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FOXO1 Regulation of Islet Cell Development in the Human Fetal Pancreas.

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Abstract

Recent studies have outlined a major role for the forkhead protein FOXO1 in β-cell differentiation, proliferation, and stress resistance in the adult islet. The objective of this study was to characterize the expression pattern and function of FOXO1 during human fetal islet cell development (8 to 21 weeks of fetal age). Using immunostaining, FOXO1 co-localized with ductal and endocrine cell markers, in addition to PDX-1 and NGN3 transcription factors throughout development. *In vitro*, nuclear export of FOXO1 occurred in islet-epithelial cell clusters (18 to 21 wks) exposed to high insulin concentrations in a PI3-Kinase/AKT dependent manner. Cells transfected with FOXO1 siRNA demonstrated an up-regulation in NGN3 and NKX6-1 mRNA levels and protein cellular distribution in parallel with an increase in the β-cell population, a down-regulation in the NGN3 inhibitory factor HES1, and no effect on PDX-1. In conclusion, these results outline a role by which FOXO1 may contribute to islet cell development.

Key words: Human fetal pancreas, Human islet-epithelial cell clusters, Islet transcription factors, nuclear FOXO1, FOXO1 siRNA

Co-Authorship

All experiments in Chapter 2 were performed by Maia Al-Masri with the assistance of several lab members. Sample collection and pancreatic tissue isolation were performed by Dr. Rennian Wang and Dr. Fraser Fellows. Technical support and solutions preparation were provided by Jinming Li, in addition to assistance with quantitative real-time RT-PCR analysis, including Real-Time PCR primers design. Assistance with western blot analysis was provided by Mansa Krishnamurthy. This manuscript was reviewed and edited by Dr. Rennian Wang, Dr. Tom Kennedy, and Dr. Andrew Watson.

"Wisdom and power follow endurance and patience"

Prophet Muhammad (PBUH)

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

%	percent
18S	ribosomal RNA
AKT	protein kinase B
ANOVA	analysis of variance
BCL-2	B-Cell CLL/Lymphoma 2
bHLH	basic helix loop helix
BIM	BCL-2 interacting mediator of cell death
bp	base pairs
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBP	cAMP response element binding protein (CREB) binding protein
cDNA	complimentary DNA
CK1	casein kinase
CK19	cytokeratin 19
cm	centimeter
CO_2	carbon dioxide
C-peptide	connecting peptide
CRM1	chromosome region maintenance 1
DAPI	4'-6' diamidino-2-phenylindole
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DSHB	developmental studies ybridoma bank
e	embryonic day
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
FasL	Fas ligand
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FKHR	forkhead in rhabdomyosarcomas
G6Pase	glucose-6-phosphatase
GLP-1	glucagon-like peptide-1
GTPase	guanosinetriphosphatase
HBSS	Hanks' balanced salt solution
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
hr	hour(s)
IGF-1	insulin growth factor 1
IgG	immuno-globulin G
InR	insulin receptor
Irs2	insulin receptor substrate 2
JNK	Jun-N-terminal kinase
kDa	kilodalton
Ki-67	proliferation index

JRL	Jackson immunoresearch labs
mg	milligrams
ml	milliliter
mM	millimol/L
Mn-SOD	Mn-superoxide dismutase
mol	mole
mA	milliamp
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NES	nuclear export signal
NLS	nuclear localization signal
ng	nanogram
nM	nanomole/L
°C	degrees Celsius
p	p value
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDK-4	pyruvate dehydrogenase kinase-4
PEPCK	phosphoenolpyruvate carboxykinase
PFA	paraformaldehyde
PI3-Kinase	phosphoinositide 3-kinase
PKB	protein kinase B
Pml	promyelocytic leukemia

PP	pancreatic polypeptide
PPARγ	peroxisome proliferator activated receptor γ
qRT-PCR	quantitative real-time reverse transcription PCR
RNA	ribonucleic acid
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
siRNA	small interfering RNA
SIRT1	sirtuin 1
SKP2	s-phase kinase-associated protein 2
SREBP-1c	sterol response element binding protein-1 c
TBS	tris buffered-saline
TRAIL	tumor-necrosis-factor-related apoptosis-inducing ligand
TRITC	tetramethyl rhodamine isothiocyanate
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
vs.	versus
wks	weeks
α	alpha
β	beta
βPdk1	β-cell specific knockout for 3-Phosphoinositide-dependent protein kinase 1
δ	delta
ε	epsilon
μg	microgram

µl	microliter
µmol	micromole

Transcription Factors

FOXA2	fork head box A 2
FOXO1	fork head box O 1
FOXO3	fork head box O 3
HES1	hairy and enhancer of split 1
ISL1	islet 1
MAFA	macrophage activating factor A
NEUROD	neurogenic differentiation
nFOXO1	nuclear FOXO1
NGN3	neurogenin 3
NKX2-2	NK2 related transcription factor related, locus 2
NKX6-1	NK6 related transcription factor related, locus 1
PAX6	paired-box homeoprotein 6
PDX-1	pancreatic duodenal homeobox gene-1

Chapter 1

Introduction

Chapter 1 – Introduction

1.1 Significance of the Study

It has long been hoped that some day individuals with type 1 diabetes will be able to rely on pancreatic islet transplantation, a cell based therapeutic approach, as a replacement for daily insulin injections (Shapiro *et al.*, 2000). However, even the most successful method of human islet transplantation could only cure a small fraction of people with type 1 diabetes through existing sources of human islets from donor pancreata (Madsen, 2005). Hence, there is a great need for alternative cell sources, and researchers have amplified their efforts to initiate or modulate organogenesis from primary cells or tissue fragments obtained from donor patients. The identification and expansion of stem cell populations, as well as the identification of regulators that control their proper differentiation into organs with highly specialized functions, are central to achieving this goal (Madsen, 2005).

My work contributes to the ultimate goal of developing protocols that promote β-cell growth *in vitro* for cell replacement therapies, with a specific focus on obtaining a comprehensive and detailed understanding of the transcription factors that govern pancreatic endocrine cell development and maintenance. As in the organogenesis of other organs, the expression patterns of specific transcription factors limit the boundaries of the developing pancreas, and several of them determine the differentiation programs of the various endocrine pancreatic cell lineages (Habener *et al.*, 2005). What makes the current research unique is that rather than employing mouse knock out models to investigate the functional role of transcription factors, as the majority of studies do, I have applied

siRNA methodology to a human pancreatic cell model to elucidate the functional role of a transcription factor during pancreatic organogenesis.

1.2 The Pancreas and Its Development

1.2.1 The Human Pancreas

The pancreas is an organ that is part of both the digestive and endocrine systems of vertebrates. It stretches across the back of the abdomen, behind the stomach, and is anatomically divided into four parts: head, neck, body and tail. The head of the pancreas is on the right side of the abdomen and is connected to the duodenum. The narrow end of the pancreas, called the tail, extends to the left side of the body (Slack, 1995). The adult mammalian pancreas is a heterogeneous organ, composed of both exocrine and endocrine tissues. Exocrine tissue, organized into dense epithelial acini, makes up over 95% of the pancreatic mass (Slack, 1995). The exocrine pancreas is mainly responsible for the production of digestive enzymes, such as trypsin and amylase, which are forced into the gut via duct cells from the acinar lumens. Eventually, these ducts merge and feed into progressively larger structures, finally forming the pancreatic duct that connects to the common bile duct at the ampulla of Vater (Slack, 1995).

Within the acinar matrix there are pockets of endocrine tissue. The endocrine pancreas is organized into globular clusters of cells called the islets of Langerhans, which in turn are made up of five different cell types (Slack, 1995). Each of the endocrine cell types produces a distinct peptide hormone: alpha (α)-cells make glucagon, beta (β)-cells produce insulin, delta (δ)-cells make somatostatin, pancreatic polypeptide (PP)-cells make pancreatic polypeptide, and a small subset of cells only recently discovered termed

epsilon (ε)-cells, make the peptide hormone ghrelin (Heller *et al.*, 2005; Ross *et al.*, 2002; Slack, 1995). The islets represent only about 1–2% of the total pancreatic mass. Within the islets, endocrine cell types are present in varying proportions. β-cells make up the majority (60–80%) and form a core around which the other cell types are arranged. α-cells comprise 15–20% of the islet mass, and the remaining cells are made of δ, PP and ε type (Heller *et al.*, 2005; Ross *et al.*, 2002; Slack, 1995). The hormones secreted by the endocrine pancreas regulate metabolic functions either systemically, regionally (in the gastrointestinal tract), or locally (in the islet itself) (Ross *et al.*, 2002). Insulin is the most abundant endocrine secretion. A blood glucose above the normal level, 70 mg/100 ml (70 mg/dl), stimulates release of insulin from β-cells, leading to uptake and storage of glucose by liver and muscle tissues (Kido *et al.*, 2001; Ross *et al.*, 2002).

1.2.2 **Development of the Human Pancreas**

General Morphological Changes during Development: During embryogenesis, the endodermal epithelium is partitioned by tightly regulated morphogenetic signals along the anterior-posterior axis, a prerequisite for the development of specialized organs at distinct regions along the gut tube. Three weeks into development, the pancreas starts to develop at the fore-mid gut boundary through the formation of a dorsal and a ventral epithelial extension on opposite sides of the gut tube (**Figure 1.1A**). As development progresses, the buds branch into the surrounding mesenchyme, while the ventral bud rotates backwards and around the developing duodenal loop. The ventral bud will eventually fuse with the dorsal bud, forming a single organ by approximately week 8 of development (Piper *et al.*, 2004; Slack, 1995). The dorsal bud gives rise to the major part of the head, body and the tail of the pancreas, whereas the ventral bud gives rise to the

(A)

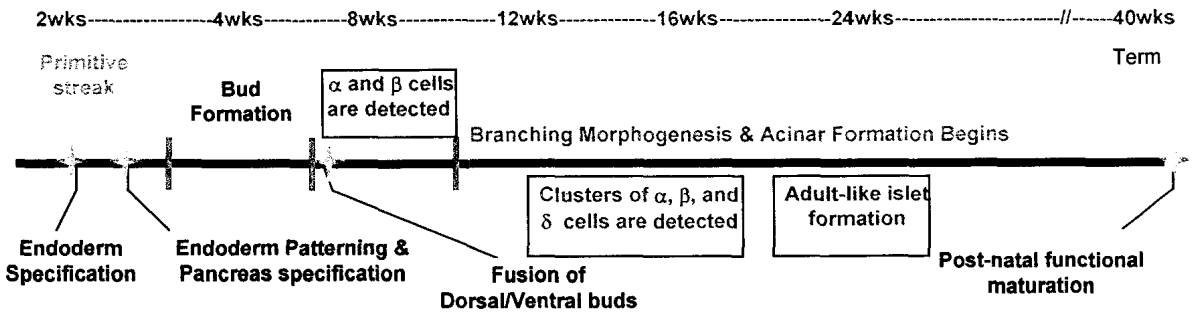
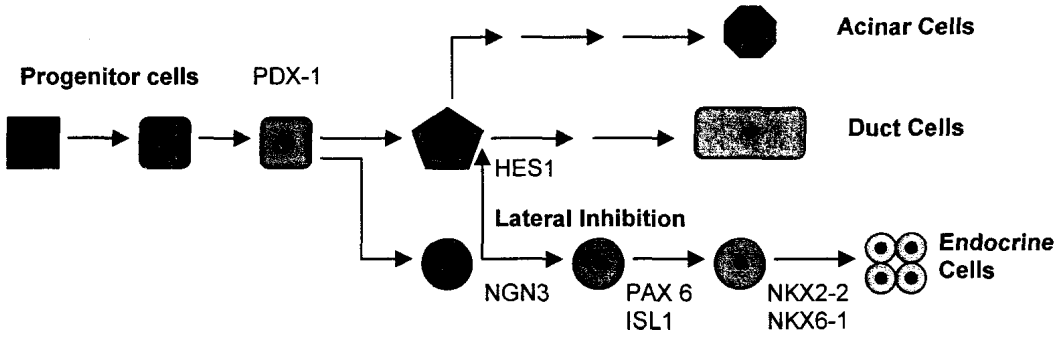


Figure 1.1. Development of the Human Fetal Pancreas. (A) A timeline of the major morphological changes occurring during human endocrine pancreatic development. Figure by Dr. Rennian Wang. Pancreatic development commences with the extension of the gut endoderm to give rise to the dorsal and ventral pancreatic buds. The two buds grow, branch, and eventually fuse by week 8 of development to form the definitive pancreas. Alpha (α) and beta (β) cells are the earliest markers defining endocrine cell development during these early stages. The approximate fetal age (in weeks) is designated for each stage of development. The symbol '//' refers to a major event during pancreatic development.

(B)



(B) A simplified model of transcription factors expressed in the developing pancreas. The figure is based on data accumulated from gene specific knockout mouse studies as summarized in the main text, and only includes factors that are addressed in this study. Although some of these factors act at more than one point during cellular differentiation, only a single point is indicated for each, and is based on the predominant timing of its function. The colored shapes are a schematic representation of differentiating cell types during the course of development. Abbreviations: PDX-1, pancreatic duodenal homeobox gene-1; HES1, hairy and enhancer of split 1; NGN3, neurogenin 3; ISL1, islet 1; PAX6, paired-box homeoprotein 6; NKX, NK homeobox. Adapted and modified from Bardeesy and DePinho, 2002.

inferior part of the head (Slack, 1995). During this period, interactions between pancreatic epithelium and mesenchyme guide organ formation and cell differentiation, allowing for the precise localization of the islets of Langerhans within a highly organized matrix of exocrine cells (Kim and Hebrok, 2001).

Early pancreatic development, 7 to 11 weeks of development, is a crucial period during which the premature pancreas begins synthesizing all of the hormones secreted by the adult pancreas (Polak *et al.*, 2000). Given the low proliferative activity of islet cells, the main increase occurring in islet cell mass during fetal pancreatic development is suggested to be brought about by islet cell differentiation from hormone negative pro-endocrine precursor cells (Bouwens *et al.*, 1997). Over the last few decades, there have been numerous histological studies aimed at outlining the early appearance and developmental pattern of endocrine cells containing all four different hormones, with detailed descriptions in some studies of the human fetal pancreas being performed as early as 7 weeks of fetal age (Lyttle *et al.*, 2008; Piper *et al.*, 2004; Polak *et al.*, 2000). At approximately 7.5 weeks of development, following the initial outgrowth of the pancreatic buds, rare epithelial cells immunoreactive for insulin first appear, with no evidence of any other endocrine hormones present. A week later, glucagon and somatostatin expressing epithelial cells appear, and PP cells emerge at approximately 10 weeks of development (Piper *et al.*, 2004). Meanwhile, insulin positive cells are more distinct as cell clusters and are more numerous than any other endocrine cell type. At approximately 18 weeks of fetal age, adult-like islets containing four endocrine cell types (β, α, δ, and PP) and an associated fine capillary network are observed. From this point onwards ontogenic changes involve mainly expansion and growth (Lyttle *et al.*, 2008;

Piper *et al.*, 2004). The above studies showed progressive developmental changes of ductal and islet structures that were paralleled by increases in β and α-cell populations during 7 to 21 weeks of human fetal pancreatic development, and therefore can serve as a critical window when endocrine cells initiate their function.

Transcriptional Regulation of Pancreatic Development: Through the use of mouse models in which specific genes have been disrupted, many transcription factors have been identified as critical regulators of pancreatic development (Habener *et al.*, 2005). In the following paragraphs I highlight the transcription factors that are addressed in this study, all of which have important roles to play in the developmental cascade leading up to endocrine cell, and more importantly β-cell, formation (**Figure 1.1B**).

