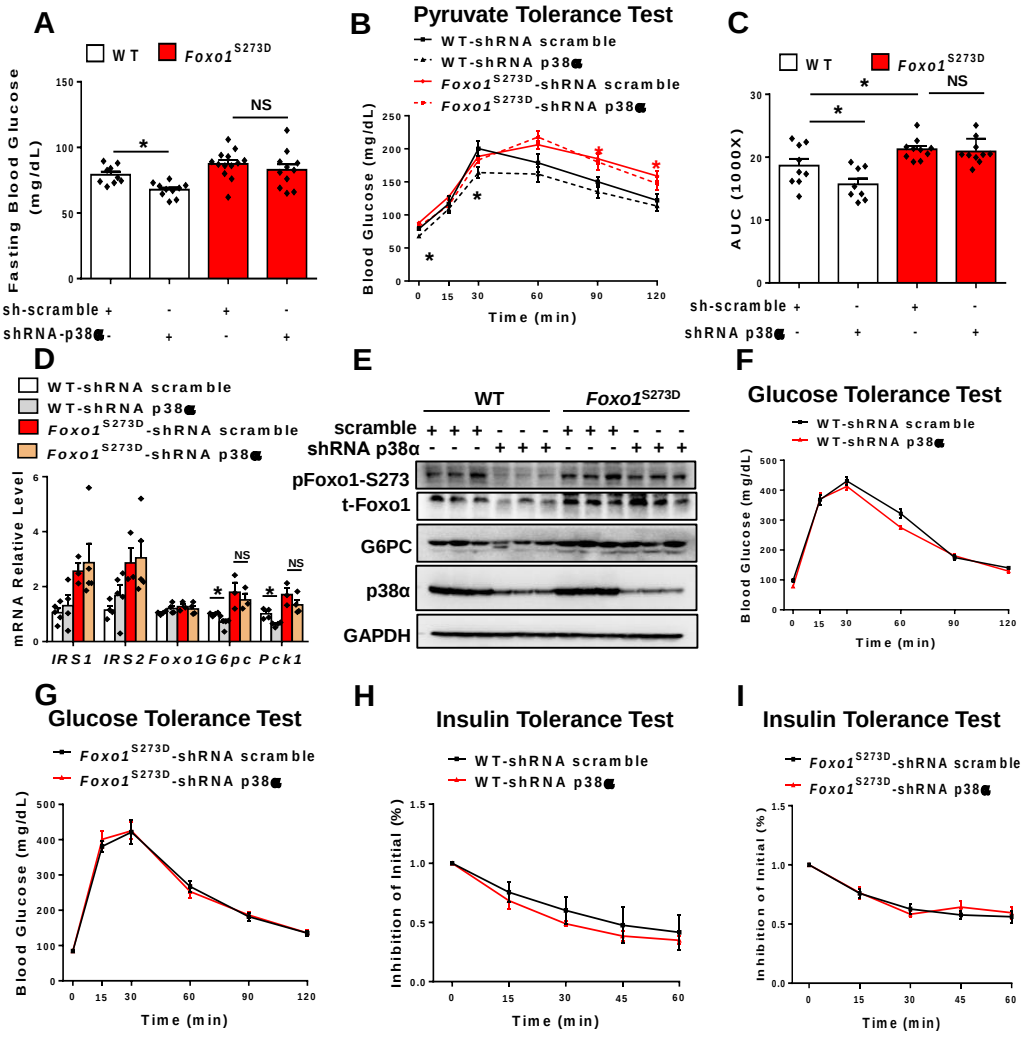



**Figure 15 Foxo1-S273D mutation blocks p38α-induced HGP in vivo**

(A) Blood glucose in 16 h fasted WT and *Foxo1*$^{S273D}$ mice administered with shRNA-p38α. *****P<0.05 versus WT-scramble, n=9-13. (B) WT and *Foxo1*$^{S273D}$ mice were transfected with shRNA-p38α for 7 days. After 16 h fasting, 2 g/kg body weight pyruvate was i.p. injected into mice, blood glucose levels were then monitored. *****P<0.05 versus WT-shRNA scramble, n=8-10. (C) AUC of pyruvate tolerance tests (B), n=5. (D) The effect of shRNA-p38α on mRNA expression levels of *IRS1*, *IRS2*, *Foxo1*, *G6pc*, and *Pck1* in the WT and *Foxo1*$^{S273D}$ mice liver, n=4-5. (E) The effect of shRNA-p38α on protein levels of pFoxo1-S273, t-Foxo1, pp38-T180/Y182, G6PC, and PCK1 in WT and *Foxo1*$^{S273D}$ mice liver. (F) Glucose tolerance test in WT mice treated with p38α shRNA. (G) Glucose tolerance test in *Foxo1*$^{S273D}$ mice treated with p38α shRNA. (H) Insulin tolerance test in WT mice treated with p38α shRNA. (I) Insulin tolerance test in *Foxo1*$^{S273D}$ mice treated with p38α shRNA. All data are presented as mean ± SEM. *****P<0.05.