PDX-1 (pancreatic duodenal homeobox gene-1): The cascade of transcription factors governing pancreatic development begins with the expression of PDX-1, otherwise known as the master regulator of both pancreatic development and the differentiation of progenitor cells into the β-cell phenotype (Habener *et al.*, 2005). Mice that carry a null *PDX-1* mutation die within days after birth (Offield *et al.*, 1996). *PDX-1* targeted gene disruption results in pancreatic agenesis, in which undeveloped pancreatic buds form, but then regress (Jonsson *et al.*, 1994). Similarly, a child born without a pancreas was found to be homozygous for an inactivating mutation in the PDX-1 encoding gene (Stoffers *et al.*, 1997). Throughout pancreatic development, PDX-1 expression is maintained as a prerequisite for the commitment of the endoderm to a pancreatic phenotype. In mouse models, PDX-1 is first detected on embryonic day (e) 8.5 within a narrow region of

foregut endoderm, and shortly after is expressed in both the ventral and dorsal pancreatic buds (Guz *et al.*, 1995; Offield *et al.*, 1996). From e11.5 to e13.5, PDX-1 is expressed throughout the developing ductal tree; however when the exocrine pancreas and the islets begin to form, between e14 and e15, the expression pattern begins to shift to the endocrine compartment. By e18.5, the expression of PDX-1 becomes primarily restricted to the mature β-cells (Guz *et al.*, 1995; Offield *et al.*, 1996). A similar expression pattern is observed in the human fetal pancreas, whereby early in development the majority of ductal precursor cells that eventually will form the ducts, acini and islets, express PDX-1. By week 14 of fetal age, the expression of PDX-1 within ductal cells starts to decrease, only to become significantly lower by 21 weeks (Lyttle *et al.*, 2008). Overall the expression pattern shows a progressive decrease in ductal islet precursors from 8 to 21 weeks. While the co-expression of PDX-1 and insulin progressively increases throughout islet development, no co-localization between PDX-1 and glucagon is ever observed, supporting the concept that PDX-1 is β-cell specific and required for establishing and maintaining its phenotype (Lyttle *et al.*, 2008; Piper *et al.*, 2004). The above studies clearly underscore the importance of PDX-1 in the development of the pancreas in mice models and humans alike.

NGN3 (neurogenin 3) and **HES1** (hairy and enhancer of split 1): The initiating step towards an endocrine cell fate depends on the expression of the basic helix loop helix (bHLH) transcription factor NGN3 (Habener *et al.*, 2005). Expression of NGN3 starts on approximately e9, peaks on e15.5 during the major wave of endocrine cell genesis, and is greatly reduced by birth and in the adult islet (Habener *et al.*, 2005). Mice that carry a

null *NGN3* gene mutation fail to generate any endocrine cells or potential endocrine precursors during development (Gradwohl *et al.*, 2000). NGN3 is necessary for the development of all pancreatic endocrine cell lineages, and therefore it has been designated as a marker of islet precursor cells (Gradwohl *et al.*, 2000; Lee *et al.*, 2001). In the human fetal pancreas the highest expression levels of NGN3 were observed at 8 to 10 weeks, where $NGN3^+$ cells co-express PDX-1, insulin and glucagon. As development progressed, the appearance of mature endocrine cell clusters coincided with a significant decrease in NGN3 expression (Lyttle *et al.*, 2008). The majority of $NGN3^+$ cells remained at the outer edges of these endocrine cell clusters, suggesting that they may be a population of endocrine cell precursors (Lyttle *et al.*, 2008).

NGN3 directs the expression of extracellular ligands that activate Notch receptors on adjacent cells (Habener *et al.*, 2005). This in turn activates a chain of signaling events that result in the downstream expression of hairy/enhancer-of-split proteins, well known inhibitory factors that bind *NGN3* and other bHLH genes, thereby silencing these genes and preventing neurogenesis (Habener *et al.*, 2005). This lateral inhibition model provides a mechanism by which a cell adopting an endocrine cell fate forces its neighboring cells to adopt a non-endocrine cell fate (Artavanis-Tsakonas *et al.*, 1999; Habener *et al.*, 2005). HES1 is the predominant mammalian hairy/enhancer-of-split homologue expressed in the developing pancreas (Jensen *et al.*, 2000). In rodents, loss of HES1 or defects in notch signaling result in increased NGN3 expression as well as premature and broader endocrine differentiation (Apelqvist *et al.*, 1999; Jensen *et al.*, 2000). HES1 represses *NGN3* expression by directly suppressing the activity of the *NGN3* promoter. HES1 targets sequences within the strong basal activation region of the *NGN3* proximal

promoter, recruits transcriptional co-repressors, and therefore promotes histone deacetylation and a closed chromatin structure (Lee *et al.*, 2001).

ISL1 (islet 1): The LIM homeodomain protein ISL1 is expressed early on in the developing mouse (e9) and human pancreas (as early as 8 weeks) (Ahlgren *et al.*, 1997; Lyttle *et al.*, 2008). In the mouse, a targeted disruption of the *ISL1* gene results in an early arrest of embryonic development on approximately e9.5 (Pfaff *et al.*, 1996). Analysis of the pancreatic remains suggests that ISL1 is not only required for the development of the mesenchyme of the dorsal pancreatic bud, but is also essential for the differentiation of its epithelium to endocrine cells (Ahlgren *et al.*, 1997). In the human fetal pancreas ISL1 co-localized with both insulin$^+$ and glucagon$^+$ cells during the 8 to 21 week developmental period, suggesting that ISL1 may be involved in the differentiation of both the β and α endocrine cell types (Lyttle *et al.*, 2008).

PAX6 (paired-box homeoprotein 6): PAX6 is expressed in the epithelium of the developing pancreas around e9.0 in both the dorsal and ventral pancreatic buds, and is eventually expressed in all four pancreatic endocrine cell types (Habener *et al.*, 2005). Mice that carry a null *PAX6* mutation die within minutes of birth (Sander *et al.*, 1997). These animals fail to form islets as a result of a lack of organization of islet cells, which could be related to the proposed function of PAX6 in the regulation of cell adhesion molecules (Chalepakis *et al.*, 1994; Sander *et al.*, 1997). Similarly, in the human fetal pancreas PAX6 is expressed as early as 8 weeks, and co-localized with both insulin$^+$ and glucagon$^+$ cells during the 8 to 21 week developmental period (Lyttle *et al.*, 2008). The

above studies implicate PAX6 as a key regulator of the terminal steps in cellular differentiation of the endocrine pancreas.

NKX Family: Two members of the NK2 family of homeoprotein transcription factors, NKX2-2 and NKX6-1, are also important regulators of the terminal differentiation of pancreatic endocrine cells (Habener *et al.*, 2005). NKX2-2 and NKX6-1 are expressed as early as 8 weeks in the developing human pancreas. NKX2-2 was expressed in both α and β-cells, while NKX6-1 expression is restricted to β-cells (Lyttle *et al.*, 2008). Mice that carry a null NKX2-2 or NKX6-1 mutation die soon after birth due to severe diabetes (Sussel *et al.*, 1998). Analysis of their pancreata showed no insulin-producing cells and very few glucagon-producing cells. NKX2-2-null mice have a large number of hormone-negative, arrested precursor cells that express endocrine pancreas-specific proteins, but not hormones. Interestingly, NKX6-1-null mice have a selective reduction of β-cells with a normal complement of other endocrine cell types (Sander *et al.*, 2000).

Recently, studies have shown that forkhead (FOX) proteins expressed in the pancreas play crucial roles in the function and development of this endocrine organ through transcriptional regulation of their targets genes (Kitamura *et al.*, 2007). Much is still to be learned about their expression patterns and requirements in the developing pancreas.

1.3 FOXO Transcription Factors

1.3.1 Molecular Structure of FOXO Transcription Factors

The forkhead box (FOX) family of transcription factors was named after its conserved "winged-helix" DNA binding domain. More than 100 DNA-binding proteins containing the forkhead domain have been identified in a wide range of species ranging from yeast to humans (Huang and Tindall, 2007). Mammalian FOXO transcription factors are O class members, one of the most diverse subclasses of the FOX family (Huang and Tindall, 2007). This subclass includes FOXO1, commonly referred to as FKHR (forkhead in rhabdomyosarcomas). The gene was identified following characterization of a chromosomal translocation t(2,13)(q35;q14) and t(1,13)(p36;q14) commonly found in alveolar rhabdomyosarcoma, a skeletal-muscle tumor that is prevalent in children (Galili *et al.*, 1993). It has orthologues that include Daf16 in *C. elegans* and dFoxo in Drosophila. In addition to FOXO1, three other O class members encoded by distinct genes have been identified in humans: these include FOXO3, FOXO4, and recently FOXO6 (Huang and Tindall, 2007).

FOXO proteins function primarily as transcription factors in the nucleus and bind as monomers to their corresponding DNA target sequences (Huang and Tindall, 2007). While the transactivation domain is located in the C-terminal end, the DNA-binding domain of these proteins is located in the N-terminal portion. It consists of three α-helices, three β-strands, a hydrophobic core, and two loops that interact with the phosphate backbone of target DNA (Boura *et al.*, 2007; Clark *et al.*, 1993). In addition, FOXO proteins contain a NLS (nuclear localization signal) in the C-terminal region of the DNA-binding domain, and a leucine-rich NES (nuclear export signal) located further downstream, that are targets to various post-transcriptional modifications forming one of the main mechanisms controlling their activity (**Figure 1.2**) (Zhao *et al.*, 2004).

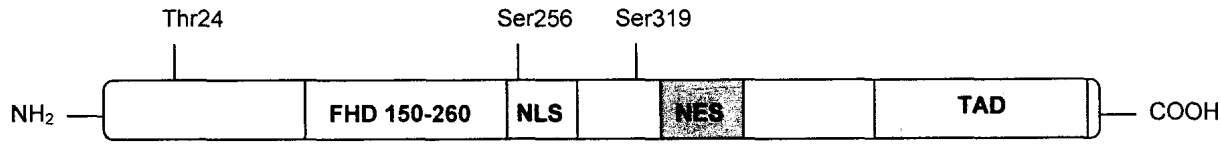


Figure 1.2. Domain Structure and AKT Phosphorylation Sites of the FOXO1 Protein. AKT phosphorylates FOXO1 at three regulatory sites (Thr24, Ser256, Ser319), which triggers the rapid relocalization of FOXO1 proteins from the nucleus to the cytoplasm. Abbreviations: FHD, Forkhead domain; NLS, nuclear localization signal; NES, nuclear export signal; TAD, transactivation domain. Adapted from Huang and Tindall, 2007.

Although the molecular basis of the DNA-binding specificity of FOXO transcription factors is poorly understood, recent high affinity DNA-binding studies have identified (G/C)(T/A)AA(C/T)AA as a consensus FOXO-recognized sequence (Furuyama *et al.*, 2000). In fact, FOXO-recognized sites that match this sequence have been identified in the promoter region of a diverse group of genes, highlighting the wide varity of signaling pathways and biological processes FOXO transcription factors are involved in (Accili and Arden, 2004).

1.3.2 **Molecular Mechanisms Regulating FOXO Transcription Factor Activity**

FOXO transcription factors are controlled by complex signaling networks triggered by environmental stimuli. Most of these environmental stimuli result in post-transcriptional modifications of FOXO factors, and include phosphorylation, ubiquitination and acetylation (Greer and Brunet, 2005; Huang and Tindall, 2007). Growth and survival signals including insulin, insulin like-growth factor 1, epidermal growth factor (EGF) and glucagon-like peptide-1 (GLP-1), negatively regulate FOXO factors' transcriptional activity through phosphorylation and nuclear export induced by the Phosphoinositide 3-Kinase (PI3-Kinase)/AKT (protein kinase B) pathway (Greer and Brunet, 2005; Huang and Tindall, 2007). Typically, it is known that AKT, once activated, targets highly conserved phosphorylation sites within the forkhead domain, causing the transport of FOXO from the nucleus to the cytoplasm (**Figure 1.2**) (Brunet *et al.*, 2001). In contrast, FOXO proteins are imported into the nucleus in response to various stress events even in the presence of growth factors (Glauser and Schlegel, 2007; Wang *et al.*, 2005). Kinases activated by oxidative stress, such as Jun-N-terminal kinase (JNK), target

different residues than growth-factor activated kinases, leading to the nuclear retention of FOXO factors and enhanced transcriptional activity, allowing for the increased expression of antioxidants (Essers *et al.*, 2004; Lehtinen *et al.*, 2006).

Given that the major consequence of the phosphorylation of FOXO transcription factors by AKT is a block in FOXO-dependent transcription in mammalian cells, current data have suggested a detailed model for FOXO nuclear export. AKT dependent phosphorylation of FOXO factors in response to growth factor stimulation generates two consensus binding sites for 14-3-3 proteins, well known members of a family of cellular chaperones that interact with their corresponding protein ligands in a phosphorylation-dependent manner (Brunet *et al.*, 2002). FOXO/14-3-3 protein binding appears to decrease the ability of FOXO/DNA binding, displacing FOXO factors from DNA (Cahill *et al.*, 2001). FOXO/14-3-3 protein binding may then promote nuclear export in a manner dependent on conformational changes within FOXO factors that expose the NES, allowing interactions with the Exportin/CRM1 (chromosome region maintenance) nuclear export complex (Brunet *et al.*, 2002). In addition, casein kinase (CK1)-mediated phosphorylation of FOXO factors at Ser322 and Ser325 further increases the interaction between FOXO factors and the nuclear export complex. Phosphorylation of FOXO at these residues allows FOXO factors to interact with the RanGTPase, a main component of the Exportin/CRM1 nuclear export complex that leads to the export of FOXO from the nucleus (Rena *et al.*, 2002). Along with its effects on FOXO factors' subcellular localization, FOXO/14-3-3 protein binding may also interfere with the function of FOXO factors NLS, hindering the rate of nuclear import (Rena *et al.*, 2001).

In addition to phosphorylation by various kinases, FOXO transcription factors may also be acetylated at several conserved lysine residues, and their acetylation status is mostly altered by stress signals, providing an additional level of regulation (Huang and Tindall, 2007). Acetylation of FOXO transcription factors by p300 and the cAMP response element binding protein (CREB) binding protein (CBP), with histone acetyltransferase activity, increases in response to oxidative stress (Brunet *et al.*, 2004 ; Kitamura *et al.*, 2005). Unlike during FOXO phosphorylation, where no specific phosphatase has been identified for the dephosphorylation of FOXO, deacetylation of FOXO proteins involves the activity of SIRT1 (sirtuin 1), a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase (Brunet *et al.*, 2004; Glauser and Schlegel, 2007; Kitamura *et al.*, 2005). Although controversial, most studies have shown that acetylation targets FOXO factors to the nucleus and prevents ubiquitin-dependent degradation, while deacetylation increases transcriptional activity of FOXO factors, a mechanism that is important for diverting FOXO-dependent responses away from apoptosis and towards cell-cycle arrest and stress resistance (Glauser and Schlegel, 2007; Greer and Brunet, 2005; Lam *et al.*, 2006). Finally the rate of FOXO degradation, which is mediated by the ubiquitin proteasome system, is favored by FOXO phosphorylation and/or deacetylation (Huang *et al.*, 2005). Recent findings indicate that ubiquitylation of the different FOXO proteins may be mediated by specific ubiquitin E3 ligases (Huang *et al.*, 2005; Kitamura *et al.*, 2005). Altogether, covalent modifications offer a large spectrum of options for the fine tuning of FOXO function in complement to the master switch operated by the control of FOXO factors' subcellular localization.

1.4 Diverse Cellular Functions of FOXO1 Proteins

Nuclear FOXO proteins regulate a wide array of cellular processes, including cell cycle progression, cell differentiation, DNA repair, glucose metabolism, and oxidative stress (Huang and Tindall, 2007). When investigating a functional role for FOXO1 in the developing human pancreas, a heterogeneous organ, we must take into account the roles identified in other cell models and consider the possibility that FOXO1 functions in the developing human pancreas may differ depending on cell-type. The following paragraphs summarize recent literature regarding the role of FOXO1 in some processes as well as key genes targeted in various cell models.

1.4.1 Apoptosis & Cell Cycle Regulation

The first activity ever attributed to FOXO proteins was the induction of programmed cell death (Greer and Brunet, 2005; Huang and Tindall, 2007; Tran *et al.*, 2003). The observation that growth factor/PI3-Kinase/AKT signaling promotes cellular survival and mediates phosphorylation-dependent inhibition of FOXO activity supported the hypothesis that FOXO factors regulate apoptosis (Brunet *et al.*, 1999). Studies have shown that FOXO-induced cell death is mediated by the transcriptional regulation of several target genes such as the death receptor ligand, Fas ligand (*FasL*) and the tumor-necrosis-factor-related apoptosis-inducing ligand (*TRAIL*) (Brunet *et al.*, 1999; Modur *et al.*, 2002). In addition to death receptor ligands, common FOXO targets also include genes which encode pro-apoptotic BCL-2 family members (Dijkers *et al.*, 2000; Huang and Tindall, 2007). For example, in human endometrial stromal cells, progesterone signaling induces cytoplasmic inactivation of FOXO1, a step necessary for survival.

Withdrawal of progesterone results in nuclear accumulation of FOXO1 due to decreased AKT signaling (Labied *et al.*, 2006). In the nucleus, FOXO1 causes the activation of BIM (BCL-2 interacting mediator of cell death), which functions in the mitochondrial apoptotic pathway, subsequently resulting in cell death. Biologically this is expressed as menstruation, the cyclic shedding of endometrial tissue. Therefore, progesterone inhibits BIM and, subsequently apoptosis through AKT mediated FOXO1 inactivation (Labied *et al.*, 2006).

The link between activation of FOXO proteins and cellular processes including apoptosis, DNA repair, and cell cycle arrest has made them attractive candidates as tumor suppressors (Greer and Brunet, 2005). A deficiency in FOXO protein function within abnormal cells that would otherwise undergo programmed cell death results in tumor development and expansion (Carter and Brunet, 2007). In fact, FOXO1 was initially isolated at sites of chromosomal breakpoints. These breakpoints result in the fusion of the transactivation domain to the DNA binding domains of other unrelated transcription factors, and are commonly found in skeletal muscle tumors and alveolar rhabdomyosarcomas (Galili *et al.*, 1993; Huang and Tindall, 2007). Furthermore, the PI3-Kinase/AKT pathway stimulated by mitogenic and survival signals, is over-activated in a variety of tumors. Given that the PI3-Kinase/AKT pathway is a negative regulator of FOXO factor activity, it may be responsible for hindering their performance as tumor suppressors, further contributing to the manifestation of tumorgenesis (Tran *et al.*, 2003). Tumor suppressor activity is achieved by FOXO1 through the transcriptional repression of positive cell cycle regulators, such as *cyclins D1* and *D2*, and the transcriptional induction of negative cell cycle regulators such as *p27* and *p130* (Tran *et al.*, 2003).

Recent studies revealed that SKP2 (s-phase kinase-associated protein 2), an oncoprotein that promotes cell proliferation through the degradation of several negative regulators of the cell cycle, directs the ubiquitylation and degradation of FOXO1, suggesting a new mechanism by which SKP2 may favor tumorgenesis (Dehan and Pagano, 2005).

1.4.2 **Metabolism**

The distribution of FOXO1 in insulin-responsive tissues, along with its regulation by growth factor-stimulated AKT phosphorylation, allow FOXO1 to mediate a variety of important metabolic functions by regulating cellular proliferation and promoting the expression of genes involved in gluconeogenesis and energy metabolism (**Figure 1.3**) (Gross *et al.*, 2008). Skeletal muscle is the major site of insulin stimulated glucose uptake in humans, which makes sustaining its mass critical for the maintenance of glucose uptake (de Lange *et al.*, 2007). Numerous studies have reported several roles for FOXO1 during skeletal muscle differentiation (Gross *et al.*, 2008). FOXO1 has been implicated in the indirect inhibition of myoblast differentiation through an increase in Notch signaling (Kitamura *et al.*, 2007). On the other hand, FOXO1 promotes the fusion of mononucleated myocytes into myotubes during muscle formation. Studies by Bois *et al.* support a feedback loop whereby, during myoblast proliferation, FOXO1 is confined to the cytoplasm. Following the initiation of the differentiation program, FOXO1 translocates to the nucleus, where it drives the expression of genes involved in myocyte fusion (Bois *et al.*, 2003; Bois *et al.*, 2005). FOXO1 also increases the expression of muscle atrophy genes, thereby promoting muscular atrophy in response to starvation (Sandri *et al.*, 2004).

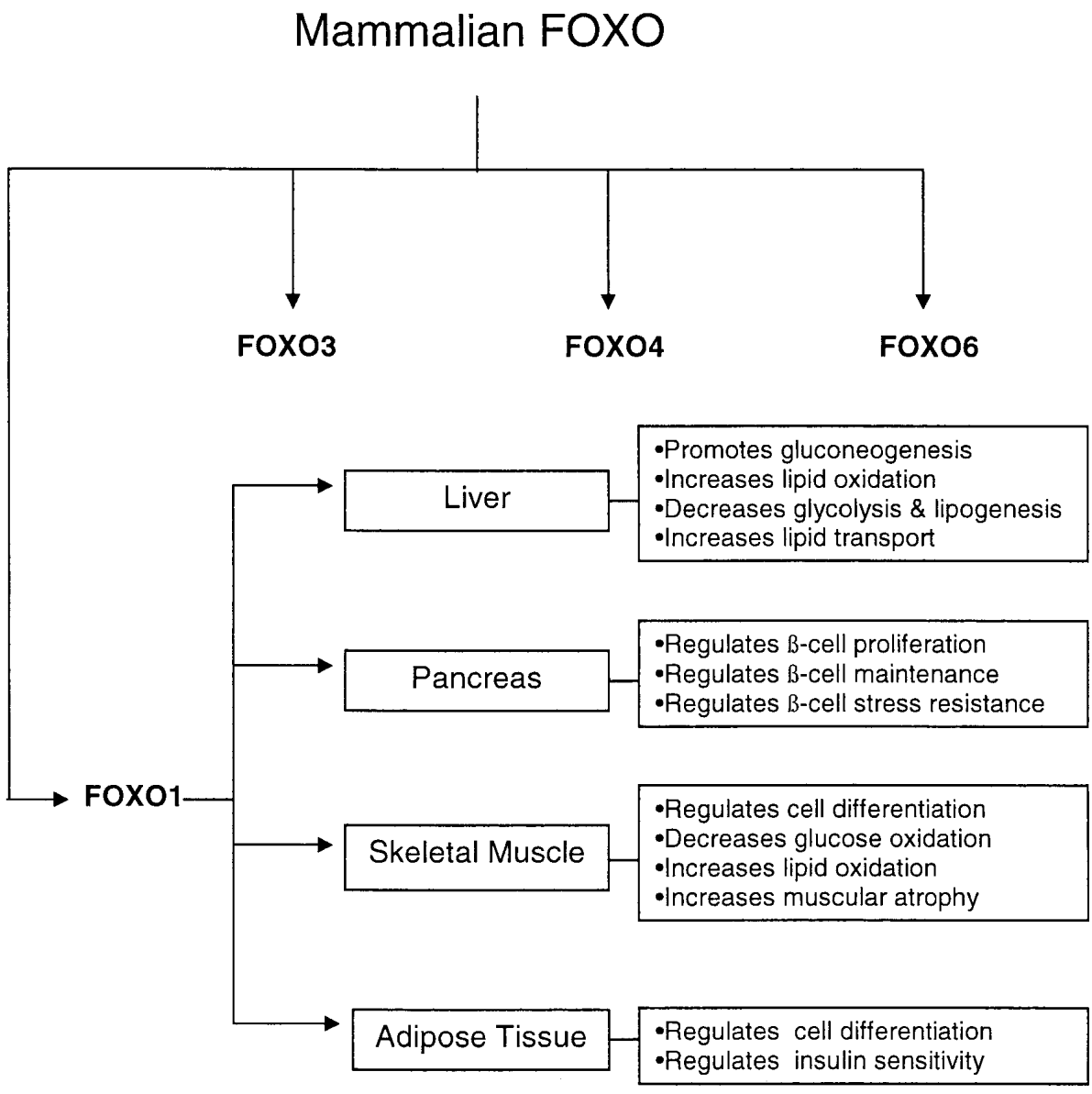


Figure 1.3. Effects of FOXO1 in Various Insulin Target Organs. The flow chart highlights the four members of the FOXO family, with specific emphasis on the main functions of FOXO1. The ability of FOXO1 to regulate cell proliferation and cell differentiation, and mediate the expression of genes involved in gluconeogenesis, energy metabolism and oxidative stress allows it to play a critical role in metabolism (Gross *et al.*, 2008).

Adipocytes, similar to skeletal muscles, take up glucose in response to insulin, storing it in the form of triglycerides, only to release it in times of energy deprivation (Ahima and Flier, 2000). Studies have shown a key role for FOXO1 in regulating adipocyte differentiation, whereby FOXO1 must be down-regulated during the initial stages of adipocyte clonal expansion, otherwise terminal differentiation would be prevented by uncontrolled FOXO1 activity. This anti-adipogenesis effect of FOXO1 is mediated mainly through the regulation of target cell-cycle inhibitors, influencing directly cell-cycle progression (Nakae *et al.*, 2003). FOXO1 may also impair adipocyte differentiation by directly repressing the expression of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ2), a main regulator of adipogenesis (Armoni *et al.*, 2006; Farmer, 2005). In addition it has been suggested that FOXO1 can alter insulin sensitivity in adipocytes through a feedback loop (Puig *et al.*, 2005). The binding of insulin to the insulin receptor (InR) results in the downstream activation of AKT and ultimately the cytoplasmic retention of FOXO1. When nutrients are limited, such as in starvation or fasting conditions, insulin levels decline and there is less PI3-Kinase/AKT signaling. Nuclear FOXO1 accumulates and promotes the transcription of *InR*, and the accumulation of InR increases sensitivity to changes in insulin concentration (Puig *et al.*, 2005).

FOXO1 also plays a role in promoting adaptation to fasting, by regulating multiple aspects of glucose and lipid metabolism (Gross *et al.*, 2008). In addition to regulating muscle mass, FOXO1 is also capable of regulating muscle function under conditions such as fasting and exercise. FOXO1 promotes the expression of lipoprotein lipase, an enzyme that hydrolyzes triglycerides into fatty acids and glycerol for uptake by

muscle cells, mediating the transition from carbohydrate oxidation to lipid oxidation (Bastie *et al.*, 2005). In addition, several studies demonstrate that FOXO1 can increase the expression of pyruvate dehydrogenase kinase-4 (PDK-4), which inhibits fatty acid synthesis and directs pyruvate towards glucose production (Dong *et al.*, 2006). In the liver, FOXO1 mediates the expression of genes involved in both glucose and lipid metabolism. Liver specific transgenic expression of constitutively active FOXO1 drives the expression of gluconeogenic enzymes, including the rate-limiting enzyme phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase), whilst suppressing the expression of genes involved in glycolysis and lipogenesis, including sterol response element binding protein-1 c (SREBP-1c), glucokinase, pyruvate kinase, and fatty acid synthase (Zhang *et al.*, 2006). Further analysis showed an increase in the hepatic uptake of glycerol, derived primarily from triacylglycerol breakdown in adipose tissue (Zhang *et al.*, 2006).

In addition to its expression in the previously mentioned insulin-sensitive tissues, FOXO1 also plays a vital role in the insulin-producing pancreatic β-cell, which will be the focus of the following section.

1.5 Cellular Functions of FOXO1 Proteins in Pancreatic β-cells

FOXO1 is the most abundant forkhead transcription factor in isolated mouse islets and in the βTC-3 β-cell line (Kitamura *et al.*, 2002). FOXO1 is also expressed in isolated human adult islets (Contreras *et al.* 2002). Even more interestingly, for unknown reasons *FOXO1* mRNA levels are elevated in islets of diabetics compared to non-diabetic patients (Del Guerra *et al.*, 2005). Furthermore, many pathways shown to regulate FOXO

functions are, in fact, active in β-cells (Glauser and Schlegel; 2007). Therefore, in the past few years, many studies have aimed to identify the role of FOXO1 factor in β-cells and its association with the type 2 diabetes (Kitamura and Ido Kitamura, 2007).

1.5.1 **Role of FOXO1 in Pancreatic β-cell Growth and Proliferation**

The maintenance of β-cell mass is critical for proper endocrine metabolic function. Many factors are capable of influencing β-cell mass through changes in cell size, proliferation and apoptosis. The insulin/IGF-1 receptor signaling pathway, enhanced by a number of growth factors and hormones such as insulin and incretins, is involved in a feedback loop controlling β-cell proliferation rate and insulin secretion. This forms an integral part of the β-cells' ability to deal with an increased demand for insulin under excessive glucose conditions (Holz and Chepurny, 2005). Mice with a homozygous null mutation in the insulin receptor substrate 2 gene (*Irs2*) develop diabetes as a result of β-cell failure and impaired proliferation (Kitamura *et al.*, 2002). As a down-stream target of the insulin/IGF-1/PI3-Kinase/AKT signaling pathway, FOXO1 factor was investigated, providing a link for its role in the regulation of β-cell function and growth.

Interestingly, FOXO1 haploinsufficiency partially improved glucose levels in $Irs2^{-/-}$ mice, mainly by restoring β-cell proliferation, and consequently insulin secretion (Nakae *et al.*, 2002). Similar results were obtained from a mouse β-cell specific knockout for 3-Phosphoinositide-dependent protein kinase 1 ($\beta Pdk1^{-/-}$) which mediates signaling downstream of PI3-Kinase; these mice were also haploinsuffiency for FOXO1 (Hashimoto *et al.*, 2006). Furthermore, the increased β-cell mass observed was most likely mediated by PDX-1, given that $FOXO1^{+/-}$ / $Irs2^{-/-}$ mice showed elevated PDX-1

levels in comparison to *Irs2*$^{-/-}$ mice (Kitamura *et al.*, 2002), and as previously mentioned, PDX-1 is commonly expressed in adult islet β-cells and is critical for their function and growth (Jonsson *et al.*, 1994).