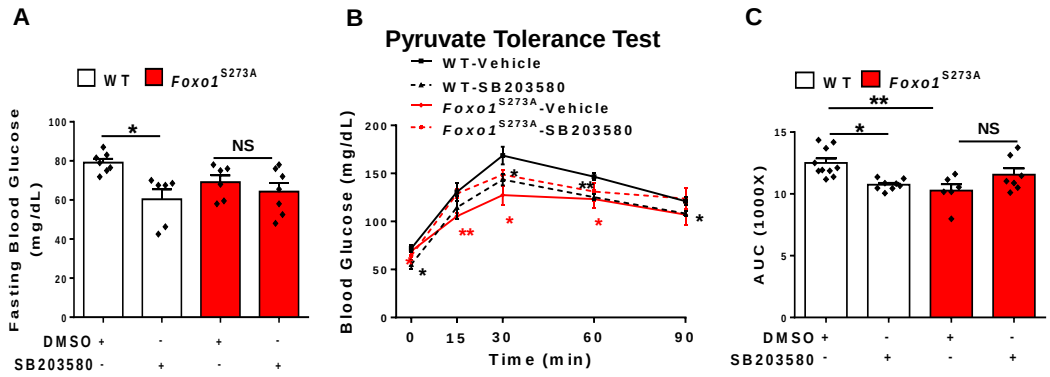



**Figure 16 Foxo1-S273A mutation abolishes p38α-induced HGP in vivo**
(A) Blood glucose levels of 16 h-fasted WT and *Foxo1*$^{S273A}$ mice injected with SB203580, n=6-7. (B) WT and *Foxo1*$^{S273A}$ mice were administered with SB203580 via i.p. injection. After 16 h fasting, 2 g/kg body weight was i.p. injected into mice and blood glucose levels were monitored. *****P<0.05 versus WT-Vehicle, n=6-10. (C) AUC of pyruvate tolerance test in (B), n=6-10. All data are presented as mean ± SEM. *****P<0.05, ******P<0.01.

## Summary

The results in this chapter reveal that glucagon activates p38α through GCGR-cAMP-EPAC signaling pathway. The activation of p38α stimulates Foxo1 phosphorylation at S273 to increase Foxo1 activity. Two mechanisms by which p38α regulates Foxo1-S273 phosphorylation are indicated: 1) p38α directly binds to Foxo1 and phosphorylates Foxo1 at S273; 2) p38α enhances glucagon-induced PKA activity through upregulation of cellular cAMP level. p38-mediated Foxo1 phosphorylation at S273 promotes gluconeogenic gene expression, thereby it increases gluconeogenesis (Figure 17).

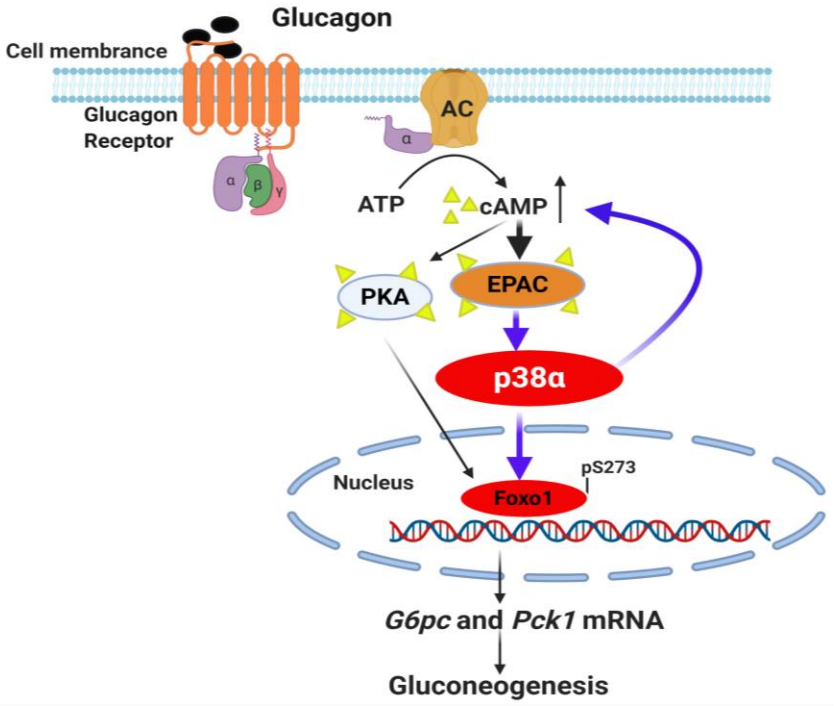



**Figure 17 Diagram of mechanism by which p38α regulates glucagon-induced hepatic glucose production**

Glucagon activatesp38 through GCGR-cAMP-EPAC pathway. Active p38α enhances PKA activity through elevation of cellular cAMP and directly binds to Foxo1 to stimulate Foxo1-S273 phosphorylation, thereby promoting glucose production.

# CHAPTER V

# P38 → FOXO1 SIGNALING IN INSULIN RESISTANCE AND INFLAMMATION

## Introduction

Chronic inflammation is believed to be the important player contributing to the pathogenesis of type 2 diabetes (230). The accumulation of adipose tissue mass chronically activates innate immune system, which results in insulin resistance. Inflammatory cytokines, such as tumor necrosis factor (TNF) α, C-reactive protein (CRP), and Interleukin (IL)-6, are believed to mediate inflammation-induced diabetes (231). Loss-function of TNFα in obese mice showed improvement in insulin sensitivity and glucose tolerance (232; 233).

Insulin stimulates insulin receptor substrates (IRS) tyrosine phosphorylation upon binding to insulin receptor and mediates insulin signaling transduction. Inflammatory factors, such as TNFα, p38α MAPK, and JNK, stimulate serine phosphorylation of IRS protein, promoting its degradation (222; 230; 234; 235). Moreover, high fat diet-induced inflammation also contributes to enhancing glucagon signaling cascade (236; 237). P38 kinase, one of subfamilies of mitogen-activated protein kinases (MAPKs), is activated by inflammatory cytokines, involving in regulation of the inflammatory response (214). In this study, we further detect the role of p38α → Foxo1 in glucose homeostasis under inflammation and insulin resistance.