Although in the adult pancreas both FOXO1 and PDX-1 are mostly expressed in adult islet β-cells, both transcription factors exhibit distinct patterns of subcellular localization (Buteau and Accili, 2007; Kitamura *et al.*, 2002). It was suggested that FOXO1 may act as a negative regulator of PDX-1 function, and as a result two mechanisms have been proposed to explain the repressive relationship observed. The first model suggests that FOXO1 functions as a regulator of *PDX-1* expression, by competing with another forkhead transcription factor, FOXA2, for a common DNA binding site within the *PDX-1* promoter (Kitamura *et al.*, 2002). Unlike FOXO1 which inhibits *PDX-1* expression upon binding to the promoter region, FOXA2 stimulates expression. Together, both forkhead factors form a regulatory mechanism controlling *PDX-1* expression and function within β-cells. The second model suggests that in response to certain stimuli, specifically oxidative stress, FOXO1 could control the nuclear localization of PDX-1 in a manner dependent on the JNK pathway (Kawamori *et al.*, 2006).

1.5.2 **Role of FOXO1 in an Anti-oxidative Stress Mechanism in β-cells**

β-cells are more sensitive to oxidative stress in comparison to other cell types, mainly due to the fact that they express low levels of antioxidant enzymes, such as catalase, Mn-superoxide dismutase (Mn-SOD) and glutathione peroxidase (Lenzen *et al.*, 1996; Welsh *et al.*, 1995). As a result, they rely on alternative mechanisms to cope with

oxidative stress. The major source of oxidative stress in β-cells comes from chronic exposure to glucose concentrations at levels exceeding their glycolytic capacity, a state commonly referred to as "glucose toxicity" (Robertson *et al.*, 2003). Unlike other cell types where FOXO1 shuttles between the nucleus and the cytoplasm, in β-cells, FOXO1 is confined to the cytoplasm due to the continuous stimulation by endogenously produced insulin (Harbeck *et al.*, 1996). However, upon exposure to oxidative stress, FOXO1 localizes to the nucleus, where it initiates the induction of MAFA (macrophage activating factor A) and NEUROD (neurogenic differentiation) expression, transcription factors that regulate *Ins2* (Kitamura *et al.*, 2005). Interestingly, under these circumstances, FOXO1 is first acetylated by p300, which targets it to nuclear promyelocytic leukemia (Pml) bodies and preventing both FOXO1 degradation and FOXO1-dependent transcription (Kitamura *et al.*, 2005). This is followed by deacetylation of FOXO1 by SIRT1 (sirtuin 1) promoting FOXO1-dependent transcription. Although this pathway activates transcription by FOXO1, it also promotes its ubiquitin-dependent degradation (Kitamura *et al.*, 2005). Therefore under oxidative stress conditions, the activity of FOXO1 is regulated by a delicate balance between acetylation and deacetylation, providing a model through which FOXO1 allows β-cells to withstand short-lived acute glucose level changes. However since this mechanism reduces the half life of FOXO1, it is unable to preserve β-cell function in the face of continuing stress (Kitamura *et al.*, 2005; Buteau and Accili, 2007).

FOXO1 is therefore central to the choice between two critical options in β-cells, promoting stress resistance and inhibiting proliferation. Therefore the balance between mechanisms that affect FOXO1 activity is crucial in determining pancreatic β-cell fate,

making it an interesting potential target to develop novel therapeutic approaches for diabetes.

1.6 Project Purpose and Outline

Previous research has focused on the role of FOXO1 in adult tissue with little published on its role during embryogenesis. Mouse embryos lacking *FOXO1* have demonstrated incomplete vascular development and do not survive beyond e10.5, indicating a crucial role for this gene in angiogenesis, as well as its requirement for embryonic development (Hosaka *et al.*, 2004). However, because of the early lethality of *FOXO1*$^{-/-}$ animals, the effect of FOXO1 on pancreatic development has yet to be examined using alternate methods. Taking into account the many findings which clearly indicate a functional role for FOXO1 in the cellular maintenance of β-cells in adult islets, my goal is to examine the mechanistic role of FOXO1 in regulating islet cell development, specifically β-cell development during human fetal pancreatic development, and whether FOXO1 plays a similar role during development to that which it has in the adult islet. In this thesis, I report the expression patterns of nuclear FOXO1 *ex vivo* in the developing human fetal pancreas during the 1st and 2nd trimester of pregnancy, and assess its subcellular localization downstream of Insulin/PI3-Kinase/AKT signaling in cultured islet epithelial cell clusters. Transfections of islet epithelial cell clusters with *FOXO1* specific siRNAs provided *in vitro* evidence of a critical role for FOXO1 in the control of β-cell differentiation.

Objective:

To characterize the expression pattern, and to identify the functional role of FOXO1 during islet cell development in the human fetal pancreas.

Hypotheses:

1. FOXO1 influences proliferation and differentiation of endocrine cells in the developing human fetal pancreas.
2. FOXO1 influences key transcriptional factors critical for the development of functional β-cells in the human fetal pancreas.

Specific Questions:

1. Is FOXO1 restricted to a specific spatial-temporal mRNA/protein distribution during islet cell development?
2. Does FOXO1 influence the subcellular localization/expression of PDX-1 in the human fetal pancreas?
3. Does FOXO1 influence cellular proliferation in the human fetal pancreas?
4. Does insulin or glucose influence the subcellular localization of FOXO1 in the human fetal pancreas?
5. Does FOXO1 influence the expression of pro-endocrine cell markers important for islet cell growth and differentiation in the human fetal pancreas?

Chapter 2

Research Design and Methods

Chapter 2 - **Research Design and Methods**

2.1. Pancreatic Tissue Collection

Human fetal pancreata (8 to 21 weeks of fetal age) were collected according to protocols approved by the Health Human Sciences Research Ethics Board at the University of Western Ontario (**Appendix I**), in accordance with the Canadian Council on Health Sciences Research Involving Human Subjects guidelines. The fetal age is typically two weeks less than the gestational age (based on the menstrual cycle), to account for the two pre-ovulation weeks that precede conception. Tissues were carefully dissected and washed with phosphate buffered saline (PBS) and immediately processed for either immunofluorescence staining, RNA and/or protein extraction, or islet-epithelial cell isolation, with at least three pancreata per age group or experimental group.

2.2. Immunofluorescence Staining & Morphometric Analyses

Pancreata were fixed in 4% paraformaldehyde (PFA) (Fisher Scientific Company; Ottawa, ON, Canada) overnight at 4°C. Samples were processed by a standard protocol of dehydration and paraffin embedding, using an automated tissue processing and embedding machine (Shandon Citadel™ Tissue Processor, Citadel 1000, Thermo Electron Corporation; Waltham, Massachusetts, USA). Some samples were embedded using a frozen tissue matrix (Shandon Cryomatrix™) (Thermo Electron Corporation) and the blocks were stored in the -80°C freezer until sectioning. A set of 5 μm consecutive paraffin sections were cut with a microtome (Leica RM2245, Vashaw Scientific Inc.; Norcross, Atlanta, USA), and a set of 5 μm consecutive frozen sections were cut with a

Cryostat (Leica CM1900), both throughout the length of the pancreas. Paraffin sections were deparaffinized in xylene, rehydrated with decreasing concentration of ethanol (from 100% to 70%) and rinsed with running water and 1xPBS. Frozen sections were allowed to dry at room temperature for at least 2 hr, after which they were fixed with 4% PFA for 30 minutes at room temperature. Nonspecific binding was blocked using 10% normal goat serum diluted in 1xPBS at room temperature for 30 minutes in a humidified chamber. Sections were first incubated with the appropriate dilutions of primary antibodies as listed in **Table 2.1** overnight at 4°C. Sections were then incubated with the appropriate dilutions of fluorescein-conjugated secondary antibodies obtained from Jackson Immunoresearch Laboratories, for 1 hr at room temperature (**Table 2.1**). Nuclei were counterstained with 4'-6'-diamidino-2-phenylindol (DAPI) (Sigma; St. Louis, MO, USA). Negative controls included the omission of the primary or secondary antibodies; these tests resulted in a negative staining reaction (**Supplementary Figure 1**). Images of sections were captured by a Leica DMIRE2 fluorescence microscope (Leica Microsystems; Bannockburn, Illinois, USA) and a digital camera (Retiga 1300, High-sensivity IEEE 1394 FireWire™ digital camera, QIMAGING; Burnaby, British Colombia, Canada) connected to a Macintosh computer. Openlab image software (Improvision; Lexington, MA, USA) was used to analyze, store and export the images.

The percentage of cells expressing nuclear FOXO1 (nFOXO1) was determined by cell counting. In brief, for samples 14 to 21 weeks of fetal age, ten random fields from the head, middle, and tail of the pancreata per pancreatic section were chosen, with a minimum of three pancreata per age group. At least 1,000 cells per ductal region, identified by cytokeratin 19 (CK19) staining, and 500 cells per endocrine region,

Table 2.1: List of Antibodies/Antisera used for Immunofluorescence Staining and Western-Blot Analyses.

Primary antibody	Dilution	Company, Location
Rabbit anti-human FOXO1	1:1000-5000[a]	Dr. Henry Dong; University of Pittsburgh, PA, USA
Mouse anti-human insulin	1:1000	Sigma; St. Louis, MO, USA
Mouse anti-human glucagon	1:1000	Sigma; St. Louis, MO, USA
Rat anti-human C-peptide	1:100	DSHB; University of Iowa, Iowa city, USA
Mouse anti-human cytokeratin 19	1:100	Dako; Mississauga, ON, CA
Mouse anti-human Ki-67	1:100	BD Biosciences; Mississauga, ON, CA
Mouse anti-human calnexin	1:2000[a]	BD Biosciences; Mississauga, ON, CA
Guinea pig anti-human PDX-1	1:500	Dr. Christopher V. Wright; University of Vanderbilt, Nashville, TN
Rabbit anti-human PDX-1	1:5000	Chemicon; Temecula, CA
Rabbit anti-human HES1	1:100-1000	Chemicon; Temecula, CA
Rabbit anti-human AKT	1:2000	Cell signaling; Danvers, MA
Mouse anti-human Phosphorylated AKT (Ser 473)	1:3000[a]	Cell signaling; Danvers, MA
Mouse anti-human NGN3	1:100	DSHB; University of Iowa, Iowa city, USA
Mouse anti-human ISL1	1:100	DSHB; University of Iowa, Iowa city, USA
Mouse anti-human PAX6	1:100	DSHB; University of Iowa, Iowa city, USA
Mouse anti-human NKX 2-2	1:100	DSHB; University of Iowa, Iowa city, USA
Mouse anti-human NKX 6-1	1:100	DSHB; University of Iowa, Iowa city, USA
	Secondary antibodies	
Goat anti-mouse	1:100	JRL; West Grove, PA, USA
Goat anti-rabbit	1:100	JRL; West Grove, PA, USA
Goat anti-guinea pig	1:100	JRL; West Grove, PA, USA
Goat anti-rat	1:100	JRL; West Grove, PA, USA

[a] dilution factor applied for western blot analysis. DSHB, Developmental Studies Hybridoma Bank; JRL, Jackson Immunoresearch Laboratories

identified by insulin or glucagon staining, per pancreata were counted. All cells were counted for samples at 8 to12 weeks of fetal age. To determine the co-localization of nFOXO1 within the different cell populations, the double-positive cells were counted and the data were expressed as a percentage of nFOXO1 positive cells within the total number of insulin, glucagon, CK19, PDX-1, NGN3, or Ki-67 (proliferation index) positive cells counted.

2.3. Treatments of Islet-Epithelial Cell Clusters with Insulin or Glucose

Human fetal pancreata at 18 to 21 weeks of fetal age, a period containing a larger endocrine cell population for analysis in comparison to earlier stages (Lyttle *et al.*, 2008), were dissected and immediately digested with a dissociation buffer (1xHBSS [Sigma], 500 ml; 25 mM HEPES [Sigma]; DNase I [Roche]) containing collagenase V (1 mg/ml) (Sigma) in a water bath at 37°C for 30 minutes. Resulting islet-epithelial cell clusters, which contain mostly undifferentiated epithelial cells with a few scattered or clustered endocrine cells (Li *et al.*, 2006), were plated onto 12-well plates (Corning/VWR; Toronto, ON, Canada). Islet-epithelial cell clusters were treated with increasing concentrations of either insulin (Sigma) (0, 10, 25, 50, or 100 nM) or glucose (Sigma) (5.5, 10, or 25 mM), and cultured for 24 hr using 1 ml of CRML-1066 medium (Invitrogen; Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Invitrogen) per well. In some cases islet-epithelial cell clusters were pre-treated with wortmannin (100 nM) (Sigma), a competitive inhibitor of PI3-Kinase (Powis *et al.*, 1994), for 1 hr before the addition of insulin (50 nM) to the medium. At least three independent human fetal pancreata

preparations were used per experimental group. Cells were harvested and processed for RNA, protein, and immunofluorescence staining as described below.

2.4. Transfection of Islet-Epithelial Cell Clusters with *FOXO1* siRNA

Freshly isolated islet-epithelial cell clusters from human fetal pancreata (18-21 wks), were incubated overnight in CRML-1066 medium containing 10% FBS. Following this overnight recovery period, cells were transiently transfected with 60 nM *FKHR/FOXO1* siRNA (sc-35382, sequences listed below) or control siRNA (sc-37007, proprietary sequence) commercially produced by Santa Cruz Biotechnology Inc. using an siRNA transfection kit (Santa Cruz; CA, USA). Six independent human fetal pancreata preparations were used per experimental group. A pool of three sequences for human *FKHR/FOXO1* siRNA (Santa Cruz) was as follows: (*1*) 5'-GCA UCC AUG GAC AAC AAC Att-3' (sense), 5'-UGU UGU UGU CCA UGG AUG Ctt-3'(antisense); (*2*) 5'-GAA GGG GAU GUG CAU UCU Att-3' (sense), 5'-UAG AAU GCA CAU CCC CUU Ctt-3' (antisense); (*3*) 5'-CCA CAC AGU GUC AAG ACA Att-3' (sense), 5'-UUG UCU UGA CAC UGU GUG Gtt-3' (antisense). For optimal transfection efficiency, Santa Cruz Biotechnology's siRNA Transfection Reagent: sc-29528, siRNA Transfection Medium: sc-36868 and siRNA Dilution Buffer: sc-29527 were used according to the manufactures' instructions. Following a 24 hr or 48 hr transfection period, islet-epithelial cell clusters were harvested and assessed for *FOXO1* mRNA and FOXO1 protein levels to determine knockdown efficiency (Li *et al.*, 2007; Wang *et al.*, 2005).

2.5. Analysis of Islet-Epithelial Cell Clusters by Immunosatining

For immunofluorescence analysis, islet-epithelial cell clusters were harvested following treatments and fixed in 4% PFA overnight at 4°C. The cell pellets were first embedded in 2% agarose, followed by paraffin embedding (Li *et al.*, 2006). A set of 5 μm consecutive sections were cut from each experimental group. The paraffin sections were then processed and analyzed as described under "Immunofluorescence Staining & Morphometric Analyses". The pro-insulin by-product, C-peptide, is secreted in the blood stream in equimolar amounts with insulin, and therefore its considered to be a reliable β-cell marker (Rebsomen *et al.,* 2008). Therefore, the anti-C-peptide antibody was employed instead of the anti-insulin antibody for immunosatining to assess the β- cell population in samples collected following *in vitro* experiments, specifically for samples treated with insulin.

2.6. Protein Extraction and Western Blot Analysis

To determine total protein levels of FOXO1, signaling molecules, and transcription factors, total human fetal pancreata and islet-epithelial cell clusters from both treated and untreated groups were harvested and incubated with a Nonidet-P40 lysis buffer (Nonidet-P40, phenylmethylsulfonyl fluoride, sodium orthovanadate; (Sigma) and complete inhibitor cocktail tablet; [Roche]) for 20 minutes. The cells were then dispersed by sonicating for 10-20 seconds (3 times) to extract the protein. Samples were then centrifuged at 12,000 rpm for 20 minutes at 4°C. The resulting supernatant was collected and frozen at -80°C until use.