## Methods

### Mice

DKO (113) and *Foxo1*$^{S273D}$ (52) mice were generated as previously described. All animal experiments were performed according to procedures approved by the Texas A&M University Institutional Animal Care and Use committee. Mice are housed at 22-24 C° and maintained on a 12 h light/12 h dark cycle with a standard chow diet. The male mice at the age of 8-12 weeks were used in all the experiments.

### Primary Mouse Hepatocytes Isolation and Culturing

Primary mouse hepatocytes were isolated from 8-12 weeks old male mice. Livers were perfused with 10 ml of HBSS with 50 mM EGTA and 1 M glucose, followed by perfusion with 15 ml HBSS supplemented with 1 M $CaCl_2$, 1 M glucose, and type II collagenase. The cells were shaken from liver and then screened through 70 μm filter and centrifuged in Percoll medium (GE Healthcare). Viable cells were seeded into low glucose DMEM Medium supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S).

### Pyruvate Tolerance Test

Recombinant adenovirus (1 x $10^{11}$ pfu/mice) was delivered into mice by retro orbital injection, and pyruvate tolerance test was performed 1 week after delivery of adenovirus. The mice were fasted for 16 h. After determining the basal blood glucose level, mice received 2 g/kg of body weight pyruvate sodium through i.p. injection. Blood glucose level were measured after 15, 30, 60, 90, and 120 min.

**Glucose Tolerance Test**

Recombinant adenovirus (1 x $10^{11}$ pfu/mice) was delivered into mice by retro orbital injection, and glucose tolerance test was performed 2 weeks after delivery of adenovirus. Mice were fasted for 16 h and then injected with 2 g/kg body weight glucose. Blood glucose levels were measured after 15, 30, 60, 90, and 120 min.

**Insulin Tolerance Test**

Recombinant adenovirus (1 x $10^{11}$ pfu/mice) was delivered into mice by retro orbital injection, and insulin tolerance test was performed 3 weeks after delivery of adenovirus. Mice were fasted for 4 h, and then injected with 1 U/kg body weight insulin via i.p. injection. Blood glucose level was monitored after 15, 30, 45, and 60 min.

**Glucose Production in Primary Hepatocytes**

Glucose production assay was performed as previously described (216). Briefly, mouse primary hepatocytes were isolated. After attachment, hepatocytes were cultured in KRB buffer containing sodium pyruvate, sodium lactate, and BSA, and then pretreated with 10μM SB203580, 10μ H89, or 10μM ESI-09 for 30 min, followed by 100 nM glucagon treatment for 3 h. Finally, 200 μl medium was collected and glucose content was measured using glucose assay kit (Thermo Fisher). For transfection and adenovirus-induced overexpression experiments, attached hepatocytes were transfected with 150 nM siRNA (per well in 6-well plate) for 12 h or treated with 100 MOI adenovirus for 12 h. Afterwards, hepatocytes were cultured in KRB medium with pretreatment of 10μM SB203580 for 30 min and following 100 nM glucagon treatment for 3 h. Cell medium was collected and glucose monitored.

**Quantitative Real-Time PCR**

Total RNAs were extracted with TRIzol reagent (Invitrogen Life Technologies). The cDNAs were synthesized using iScript$^{TM}$ Reverse Transcription Supermix (Bio-Rad). Quantitative real-time PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The primers are listed in APPENDIX Table 1.

**Statistical Analysis**

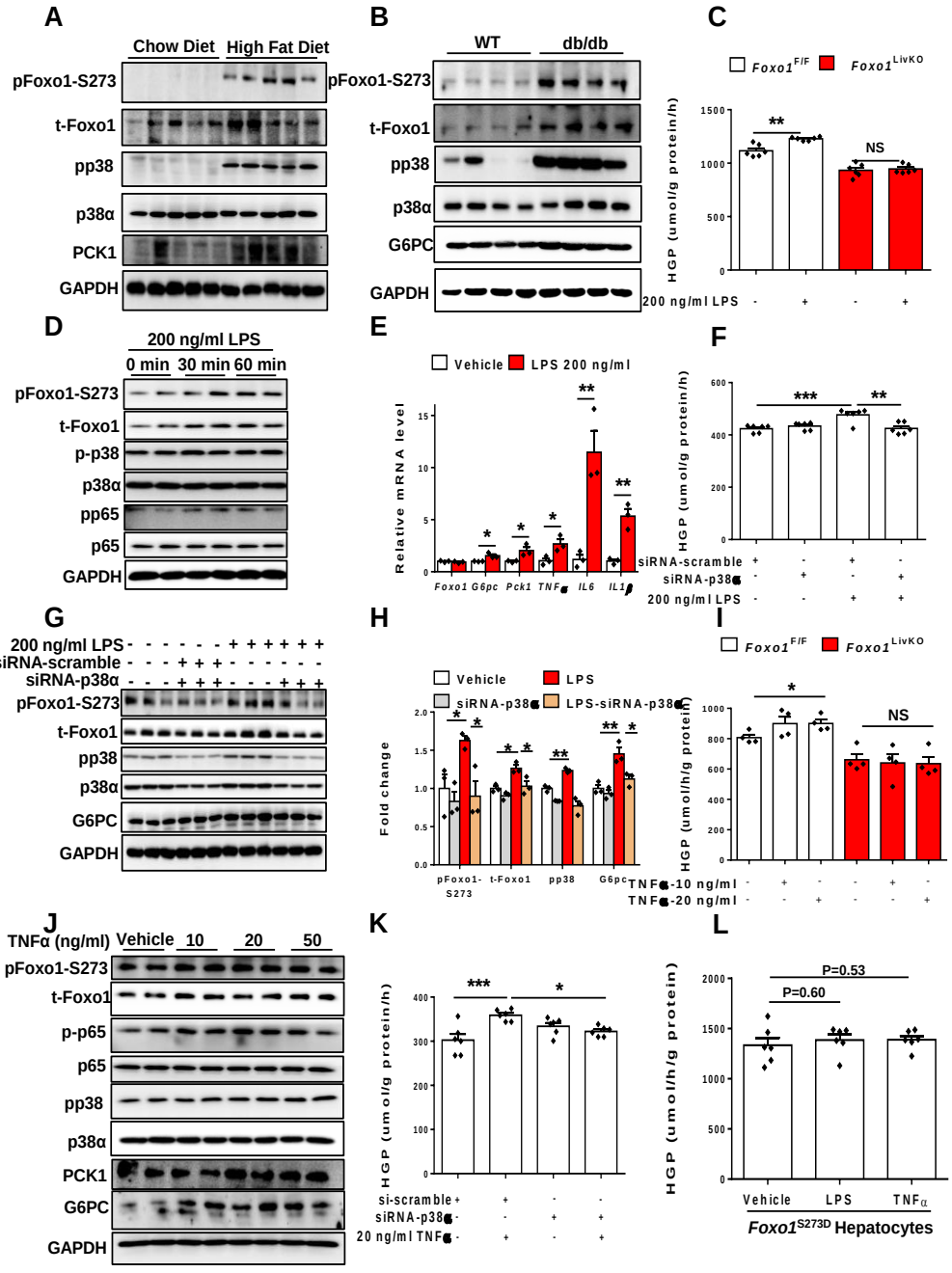
All results are presented as mean ± SEM. P values were calculated using the Student-t test for the comparison of difference between two groups. Significance among multiple groups was tested using one-way and two-way ANOVA followed by Tukey's multiple comparison test in GraphPad Prism. $P < 0.05$ was considered statistically significant.