The protein concentration was measured by a protein assay using Bradford dye (BioRad Laboratories; Mississauga, ON, Canada), with bovine serum albumin (fraction V) as the standard. An equal amount (35 μg) of lysate protein from each sample or experimental group was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophesis (SDS-PAGE) and transferred to a nitrocellulose membrane by electroelution (Bio-Rad Laboratories). For each transfer a sandwich (sponge - filter paper - gel - membrane - filter paper – sponge) was assembled for Bio-Rad's Transblot. Blots were transferred at 250 mA for 2 hr at 4°C on a stir plate. Following transfer, the membranes were washed in Tris buffer-saline (TBS) containing 0.1% Tween 20, and blocked with 5% nonfat dry milk overnight at 4°C. Immunoblotting was performed with the appropriate dilutions of the primary antibodies listed in **Table 2.1** and incubated for 1 hr at room temperature. The secondary antibodies were goat anti-rabbit IgG or anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) diluted at 1:3000-5000 and incubated for 1 hr at room temperature. Anti-calnexin (BD Biosciences; Mississauga, ON, Canada) was used as a loading control. Proteins were detected by enhanced chemiluminescence reagents (Perkin Elmer; Wellesley, MA, USA), and exposed to BioMax MR Film (Kodak; Rochester, NY, USA). Densitometric quantification of bands at subsaturating levels was performed using the Syngenetool gel analysis software (Syngene; Cambridge, UK) and normalized to the loading control, calnexin.

2.7. Quantitative real-time RT-PCR (qRT-PCR) Analysis

Total RNA was extracted from pancreatic tissues using TRIZOL reagent (Invitrogen), and from cultured islet-epithelial cell clusters using the RNAqueous-4PCR kit (Ambion; Austin, TX, USA) according to the manufacturers' instructions, which also include a DNase I treatment step. RNA quality was verified by 1% agarose gel electrophoresis using eithidium bromide staining (**Supplementary Figure 2**). For each co-application RT reaction mixture the following components were added; 2 μg of DNA-free RNA, 1 ul random hexamers (3000 ng/ml), 1 ul Oligo-(dT) (500 μg/ml), and sterile distilled water up to 12 μl. Tubes were then heated in the PCR machine to 65°C for 5 minutes, and then cooled to 4°C for 5 minutes (Zhu and Altmann, 2005). For each reaction mixture the following components were then added; 4 ul 5x Buffer, 2 ul 0.1 M DTT, and 2 ul dNTP (10 mM) mix. Tubes were then incubated in the PCR machine for 2 minutes at 42°C before the addition of 1 ul SuperScript™ II reverse transcriptase (200 units) (Invitrogen). Tubes were then incubated at 42°C for 90 minutes. The reactions were inactivated by heating the tubes at 94°C for 5 minutes. PCR analyses were carried out in a T-gradient Biometra PCR thermal cycler (Montreal Biotech; Kirkland, QC, Canada) to determine the optimal annealing temperature for each primer and their specificity (Wang *et al.*, 2005). The amplified products were analyzed on 1% agarose gels and visualized using eithidium bromide staining (**Supplementary Figure 3**). Real-time PCR analyses were performed with 2 μl cDNA (50 ng/μl) using the iQ SYBR Green Supermix kit (10 μl) in Chromo4 Real-time PCR (Bio-Rad Laboratories). The PCR primers (10 μM) used are listed in **Table 2.2** (2 μl per reaction mix). Total volume per reaction was 20 μl. Thermal cycler conditions are listed in **Table 2.3**. Relative gene mRNA levels were

Table 2.2: Sequences of Real-time PCR Primers

Primer Name	Accession Number& Definition	Primer Pair Sequence 5'----3' (*Sense/Antisense*)	Location(*nt*)	Fragment Size (bp)	Annealing Temperature (^{o}C)
FOXO1A	NM_002015.2	GGG CCC TAA TTC GGT CAT GT	218-237	115	57
		TTG GGT CAG GCG GTT CAT AC	332-313		
HES1	NM_005524.2	GTC AAC ACG ACA CCG GAT AAA	297-317	351	57
		AGT GCG CAC CTC GGT ATT AAC	647-627		
Insulin	NM_000207.1	GCA TCA GAA GAG GCC ATC AAG C	4-25	178	59
		CGT TCC CCG CAC ACT AGG TA	181-162		
NGN3	NM_020999.2	AGC CGG CCT AAG AGC GAG TT	363-382	158	59
		TTG GTG AGC TTC GCG TCG TC	520-501		
NKX2-2	NM_002509.2	TGA CTC TCG GCT CCA CTA GG	1304-1323	295	59
		CGG CTG ACA ATA TCG CTA CTC A	1598-1577		
NKX6-1	NM_006168.1	ACA CGA GAC CCA CTT TTT CCG	719-739	336	59
		TGC TGG ACT TGT GCT TCT TCA AC	1054-1032		
PAX6	NM_000280.2	CCC GGC AGA AGA TTG TAG AG	586-605	323	57
		GCT AGC CAG GTT GCG AAG AA	908-889		
PDX-1	NM_000209.1	TGA TAC TGG ATT GGC GTT GT	1207-1226	270	53
		GCA TCA ATT TCA CGG GAT CT	1476-1457		
18S	M11188.1	GTA ACC CGT TGA ACC CCA TTC	1577-1597	153	57
		CCA TCC AAT CGG TAG TAG CG	1729-1710		

Table 2.3: Thermal Cycler Conditions (Number of cycles: 34)

Step	Time (s)	Temperature
Denaturation	30	94°C
Annealing	15	53-59°C
Extension	30	72°C

*Annealing temperature is approximately 5°C below Tm of primers. Simplified formula for estimating melting temperature (T_m): T_m= 2°C x (A+T) + 4°C x (G+C)

calculated using the arithmetic formula "$2^{-\Delta\Delta C}{}_T$", where $^{\Delta}$CT is the difference between the threshold cycle of a given target cDNA and the internal standard gene 18S rRNA subunit cDNA, with at least five repeats per age or experimental group. The melting curves were used to indicate if the qRT-PCR assays had amplified target sequences with excellent specificity (**Supplementary Figure 4**). Controls involved omitting reverse transcriptase, cDNA or DNA polymerase and showed no reaction bands (**Supplementary Figure 3**) (Wang *et al.,* 2005).

2.8. Statistical Analysis

Data were expressed as means ± SEM. Statistical significance was determined using either the paired Student's *t*-test or one-way ANOVA followed by the Post-Hoc Bonfferroni comparison test. GraphPad prism version 4 was used (GraphPad software; La Jolla, CA, USA). Differences were considered to be statistically significant when $p<$ 0.05.

Chapter 3

Results

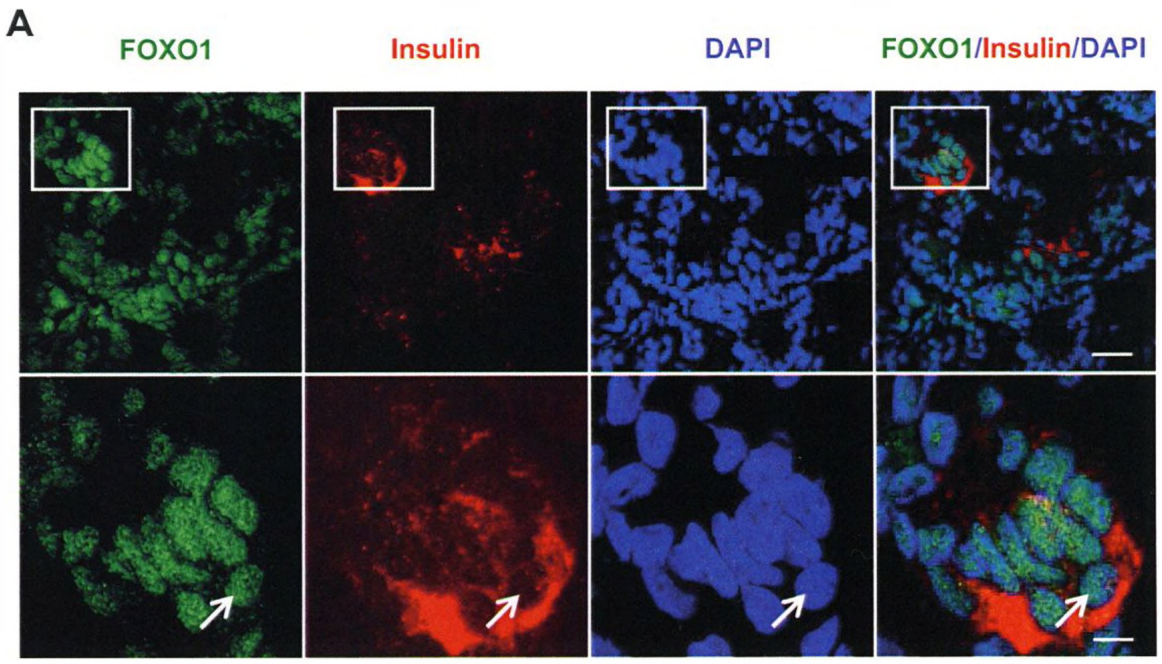
Chapter 3 - **Results**

3.1. **Characterization of nFOXO1 Expression in the Developing Human Fetal Pancreas.**

To determine whether FOXO1 cellular distribution is restricted to a specific spatial/temporal pattern during islet cell development in the human fetal pancreas, I examined the levels of nuclear FOXO1 (nFOXO1) protein in both ductal ($CK19^+$) and islet endocrine ($insulin^+$ or $glucagon^+$) cells, within human fetal pancreata isolated during 8 to 21 weeks of fetal age. Pancreatic samples were categorized into three developmental stages: (I) early (8 to 12 weeks of fetal age) when pancreata contain numerous undifferentiated $PDX\text{-}1^+$ ductal cells with a few scattered single endocrine cells; (II) middle (14 to 16 weeks) when islet clusters assemble, and (III) late (18 to 21 weeks) when adult-like islet structures appear with associated capillary networks (Lyttle *et al.*, 2008). Double immunostaining revealed that nFOXO1 localization within pancreatic cells was apparent from the earliest developmental stage studied (8 wks), not only within newly formed single endocrine ($insulin^+$ or $glucagon^+$) cells budding off from the ducts (**Figure 3.1; Figure 3.2**) but also within the ductal epithelium (**Figure 3.3**). Morphometric analysis showed that the majority of cytokeratin 19^+ (CK19) cells (>80%) stained positive with nFOXO1 (**Figure 3.3B**). As the islets developed it was also apparent that many single insulin- or glucagon-positive cells or endocrine cells in small clusters localized with nFOXO1 (**Figure 3.1A; Figure 3.2A**). A high proportion of nFOXO1 and insulin (>80%) localization was observed with no changes in the co-localization level throughout all three developmental stages (8 to 21 wks) (Figure 3.1B). A similar co-localization pattern of nFOXO1 with glucagon (>80%) was observed (Figure 3.2B).

Figure 3.1. Co-localization of nFOXO1 with Insulin during Human Fetal Pancreatic Development.

Human fetal pancreatic sections (frozen) from three developmental stages (8-12 wks, 14-16 wks, and 18-21 wks) were co-stained with anti-FOXO1 and anti-insulin antibodies. The anti-FOXO1 antibody was visualized with FITC-conjugated anti-rabbit IgG (green), and the anti-insulin with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section is shown. **(A)** The top panel of images shows double immunostaining for FOXO1, insulin, DAPI, and an overlay of the images as stated in a human fetal pancreas section at 14 wks. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a $nFOXO1^+/insulin^+$ cell. **(B)** Quantitative analysis of $nFOXO1^+/insulin^+$ cells relative to the total number of $insulin^+$ cells counted. Data were determined by morphometric analysis and are expressed as means ± SEM (n = 3-6 pancreata per age group).


A
FOXO1
Insulin
DAPI
FOXO1/Insulin/DAPI

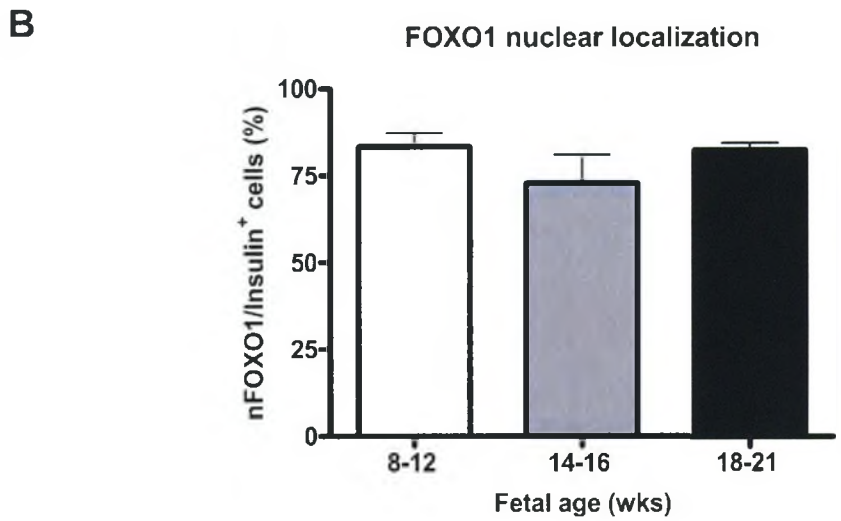

B
FOXO1 nuclear localization
nFOXO1/Insulin+ cells (%)
100
75
50
25
0
8-12
14-16
18-21
Fetal age (wks)

Figure 3.1

Figure 3.2. Co-localization of nFOXO1 with Glucagon during Human Fetal Pancreatic Development.

Human fetal pancreatic sections (frozen) from three developmental stages (8-12 wks, 14-16 wks, and 18-21 wks) were co-stained with anti-FOXO1 and anti-glucagon antibodies. The anti-FOXO1 antibody was visualized with FITC-conjugated anti-rabbit IgG (green), and the anti-glucagon with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section is shown. **(A)** The top panel of images shows double immunostaining for FOXO1, glucagon, DAPI, and an overlay of the images as stated in a human fetal pancreas section at 16 wks. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a $nFOXO1^{+}$/$glucagon^{+}$ cell. **(B)** Quantitative analysis of $nFOXO1^{+}$/$glucagon^{+}$ cells relative to the total number of $glucagon^{+}$ cells counted. Data were determined by morphometric analysis and are expressed as means ± SEM (n = 3-6 pancreata per age group).

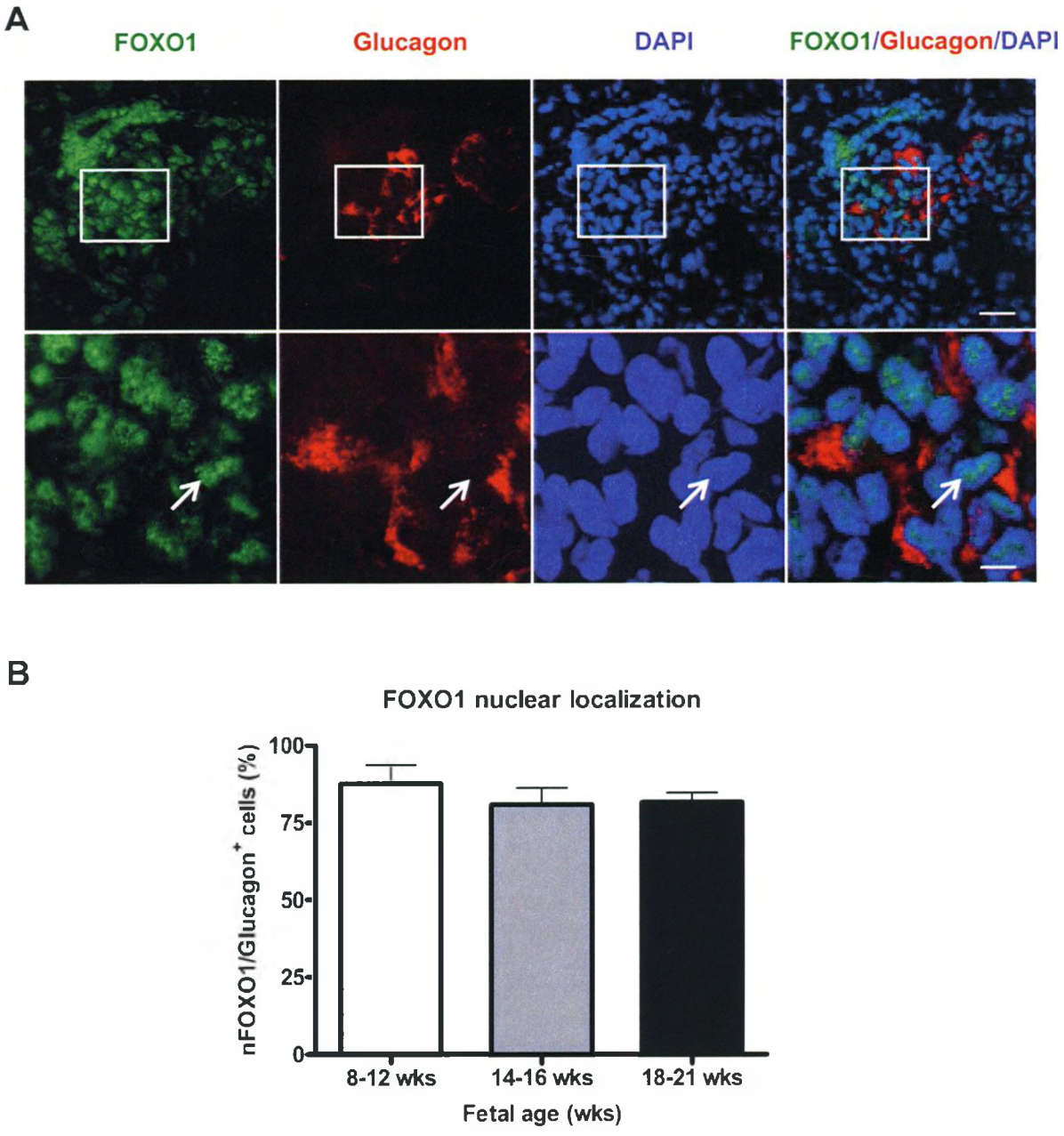
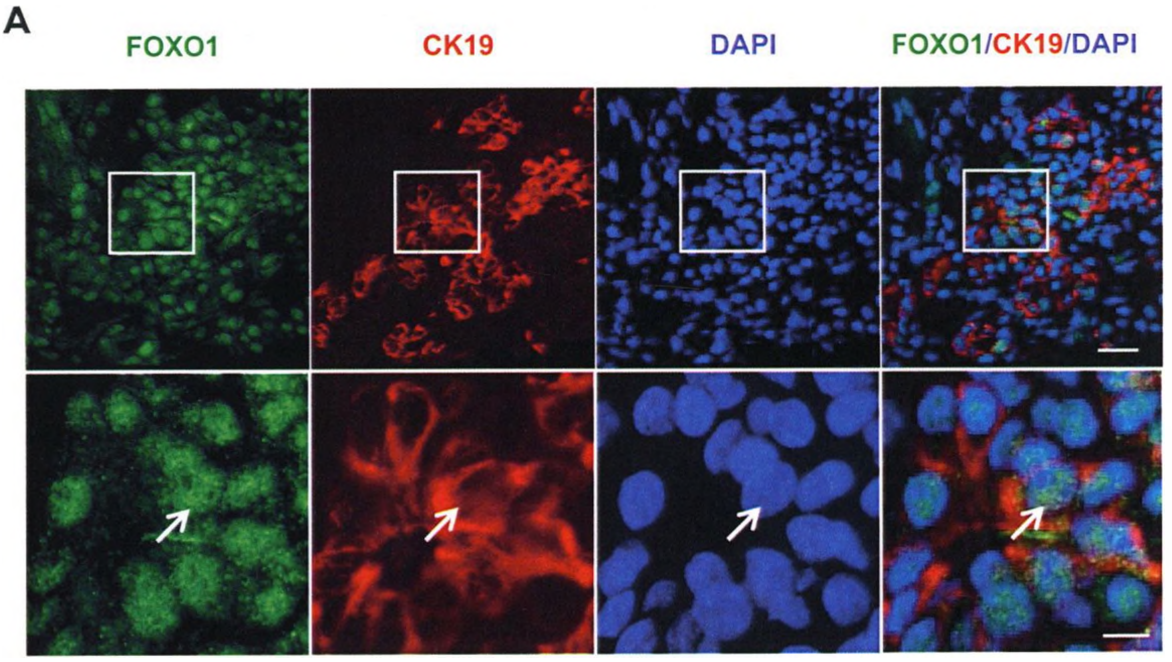

A
FOXO1
Glucagon
DAPI
FOXO1/Glucagon/DAPI
B
FOXO1 nuclear localization
nFOXO1/Glucagon+ cells (%)
100
75
50
25
0
8-12 wks
14-16 wks
18-21 wks
Fetal age (wks)

Figure 3.2

Figure 3.3. Co-localization of nFOXO1 with CK19 during Human Fetal Pancreatic Development.

Human fetal pancreatic sections (frozen) from three developmental stages (8-12 wks, 14-16 wks, and 18-21 wks) were co-stained with anti-FOXO1 and anti-CK19 antibodies. The anti-FOXO1 antibody was visualized with FITC-conjugated anti-rabbit IgG (green), and the anti-CK19 with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section is shown. **(A)** The top panel of images shows double immunostaining for FOXO1, CK19, DAPI, and an overlay of the images as stated in a human fetal pancreas section at 14 wks. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a $nFOXO1^{+}/CK19^{+}$ cell. **(B)** Quantitative analysis of $nFOXO1^{+}/CK19^{+}$ cells relative to the total number of $CK19^{+}$ cells counted. Data were determined by morphometric analysis and are expressed as means ± SEM (n = 3-6 pancreata per age group).


A
FOXO1
CK19
DAPI
FOXO1/CK19/DAPI

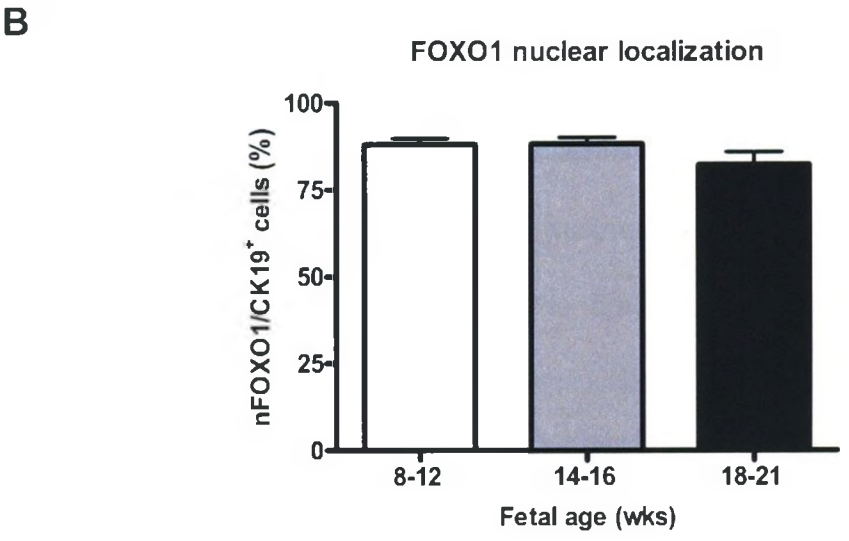

B
FOXO1 nuclear localization
nFOXO1/CK19+ cells (%)
100
75
50
25
0
8-12
14-16
18-21
Fetal age (wks)

Figure 3.3

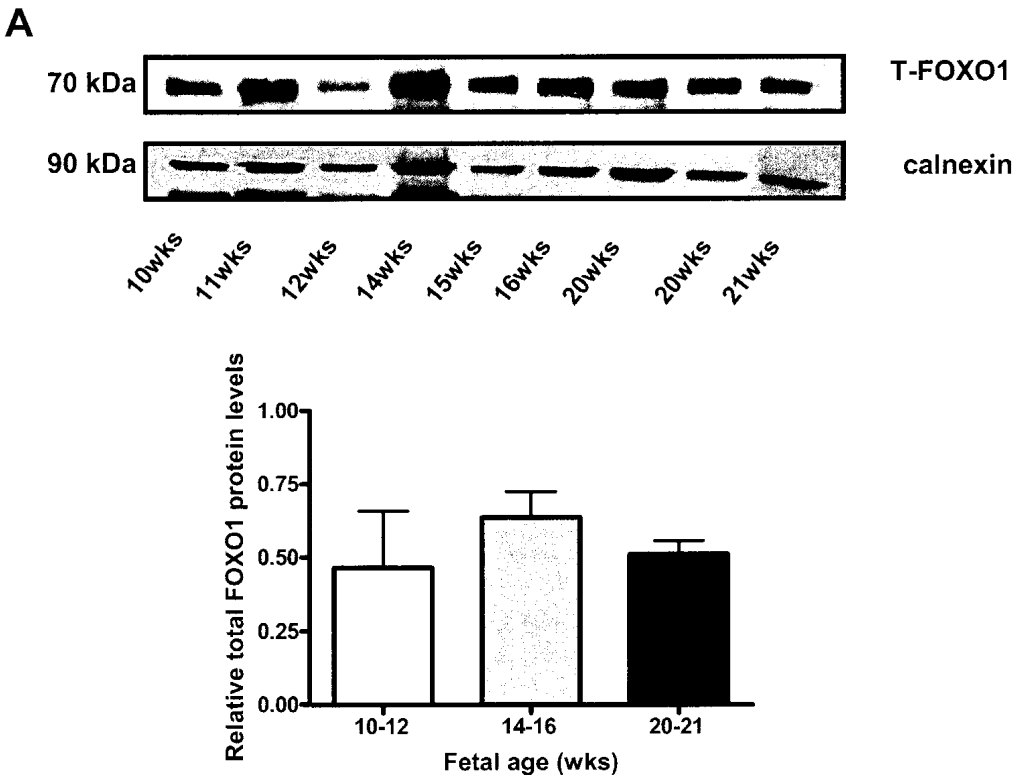
Total FOXO1 protein levels were quantified by western blot analysis of intact pancreata, and showed no changes from 8 to 21 weeks of development (**Figure 3.4A**). Finally, analysis of total *FOXO1* mRNA levels of intact pancreata by qRT-PCR showed no changes during 8 to 16 weeks of development, and a 2-fold increase during 18 to 21 weeks of development (**Figure 3.4B**).

3.2. Co-localization of nFOXO1 with Transcription Factors in the Developing Human Fetal Pancreas.

To investigate the repressive relationship observed in adult islet β-cells between FOXO1 and PDX-1, I next analyzed the subcellular distribution of FOXO1 in PDX-1 positive cells throughout 8 to 21 weeks of human fetal pancreatic development. Double immunostaining for FOXO1 and PDX-1 displayed co-stained nuclei (**Figure 3.5A**), and morphometric analysis revealed that approximately 50% of PDX-1$^+$ cells co-localized with nFOXO1 throughout the examined developmental period (8 to 21 wks) (**Figure 3.5B**). To understand the functional role of FOXO1 in islet cell assembly and development, double immunostaining was performed for nFOXO1 with NGN3. NGN3 is highly expressed during early human fetal pancreatic development, and is required for the development of all endocrine lineages in the mouse (**Figure 3.6A**) (Habener *et al.*, 2005; Lyttle *et al.*, 2008). According to morphometric analysis, nFOXO1 co-localized with more than 80% of NGN3$^+$ cells, which represent a population of islet progenitors, at a constant level during all three stages of human fetal pancreatic development (8 to 21 wks) (**Figure 3.6B**).

Figure 3.4. Total Protein and mRNA Levels of FOXO1 during Human Fetal Pancreatic Development.

(A) Western blot analysis of total FOXO1 (molecular weight of bands 70 kDa) and calnexin (molecular weight of bands 90 kDa) protein levels in human fetal pancreatic tissues during three developmental stages (10-12 wks, 14-16 wks, and 20-21 wks). Densitometric quantifications were performed. Data were normalized to calnexin and are expressed as means ± SEM. A blot of $n = 3$ pancreata per age group is displayed. **(B)** qRT-PCR analysis of total *FOXO1* mRNA levels in human fetal pancreatic tissues during three developmental stages (8-12 wks, 14-16 wks, and 18-21 wks). Data were normalized to 18S rRNA and are expressed as means ± SEM ($n = 5$ pancreata per age group).


A
70 kDa
T-FOXO1
90 kDa
calnexin
10wks
11wks
12wks
14wks
15wks
16wks
20wks
20wks
21wks
Relative total FOXO1 protein levels
1.00
0.75
0.50
0.25
0.00
10-12
14-16
20-21
Fetal age (wks)

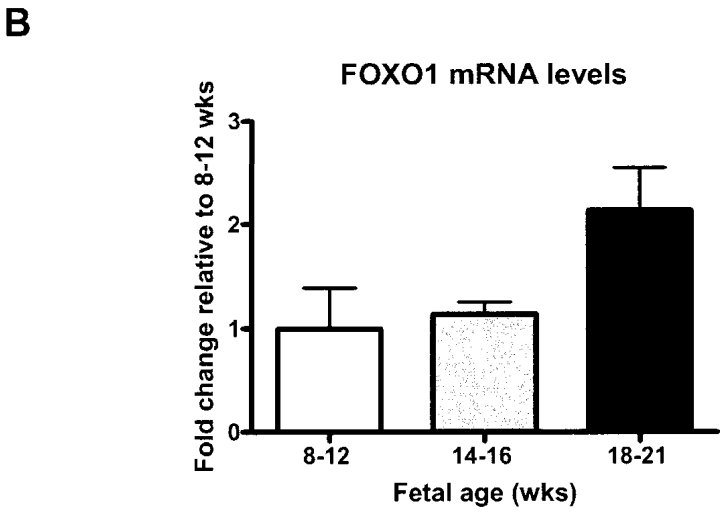
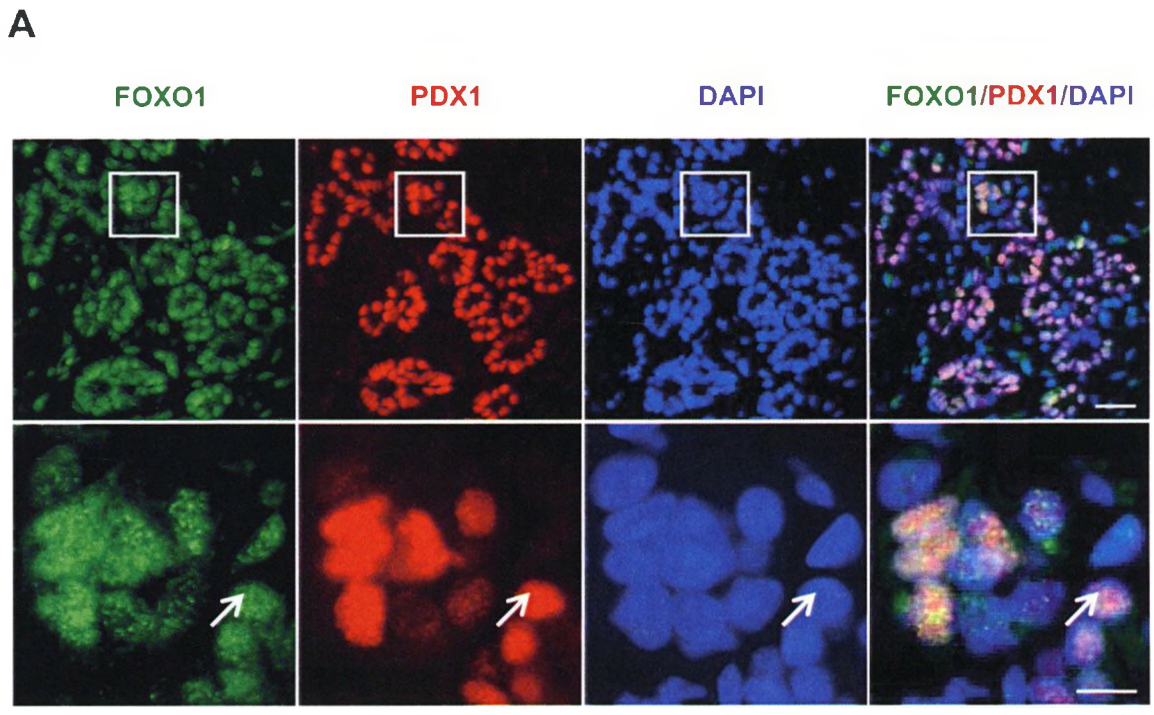

B
FOXO1 mRNA levels
Fold change relative to 8-12 wks
3
2
1
0
8-12
14-16
18-21
Fetal age (wks)

Figure 3.4

Figure 3.5. Co-localization of nFOXO1 with PDX-1 during Human Fetal Pancreatic Development.