## Results

Chronic inflammation contributes to insulin resistance and the dysregulation of glucose homeostasis (230). p38α is a key player in promotion of inflammation (238). Indeed, the diet-induced obese mice and *db*/*db* mice showed increases in pFoxo1-S273, pp38-T180/Y182, t-Foxo1, G6PC, and PCK1 protein levels in liver (Figures 18A and 18B). It is shown that LPS and TNFα levels increased in the liver of HFD or *db*/*db* mice (239; 240). We next determined whether the p38α → Foxo1 pathway plays a role in the inflammatory state. We treated WT hepatocytes with LPS and TNFα, and then measured glucose production. LPS treatment resulted in an 11% increase in glucose production in control *Foxo1*$^{F/F}$ hepatocytes, while Foxo1 deficiency diminished this effect of LPS (Figure 18C). LPS treatment resulted in increases in pFoxo1-S273 and t-Foxo1 protein levels (Figure 18D, top two blots). LPS treatment also induced phosphorylation of NFκB-p65 (pp65) and p38 (Figure 18D, bottom five blots). Consistently, mRNA levels of inflammatory factors, including *TNFα*, *IL6*, and *IL1β*, were increased significantly in hepatocytes treated with LPS. In addition, LPS dramatically increased *G6pc* and *Pck1* mRNA levels without effect on *Foxo1* mRNA level (Figure 18E). Moreover, RNAi-mediated p38α suppression significantly attenuated LPS-induced HGP (Figure 18F), as well as LPS-induced pFoxo1-S273, pp38-T180/Y182, t-Foxo1, and G6PC protein levels (Figures 18G and 18H). TNFα is a major mediator of inflammation (241). *Foxo1*$^{F/F}$ hepatocytes showed an 11% increase in HGP upon TNFα treatment. However, TNFα-induced HGP was abolished in *Foxo1*$^{Livko}$ cells (Figure 18I). Protein analyses revealed

**Figure 18 p38α→ Foxo1 signaling regulates HGP in the inflammatory state**

(A-B) pFoxo1-S273, t-Foxo1, pp38-T180/Y182, G6PC, and PCK1 protein levels were detected in liver from HFD fed (A) and db/db (B)mice. (C) Hepatocytes were isolated from *Foxo1*$^{F/F}$ and *Foxo1*$^{LivKO}$ mice, then treated with 200 ng/ml LPS for 3 h. Glucose content was measured by glucose kit, n=3. (D) Primary hepatocytes were treated with 200 ng/ml LPS for 30 min and 60 min, respectively. pFoxo1-S273, t-Foxo1, pp38-T180/Y182, and pp65-S536 were analyzed by western blotting. (E) Hepatocytes were treated with 200 ng/ml LPS for 3 h, respectively. Gene mRNA levels were detected by Q-PCR, n=3. (F) Primary hepatocytes were transfected with siRNA-p38α for 12 h, then treated with 200 ng/ml LPS for 3 h. Glucose content was measured by glucose kit, n=3. (G) siRNA-p38α was transfected into hepatocytes, followed by 200 ng/ml LPS treatment for 3 h. pFoxo1-S273, t-Foxo1, pp38-T180/Y182, and G6pc levels were analyzed by western blotting. (H) Quantification of (G), n=3. (I) *Foxo1*$^{F/F}$ and *Foxo1*$^{LivKO}$ hepatocytes were treated with 10 and 20 ng/ml TNFα for 3 h. Glucose content was measured by glucose kit, n=3. (J) hepatocytes were treated with 10, 20, and 50 ng/ml TNFα for 3 h. pFoxo1-S273, t-Foxo1, pp38-T180/Y182, PCK1 and G6PC levels were detected. (K) hepatocytes were transfected with siRNA-p38α for 12 h, then treated with 20 ng/ml TNFα. Glucose content was measured, n=3. (L) *Foxo1*$^{S273D}$ hepatocytes were treated with 200 ng/ml LPS and 20 ng/ml TNFα for 3 h. Glucose content was measured by glucose kit, n=6. All data are presented as mean ± SEM. *****P<0.05, ******P<0.01.


A
Chow Diet
High Fat Diet
pFoxo1-S273
t-Foxo1
pp38
p38α
PCK1
GAPDH
B
WT
db/db
pFoxo1-S273
t-Foxo1
pp38
p38α
G6PC
GAPDH
C
Foxo1F/F
Foxo1LivKO
HGP (umol/g protein/h)
**
NS
200 ng/ml LPS
D
200 ng/ml LPS
0 min
30 min
60 min
pFoxo1-S273
t-Foxo1
p-p38
p38α
pp65
p65
GAPDH
E
Vehicle
LPS 200 ng/ml
Relative mRNA level
Foxo1
G6pc
Pck1
TNFα
IL6
IL1β
F
HGP (umol/g protein/h)
***
**
siRNA-scramble
siRNA-p38α
200 ng/ml LPS
G
200 ng/ml LPS
siRNA-scramble
siRNA-p38α
pFoxo1-S273
t-Foxo1
pp38
p38α
G6PC
GAPDH
H
Vehicle
LPS
siRNA-p38α
LPS-siRNA-p38α
Fold change
pFoxo1-S273
t-Foxo1
pp38
G6pc
I
Foxo1F/F
Foxo1LivKO
HGP (umol/h/g protein)
*
NS
TNFα-10 ng/ml
TNFα-20 ng/ml
J
TNFα (ng/ml)
Vehicle
10
20
50
pFoxo1-S273
t-Foxo1
p-p65
p65
pp38
p38α
PCK1
G6PC
GAPDH
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HGP (umol/g protein/h)
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si-scramble
siRNA-p38α
20 ng/ml TNFα
L
HGP (umol/h/g protein)
P=0.53
P=0.60
Vehicle
LPS
TNFα
Foxo1S273D Hepatocytes

that TNFα increased the levels of pFoxo1-S273, pp65-S536, p38-T180/Y182, t-Foxo1, G6PC, and PCK1 proteins (Figure 18J). Importantly, knockdown of p38α by siRNA blocked TNFα-induced HGP in WT hepatocytes (Figure 18K). However, Foxo1-S273D mutation blocked both LPS and TNFα- induced HGP (Figure 18L).

We next detected the role of p38α → Foxo1 pathway in insulin resistant (DKO) mice. DKO mice showed hyperglycemia in feeding and fasting state and p38α knockdown rescued the blood glucose to normal level (Figures 19A and 19B). As compared to control mice, DKO mice had a 57% increase in hepatic glucose production during ipPTT, severe glucose intolerance and insulin resistance; importantly, p38α knockdown markedly attenuated glucose production and improved glucose and insulin tolerance in DKO mice (Figures 19C-G). Further analysis of gene expression showed that DKO mice showed significant increases in inflammatory factors, including *TNFα, IL-1b*, *MCP1*, and *HO1*, as well as gluconeogenic genes, including *G6pc* and *Pck1* in livers. p38α deficiency blocked the upregulation of *G6pc*, *Pck1*, *IL-1b*, and *HO1* in DKO mice livers. As expected, IRS1 and IRS2 levels were remarkedly decreased in DKO mice livers (Figure 19H). DKO mice liver also showed increases in pFoxo1-S273, t-Foxo1, pp38, PCK1, and G6PC proteins abundance, which are attenuated by p38α ablation (Figure 19I). These data indicate that p38α → Foxo1 pathway regulates glucagon homeostasis in insulin resistance condition.