Human fetal pancreatic sections (paraffin) from three developmental stages (8-12 wks, 14-16 wks, and 18-21 wks of fetal age) were co-stained with anti-FOXO1 and anti-PDX-1 antibodies. The anti-FOXO1 antibody was visualized with FITC-conjugated anti-rabbit IgG (green) and the anti-PDX-1 antibody with TRITC-conjugated anti-guinea pig IgG (red). Nuclei were stained with DAPI (blue). A representative section is shown. **(A)** The top panel of images shows double immunostaining for FOXO1, PDX-1, DAPI, and an overlay of the images as stated in a human fetal pancreas section at 18 wks. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Arrows indicate a $nFOXO1^{+}/PDX\text{-}1^{+}$ cell. Scale bar corresponds to 10 μm. **(B)** Quantitative analysis of $nFOXO1^{+}/PDX\text{-}1^{+}$ cells relative to the total number of $PDX\text{-}1^{+}$ cells counted. Data were determined by morphometric analysis and are expressed as means ± SEM (n = 3-4 pancreata per age group).


A
FOXO1
PDX1
DAPI
FOXO1/PDX1/DAPI

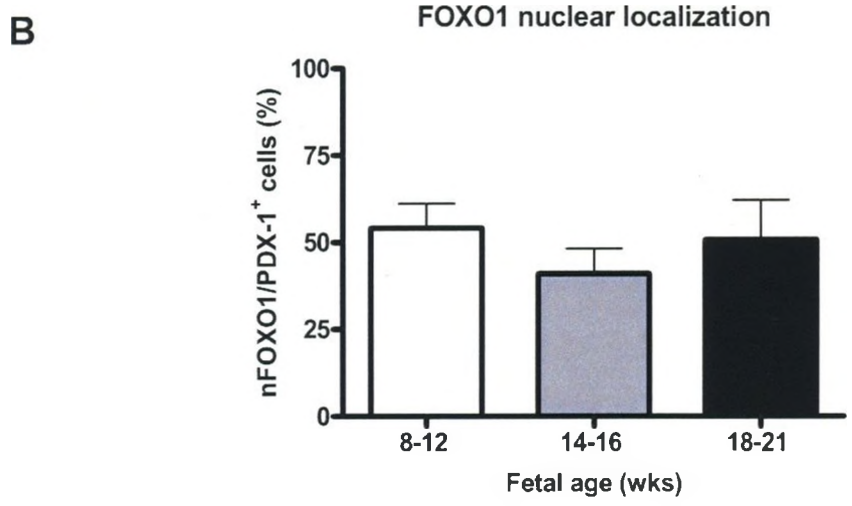

B
FOXO1 nuclear localization
nFOXO1/PDX-1+ cells (%)
100
75
50
25
0
8-12
14-16
18-21
Fetal age (wks)

Figure 3.5

Figure 3.6. Co-localization of nFOXO1 with NGN3 during Human Fetal Pancreatic Development.

Human fetal pancreatic sections (frozen) from three developmental stages (8-12 wks, 14-16 wks, and 18-21 wks of fetal age) were co-stained with anti-FOXO1 and anti-NGN3 antibodies. The anti-FOXO1 antibody was visualized with FITC-conjugated anti-rabbit IgG (green) and the anti-NGN3 antibody with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section is shown. **(A)** The top panel of images shows double immunostaining for FOXO1, NGN3, DAPI, and an overlay of the images as stated in a human fetal pancreas section at 14 wks. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Arrows indicate a $nFOXO1^{+}$/NGN3 cell. Scale bar corresponds to 10 μm. **(B)** Quantitative analysis of $nFOXO1^{+}/NGN3^{+}$ cells relative to the total number $NGN3^{+}$ cells counted. Data were determined by morphometric analysis and are expressed as means ± SEM (n = 3-4 pancreata per age group).

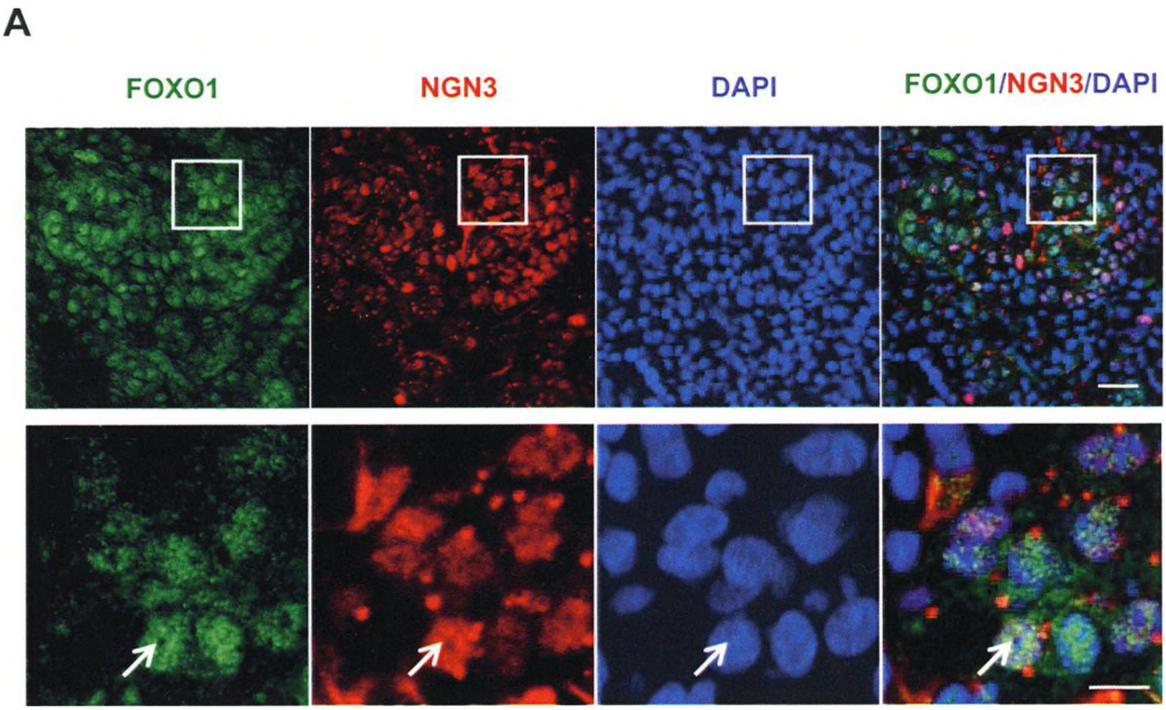

A
FOXO1
NGN3
DAPI
FOXO1/NGN3/DAPI
B
FOXO1 nuclear localization
nFOXO1/NGN3+ cells (%)
100
75
50
25
0
8-12
14-16
18-21
Fetal age (wks)

Figure 3.6

3.3. Co-localization Pattern of nFOXO1 with Proliferating (Ki-67$^+$) Cells in the Developing Human Fetal Pancreas.

The majority of studies have characterized FOXO1 as having a negative role during cell proliferation in various cells and tissues (Greer and Brunet; 2005; Huang and Tindall, 2007; Tran *et al.*, 2003). To begin to investigate if FOXO1 is a negative regulator of cell proliferation during human fetal pancreas development, FOXO1 co-localization with Ki-67, a proliferative index, was assessed (Scholzen and Gerdes, 2000). Double immunostaining revealed that nFOXO1 co-localized with Ki-67$^+$ cells during development (8 to 21 wks) (**Figure 3.7A**). Morphometric analysis showed a high proportion of Ki-67$^+$ cells co-stained with nFOXO1 during early (8 to 12 wks) development (68 ± 6.5%), followed by a significant decrease in middle (14 to 16 wks) (31 ± 5.7%, $p<0.01$ vs. early) and late (18 to 21 wks) stages (22 ± 4.2%, $p<0.001$ vs. early) of human fetal pancreatic development (**Figure 3.7B**).

3.4. The Effects of Insulin and Glucose on the Subcellular Localization of FOXO1 in Islet-Epithelial Cell Clusters.

In most cell types FOXO1 shuttles between the nucleus and cytoplasm, a well established regulatory mechanism that is regulated mainly through the PI3-Kinase/AKT signaling pathway (Martinez *et al.*, 2006). I sought to determine if growth factors can induce a change in the subcellular localization of FOXO1 in the developing human fetal pancreas. Islet-epithelial cell clusters, isolated from 18 to 21 weeks of fetal age, were challenged with increasing concentrations of either insulin or glucose (to induce endogenous release of insulin), and cultured for 24 hr. However, changes in the

Figure 3.7. Co-localization of nFOXO1 with Proliferating Cells (Ki-67$^+$) During Human Fetal Pancreatic Development.

Human fetal pancreatic sections (frozen) from three developmental stages (8-12 wks, 14-16 wks, and 18-21 wks of fetal age) were co-stained with anti-FOXO1 and anti-Ki-67 antibodies. The anti-FOXO1 antibody was visualized with FITC-conjugated anti-rabbit IgG (green), and the anti-Ki-67 antibody with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section is shown. **(A)** Top panel of images shows double immunostaining for FOXO1, Ki-67, DAPI, and an overlay of the images as stated in a human fetal pancreas section at 12 wks. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Arrows indicate a nFOXO1$^+$/Ki-67$^+$cell. Scale bar corresponds to 10 μm. **(B)** Quantitative analysis of nFOXO1$^+$/Ki-67$^+$ cells relative to the total number of Ki-67$^+$ cells counted. Data were determined by morphometric analysis and are expressed as means ± SEM (n = 3-6 pancreata per age group). Asterisks indicate $^{**}p< 0.01$, $^{***}p< 0.001$ vs. respective early group by one-way ANOVA.

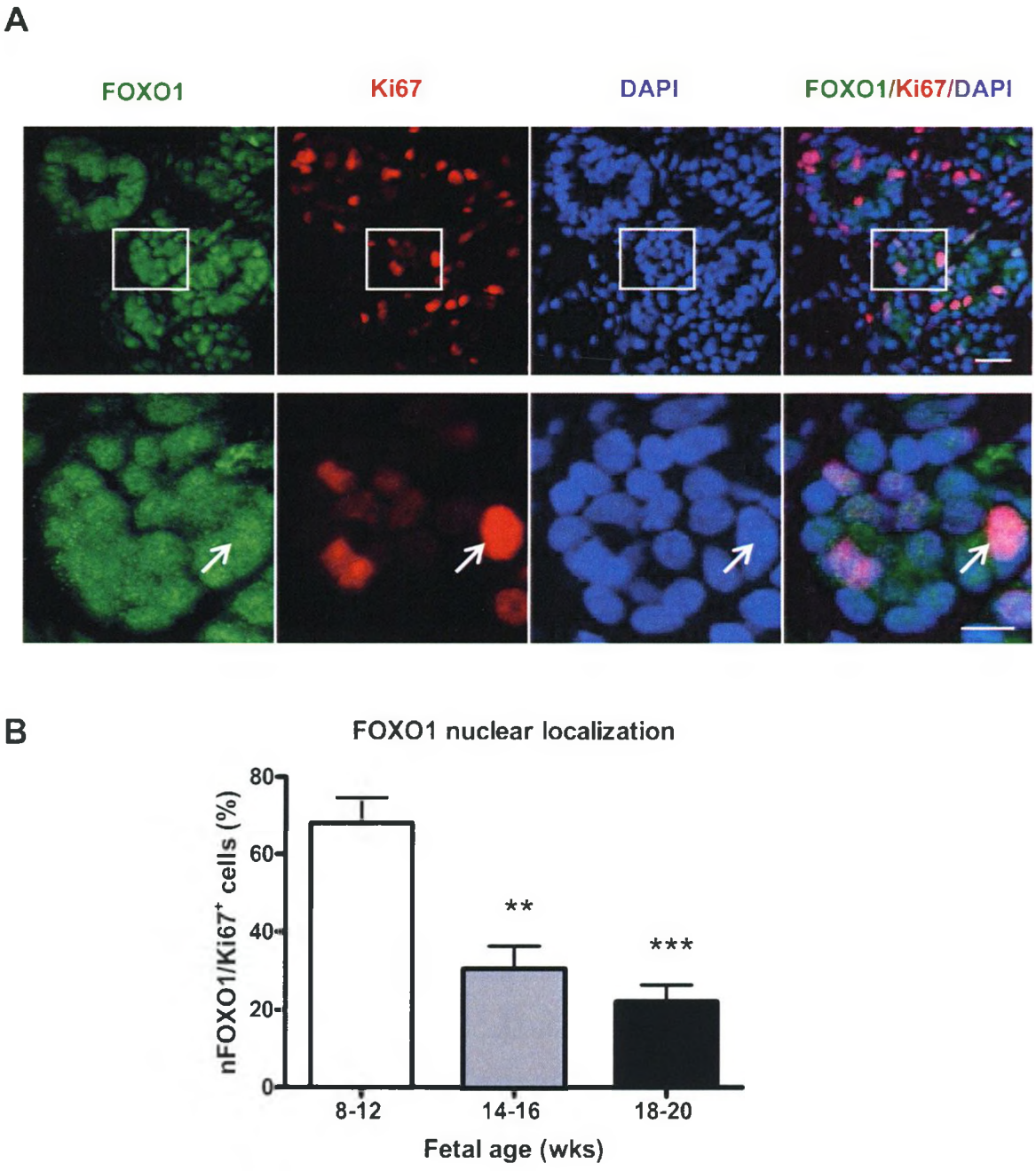

A
FOXO1
Ki67
DAPI
FOXO1/Ki67/DAPI
B
FOXO1 nuclear localization
nFOXO1/Ki67+ cells (%)
80
60
40
20
0
**
***
8-12
14-16
18-20
Fetal age (wks)

Figure 3.7

subcellular localization of FOXO1 within ductal cells ($CK19^+$ cells), which make up the majority of islet-epithelial cell clusters (Li *et al.*, 2006), was observed only when the cell clusters were treated with at least a 50 nM concentration of insulin (55 ± 2.8%, $p<0.01$ vs. control 76.2 ± 2.5%) (**Figure 3.8A**), with no prominent effects in the cells treated with glucose (**Figure 3.8B**). Insulin-induced FOXO1 nuclear reduction was significantly enhanced when the cells were exposed to a higher insulin concentration (100 nM) (50 ± 1.2%, $p<0.001$ vs. control 76.2 ± 2.5%) (**Figure 3.8A**). Consistent with the results obtained from ductal cells, FOXO1 nuclear localization with β-cells ($C\text{-}Peptide^+$ cells), which make up a small cellular population of islet-epithelial cell clusters (Li *et al.*, 2006), was also significantly reduced by >1.5-fold ($p<0.05$ vs. glucose) when treated with insulin (50 nM), but not glucose (25 mM) following a 24 hr culture period (**Figure 3.8C**). These data suggest that insulin may influence the function of FOXO1, by affecting its subcellular localization, in the developing human fetal pancreas.