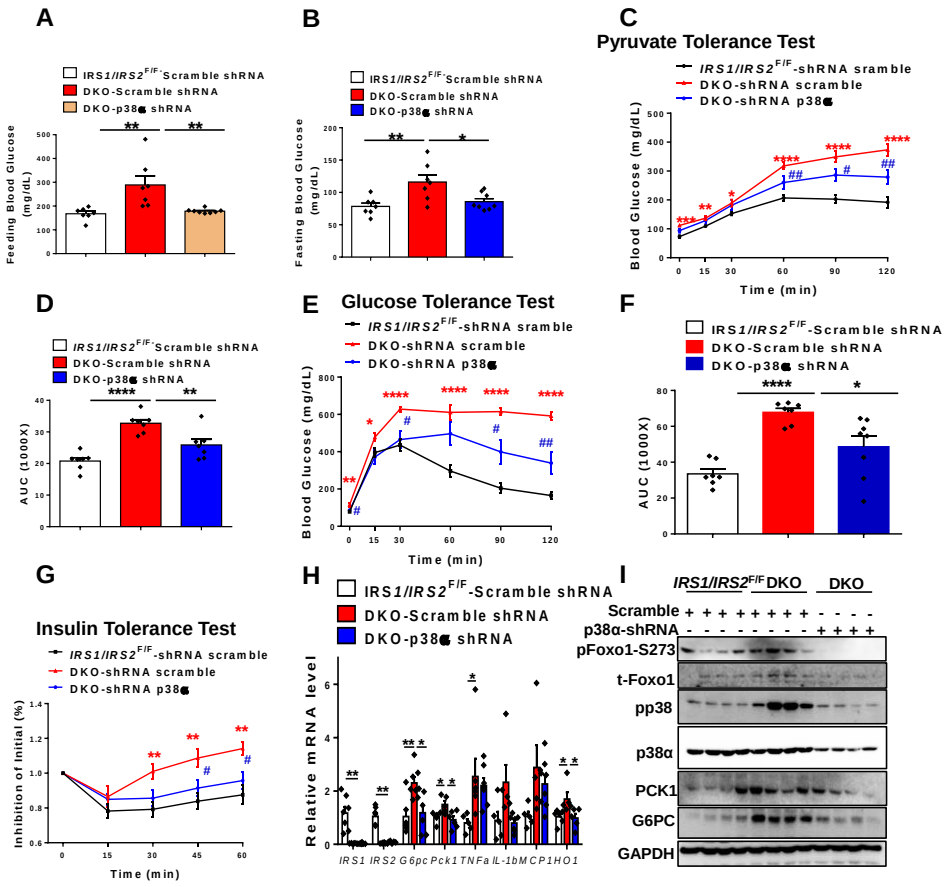


**Figure 19 p38α→ Foxo1 signaling contributes to glucose dysregulation in insulin resistant mice**

(A) Feeding blood glucose level in DKO mice treated with p38α shRNA, n=7-8. (B) Fasting blood glucose level in DKO mice treated with p38α shRNA, n=7-8. (C) Pyruvate tolerance test in DKO mice treated with p38α shRNA. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 versus IRS1/IRS2F/F-Scramble. #P<0.05, ##P<0.01 versus DKO, n=7. (D) AUC of pyruvate tolerance test in (C), n=7. (E) Glucose tolerance test in DKO mice treated with p38α shRNA. *P<0.05, **P<0.01, ****P<0.0001 versus IRS1/IRS2F/F-Scramble. #P<0.05, ##P<0.01 versus DKO, n=7. (F) AUC of glucose tolerance test in (E), n=7-8. (G) Insulin tolerance test in DKO mice treated with p38α shRNA. *P<0.05 versus IRS1/IRS2F/F-Scramble. #P<0.05 versus DKO, n=7. (H) Gluconeogenic and inflammatory genes expression in the livers from DKO mice treated with p38α shRNA, n=5-7. (I) pFoxo1-S273, t-Foxo1, pp38, G6PC, and PCK1 protein levels in livers from DKO mice treated with p38α shRNA, n=4. All data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

## Summary

The results in this chapter show that p38α-Foxo1 signaling is activated in obese and insulin resistance mice. Suppression of p38α-Foxo1 signaling largely improves glucose tolerance and insulin sensitivity, as well as attenuates HGP. Moreover, TNFα or LPS-induced HGP is blocked by the disruption of p38α-Foxo1 signaling. These results indicate that p38α-Foxo1 signaling is activated in both inflammatory and insulin resistant conditions and plays a key role in regulation of glucose homeostasis.

# CHAPTER VI

# FINAL SUMMARY

Glucagon plays an essential role in regulation of HGP in both physiological and pathological conditions. In this study, we provided genetic and biochemical evidence that p38α mediates the action of glucagon in control of HGP and blood glucose via Foxo1-S273 phosphorylation. In addition to PKA, we established a new connection among glucagon, p38α, and Foxo1 where p38α is activated by glucagon-increased cAMP-EPAC signaling, increasing Foxo1 stability and promotes Foxo1 nuclear localization via stimulation of Foxo1-S273 phosphorylation, mediating the action of glucagon for induction of HGP and blood glucose. Importantly, the results in this study indicate that p38α→Foxo1 is an important link between inflammation and HGP. The pathway summarized as glucagon→ cAMP → EPAC → p38α → Foxo1 → G6pc/Pck1 in this study will provide new understanding into the mechanism of glucagon action in control of glucose homeostasis (Figure 20).

Glucagon exerts its action via the glucagon receptor (GCGR)-induced G coupled proteins. Three major signaling pathways are believed to fulfill glucagon-induced gluconeogenic effect: 1) $G_S$α-adenylate cyclase → cAMP → PKA → FOXO1/CREB; 2) $G_S$α-adenylate cyclase → cAMP → PKA → Calcium; 3) $G_q$-adenylate cyclase → phospholipase C → inositol 1,4,5-triphosphate → Calcium (49; 59; 206). In this study, our data suggest that GCGR-cAMP plays a role in the stimulation of p38 in liver. Although cAMP also activates p38 in adipose tissue and immune system, especially T cell (242-

244), p38 phosphorylation is not changed by increased cAMP in muscle (245); this suggests that cAMP mediates p38 activation in a tissue-specific manner. The link between cAMP and p38 may be attributed to cAMP-PKA-CaMKII pathway (60). Based on our data, p38 enhances glucagon-induced PKA activity, whereas PKA activation is barely required for activation of p38 in the action of glucagon or cAMP; this is also consistent with a previous report (208). Therefore, p38 acts partially an upstream regulator of PKA in the glucagon signaling pathway. Ozcan *et al.* reported that $Ca^{2+}_{i}$ -sensing enzyme CaMKII, downstream of PKA, stimulates p38 activity in serum-starved primary hepatocytes. Thus, there may be a positive feedback loop by PKA-Calcium-p38 that amplifies glucagon action. Indeed, we observed that CaMKII regulates the basal p38 activity, but it is not required for glucagon-induced p38 activity; this suggests that there is another mechanism stimulated by glucagon or cAMP which thereby activates p38. The actions of cAMP are mediated by two intracellular cAMP targets: PKA and exchange protein directly activated by cAMP (EPAC) (53); these two mediators of cAMP transduce signal cascades independently (246). EPAC mediates p38 activation by cAMP to regulate neuronal function and brain damage (247; 248), indicating a potential role of EPAC in p38 activation in other tissues, such as liver. Our data demonstrate that inhibition of EPAC significantly and largely blocks glucagon-induced p38 activation and Foxo1-S273 phosphorylation. However, the mechanism whereby EPAC activates p38 remains to be further investigated. Moreover, our data suggest that p38α induces Foxo1-S273 phosphorylation and increases Foxo1 activity. Taken together, p38α is involved in glucagon action on HGP by the signaling pathway: Gsα-adenylate cyclase → cAMP →

EPAC → p38α → FOXO1. In addition to liver, extrahepatic tissues also regulate HGP, including adipose tissue, pancreas, and central nervous system (125). We do not rule out the potential role of the cAMP-EPAC-p38α-FOXO1 pathway in these tissues; but our results from mouse primary hepatocytes indicate that the p38α→Foxo1 pathway governs HGP in a cellular autonomous manner. Since these mechanistic studies were performed *in vitro*, there are limitations to mimic the *in vivo* conditions. To improve the cell study to mimic the physiological and pathological states properly, we should adjust the glucose, glucagon, insulin levels in the cell medium to the equivalent concentrations *in vivo*. Especially in obese condition, free fatty acids (FFAs) and inflammatory cytokines should be considered in the cell medium. Besides, compared to the traditional 2-dimensional culture, 3-dimenional platforms can mimic human physiology with better accuracy.

Foxo1 has several phosphorylation sites targeted by multiple protein kinases. Insulin phosphorylates Foxo1 at T24, S253, and S316 via Akt, promoting Foxo1 degradation and nuclear exclusion and suppressing insulin-regulated gene expression (63). Foxo1 also has several non-Akt phosphorylation sites, such as S246, S284, S295, S326, and T553 through p38, ERK, and CaMKII (60; 170). Recently, we have identified a new phosphorylation site of Foxo1 by PKA at S273 and Foxo1-S273 phosphorylation increases Foxo1 protein stability and nuclear translocation (52). In this study, we established that p38α also stimulates Foxo1-S273 phosphorylation to regulate HGP in response to glucagon, two potential mechanisms of which are revealed: 1) p38α binds to Foxo1 and stimulates Foxo1-S273 phosphorylation directly; 2) p38α partially enhances the activity of PKA in the action of glucagon. p38α also phosphorylates Foxo1 at other serine sites,

including S284, S295, S326, S413, S429, S467 and S475 in vitro; these sites potentially impair Foxo1 nuclear translocation (60). However, future work is needed to assess if the individual phosphorylation site is involved in regulation of Foxo1 activity and nuclear translocation.

CREB and Foxo1 are two well-studied transcriptional factors in regulation of hepatic gluconeogenesis (57). CREB is involved in p38α-mediated HGP (205). While there is no evidence that p38α phosphorylates CREB directly, MSK1 potentially mediates p38-induced CREB activation (243). Here, we provide evidence that PKA is another mediator in activating CREB via p38α. p38α enhances PKA activity potentially through AMPK, which is very weak compared to that of glucagon. Hepatic p38α suppresses AMPK signaling (206), which lowers cAMP levels via activation of hepatic cyclic nucleotide phosphodiesterase (PDE) isoform PDEB to antagonize glucagon action (152). Hence, AMPK → PDEB → cAMP → PKA is the potential mechanism by which p38α partially enhances PKA activity.

Plasma glucagon significantly increases in both type 1 and type 2 diabetic patients (123). In the obese mice model, plasma glucagon level is significantly increased (52). Both the cellular cAMP level and the activity of PKA are markedly increased in obese mice livers (249). Therefore, glucagon signaling is extremely enhanced under overnutrition-induced obese condition, contributing to hyperglycemia. In this study, we show that p38α→Foxo1 signaling is remarkably activated in the livers from HFD-induced obese mice, which is indicated by increases in pp38-T180/Y182, ppFoxo1-S273, and t-Foxo1. Indeed, p38 expression level and activity are increased in the livers of patients with

NAFLD (250), suggesting that the potential role of p38 signaling in regulation of glucose homeostasis in both mice and human. Free fatty acids (FFAs) levels are elevated in both serum and livers from obese mice. Considering FFAs stimulates p38 activity (251; 252), hyperglucagonemia and increased FFAs together contributes to the activation of p38 and subsequent activation of Foxo1, thereby enhancing hyperglycemia. Many dietary compounds have been indicated to improve diabetic syndrome, such as curcumin. The administration of curcumin improves the glycemic control in both obese human and mice (253; 254). Previous study indicates that curcumin suppresses p38 activation (255). Thus, p38α→Foxo1 signaling might be the potential target of curcumin in regulation of glucose homeostasis.

The discovery of the association of p38α and Foxo1 provides new insight into the link between inflammation and hyperglycemia. Inflammatory factors disrupt insulin signaling via inhibitory serine phosphorylation of IRS1 (234; 235; 256). On the other hand, inflammatory factors, such as NF-κB–inducing kinase (NIK) also lead to hyperglycemia and glucose intolerance via augmenting the glucagon action (236; 237). p38 MAPK is activated by environmental stress and inflammatory cytokines through dual phosphorylation of Thr-Gly-Tyr motif mediated by upstream protein kinases MKK3 and MKK6 (199). As we showed in this study, activation of p38 enhances glucagon signaling and increases HGP, thereby contributing to hyperglycemia. Deletion of p38α in liver significantly decreases blood glucose level and HGP, as well as improves glucose tolerance in diet-induced obese mice model (206; 209); this suggests that p38α is a critical player in the connection between inflammation and hyperglycemia. The p38α impairs

insulin sensitivity via degradation of IRSs and activation of PERK-ATF4-TRB3 signaling (209; 222). Moreover, p38α enhances glucagon action to promote HGP through Foxo1 in an insulin signaling-independent manner. Thus, p38α is involved in the development of type 2 diabetes by interrupting insulin action and stimulating glucagon action. The dysregulation of glucagon action is important in the diabetic pathogenesis, and suppression of glucagon function improves diabetic syndrome in mice (257-260). The identification of the importance of the p38α → Foxo1 pathway in glucagon action will provide the fundamental evidence for future therapeutic applications to prevent or cure type 2 diabetes.

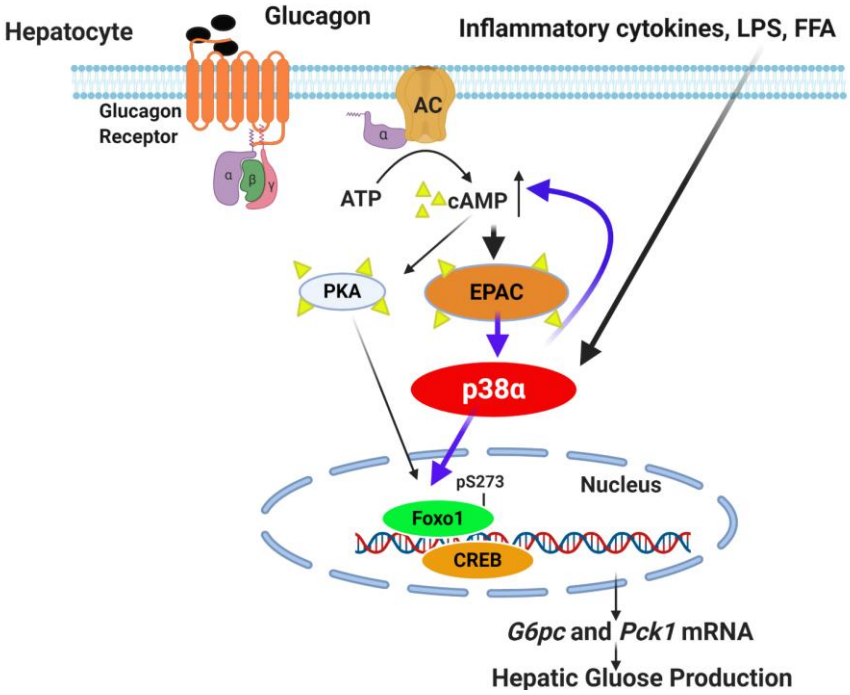


**Figure 20 Diagram of the mechanisms by which p38α regulates glucagon-induced hepatic glucose production**

Glucagon activates p38α through cAMP-EPAC signaling. The p38α stimulates phosphorylation of Foxo1 at S273, involving in the glucagon-induced hepatic glucose production. Under pathological condition, inflammatory cytokines, LPS, and Free fatty acids (FFA) enhance the activity of p38α to further increase hepatic glucose production, contributing to hyperglycemia.

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# APPENDIX

## TABLES

**Table 1. Mouse Primer List**

| Gene Name | Forward 5'-3' | Reverse 5'-3' |
|---|---|---|
| *Foxo1* | agatgagtgccctgggcagc | gatggactccatgtcacagt |
| *G6pc* | cattgtggcttccttggtcc | ggcagtatgggataagactg |
| *Pck1* | ccatcggctacatccctaag | gacctggtcctccagata |
| *IRS1* | cccgttcggtgccaaatagc | gccactggtgaggtatccacatagc |
| *IRS2* | acttcccagggtcccactgctg | ggctttggaggtgccacgatag |
| *p38α* | gccatgaggcaagaaactacat | ctcatcatcagggtcgtggt |
| *p38β* | acctccttggaagaatgctgg | aagtaagctccttccactcctc |
| *p38γ* | gcagaggccaagaactacatgga | tgctgtcacccgctgttccg |
| *p38δ* | acactgagatgacgggctatg | ggatttggccgccttgtctt |
| *TNFα* | gagaaagtcaacctcctctctg | gaagactcctcccaggtatatg |
| *IL-1β* | tgttctttgaagttgacggaccc | tcatctcggagcctgtagtgc |
| *MCP1* | caggtgtcccaaagaagctgtag | gggtcagcacagacctctctct |
| *HO1* | gccgagaatgctgagttcatg | tggtacaaggaagccatcacc |
| *Cyclophilin* | actgaatggctggatggcaag | tgcccgcaagtcaaaagaaat |

**Table 2. Antibody List**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Anti-Foxo1 | Cell signaling technology | Cat#2880S |
| Anti-pFoxo1-S273 | Covance (Custom Immunology Service,) | N/A |
| Anti-pp38-T180/182 | Cell signaling technology | Cat#4511S |
| Anti-p38 | Cell signaling technology | Cat#8690S |
| Anti-p38α | ABclonal | Cat#A14401 |
| Anti-p38β | ABclonal | Cat#A7717 |
| Anti-p38γ | ABclonal | Cat#A13046 |
| Anti-p38δ | ABclonal | Cat#A7496 |
| PCK1 | Cell signaling technology | Cat#12940S |
| G6PC | Abcam | Cat#Ab83690 |
| Anti-pCREB-S133 | Cell signaling technology | Cat#9198S |
| Anti-CREB | Cell signaling technology | Cat#9197S |
| Anti-PKAc | Cell signaling technology | Cat#4782S |
| Anti-pPKA substrates | Cell signaling technology | Cat#9624S |
| Anti-pCaMKII-T286 | Cell signaling technology | Cat#12716S |
| Anti-CaMKII | Cell signaling technology | Cat#4436S |
| Anti-pp65-S536 | Cell signaling technology | Cat#3031S |
| Anti-p65 | Cell signaling technology | Cat#8242S |
| Anti-GAPDH | Cell signaling technology | Cat#5174S |