3.5. Insulin Influences FOXO1 Nuclear Localization via the PI3-Kinase/AKT Signaling Pathway in Human Fetal Islet-Epithelial Cell Clusters.

The nuclear export of FOXO1 is mediated primarily through the PI3-Kinase/AKT signaling pathway (Brunet *et al.*, 1999). Similar alterations in the PI3-Kinase pathway were observed in the present study; insulin stimulation resulted in more than a 3-fold increase in AKT phosphorylation in comparison with control groups (**Figure 3.9A**). In order to verify that the PI3-Kinase signaling cascade is responsible for the insulin-induced reduction of nFOXO1 localization, human fetal islet-epithelial cell clusters were pre-treated with wortmannin (100 nM), a competitive inhibitor of

Figure 3.8. Concentration Effects of Insulin or Glucose on the Subcellular Distribution of Endogenous FOXO1 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters, isolated from human fetal pancreata (18-21 wks of fetal age) were cultured with insulin (0, 10, 25, 50 or 100 nM) or glucose (5.5, 10, 25 mM) for 24 hr. Cells were then harvested and co-stained with anti-FOXO1 and anti-CK19, or anti-C-peptide antibodies. Nuclear localization of endogenous FOXO1 in response to insulin or glucose was quantified by morphometric analysis. **(A)** Quantitative analysis of $nFOXO1^{+}/CK19^{+}$ cells relative to the total number of $CK19^{+}$ cells counted treated with the indicated concentrations of insulin. Data are expressed as means ± SEM (n = 3 pancreata per experimental group). Asterisks indicate $^{**}p< 0.01$, $^{***}p< 0.001$ vs. control group by one-way ANOVA. **(B)** Quantitative analysis of $nFOXO1^{+}/CK19^{+}$ cells relative to the total number of $CK19^{+}$ cells counted treated with the indicated concentrations of glucose. Data are expressed as means ± SEM (n = 3 pancreata per experimental group). **(C)** Quantitative analysis of $nFOXO1^{+}$/C-peptide^{+} cells relative to the total number of C-peptide^{+} cells counted in the indicated treatment (control, glucose (25 mM), or insulin (50 nM)). Data were normalized to control and are expressed as means ± SEM (n = 3 pancreata per experimental group). Asterisk indicates $^{*}p< 0.05$ vs. glucose treatment by one-way ANOVA.

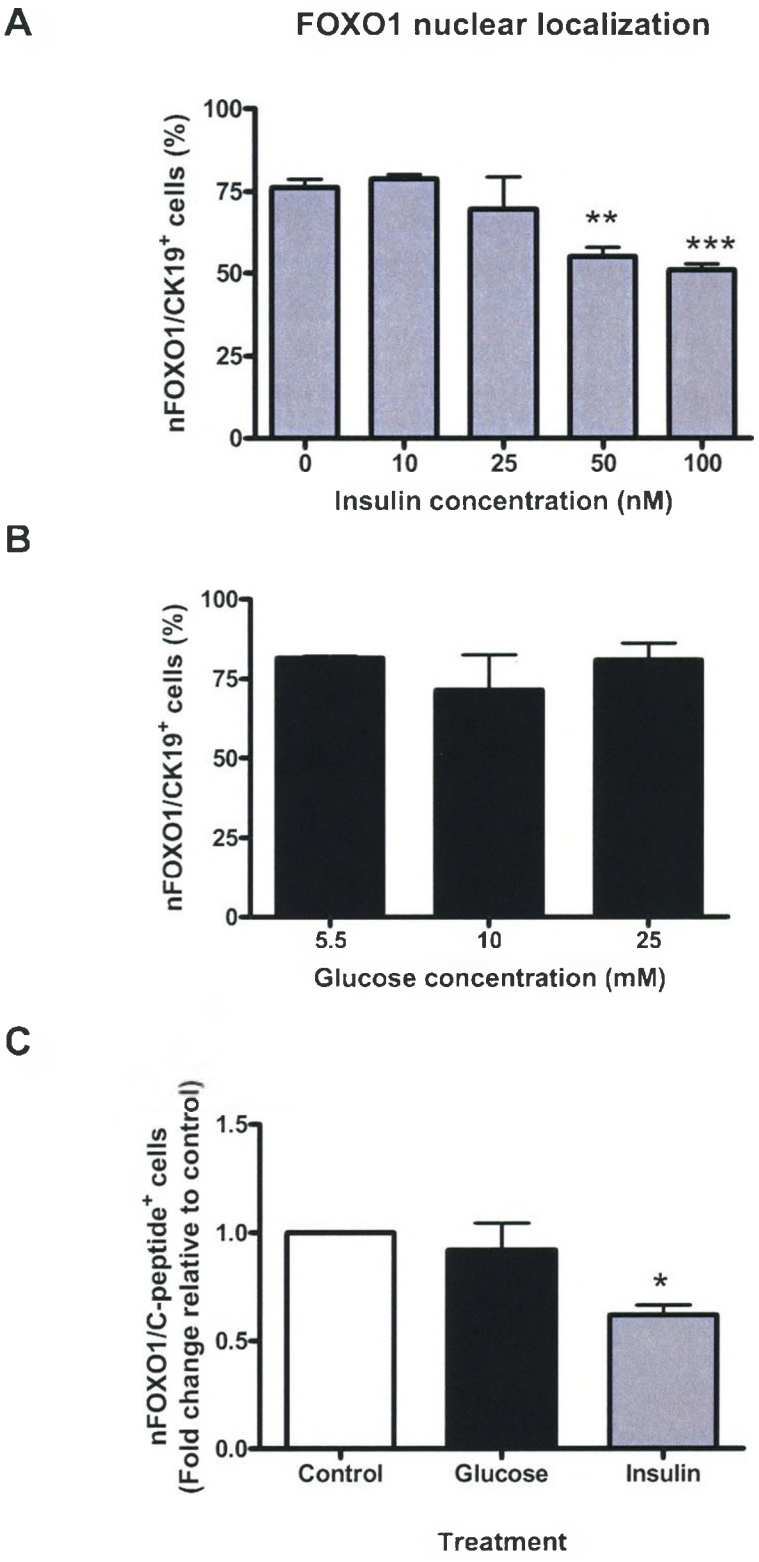
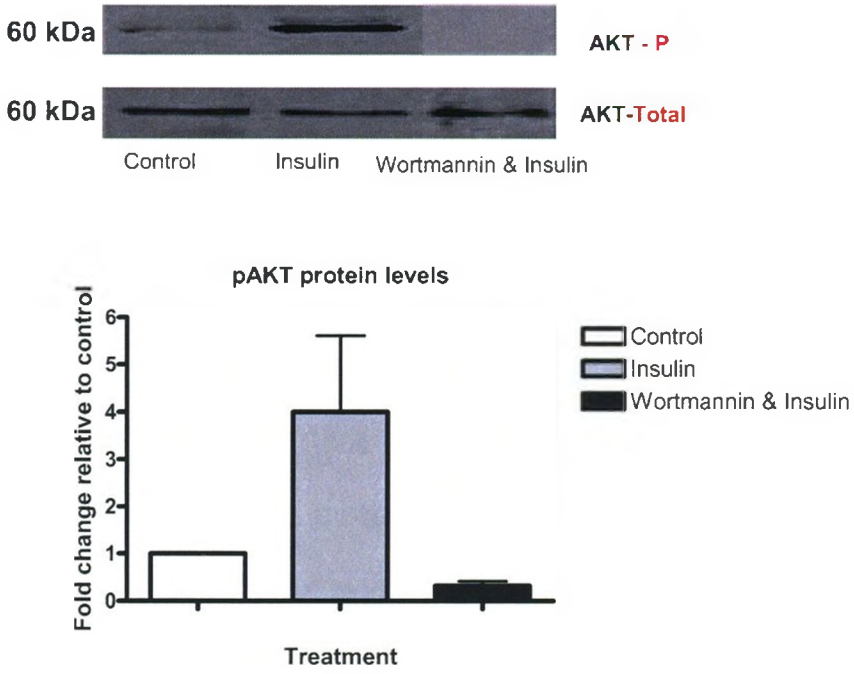

A
FOXO1 nuclear localization
nFOXO1/CK19+ cells (%)
100
75
50
25
0
**
***
0
10
25
50
100
Insulin concentration (nM)
B
nFOXO1/CK19+ cells (%)
100
75
50
25
0
5.5
10
25
Glucose concentration (mM)
C
nFOXO1/C-peptide+ cells
(Fold change relative to control)
1.5
1.0
0.5
0.0
*
Control
Glucose
Insulin
Treatment

Figure 3.8

Figure 3.9. Effect of Insulin and Wortmannin on AKT Protein Levels and nFOXO1 Cellular Distribution in Islet-Epithelial Cell Clusters.

(A) Western blot analysis of total phosphorylated AKT (AKT-p) (molecular weight of band 60 kDa) and total AKT (molecular weight of band 60 kDa) in islet-epithelial cell clusters treated with or without insulin (50 nM), or wortmannin (100 nM) and insulin (50 nM) for 24 hr. Densitometric quantifications were performed. Data were normalized to total AKT and are expressed as means ± SEM. A representative blot of $n = 3$ per experimental group is displayed. **(B)** Quantitative analysis of $nFOXO1^{+}$/C-$peptide^{+}$ cells relative to the total number of C-$peptide^{+}$ cells counted in each experimental group. Data were determined by morphometric analysis and are expressed as means ± SEM ($n = 4$ per experimental group). Asterisk indicates $*p < 0.05$ vs. wortmannin/insulin group by one-way ANOVA.

A


60 kDa
AKT - P
60 kDa
AKT-Total
Control
Insulin
Wortmannin & Insulin
pAKT protein levels
Fold change relative to control
0
1
2
3
4
5
6
Treatment
Control
Insulin
Wortmannin & Insulin

B

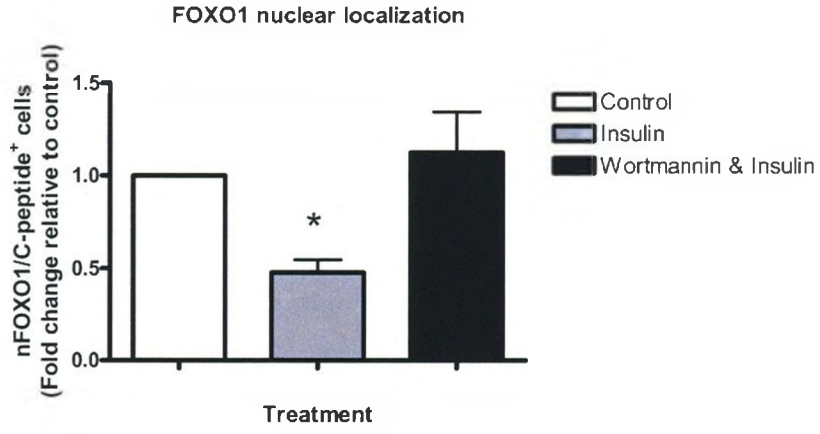

FOXO1 nuclear localization
nFOXO1/C-peptide+ cells
(Fold change relative to control)
0.0
0.5
1.0
1.5
*
Treatment
Control
Insulin
Wortmannin & Insulin

Figure 3.9

PI3-Kinase (Powis *et al.*, 1994). Double immunostaining (**Figure 3.10ABC**) and morphometric analyses (**Figure 3.9B**) revealed that the insulin-induced reduction in FOXO1 nuclear localization was inhibited by wortmannin blockage of PI3-Kinase signaling in β-cells (C-peptide^{+}) as more cells co-stained with nFOXO1 (**Figure 3.9B; Figure 3.10C**). Furthermore, western blot analysis showed a decrease in phosphorylated AKT upon wortmannin treatment (**Figure 3.9A**). These results suggest that the insulin-induced reduction in FOXO1 nuclear localization within human fetal islet-epithelial cell clusters is in part PI3-Kinase/AKT dependent.

3.6. Knockdown in *FOXO1* mRNA Levels Promotes β-Cell Differentiation in Human Fetal Islet-Epithelial Cell Clusters.

Using specific human *FOXO1* small interfering (si) RNAs, I examined the effects of down-regulating FOXO1 expression on β-cell development in isolated human fetal islet-epithelial cell clusters. Following transient transfection of *FOXO1* siRNA, qRT-PCR analysis was initially used to quantify knockdown levels of *FOXO1* mRNA in cell clusters following either 24 or 48 hr of culture. My results showed a significant 2-fold decrease (50%) in the levels of *FOXO1* mRNA 48 hr following the transfections, in comparison to both 24 hr treated and control groups ($p<0.01$, **Figure 3.11A**). Immunostaining revealed that several cell clusters treated with *FOXO1* siRNA were negative for nFOXO1 protein localization (**Figure 3.12B**) in comparison to cell clusters exposed to control siRNA, which stained positive for nFOXO1 (**Figure 3.12A**). Western

Figure 3.10. Effect of Insulin and Wortmannin on the Subcellular Distribution of Endogenous FOXO1 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters isolated from human fetal pancreata (18-21 wks of fetal age) were cultured for 24 hr in the absence or presence of insulin (50 nM), or islet cell clusters were pretreated for 1 hr with wortmannin (100 nM) before the addition of insulin (50 nM) to the medium. Cells were then harvested and co-stained (paraffin cell sections) with anti-FOXO1 and anti-C-peptide antibodies. The anti-FOXO1 antibody was visualized with FITC-conjugated anti-rabbit IgG (green), and the anti-C-peptide antibody with TRITC-conjugated anti-rat IgG (red). Nuclei were stained with DAPI (blue). A representative section from each experiment is shown. For each set the top panel of images shows double immunostaining for FOXO1, C-peptide, DAPI, and an overlay of the images as stated, of islet-epithelial cell clusters cultured in **(A)** control, **(B)** insulin, or **(C)** wortmannin & insulin. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Arrows indicate a $nFOXO1^{+}$/C-$peptide^{+}$cell, and dashed arrows indicate a $nFOXO1^{-}$/C-$peptide^{+}$ cell. Scale bar corresponds to 10 μm.

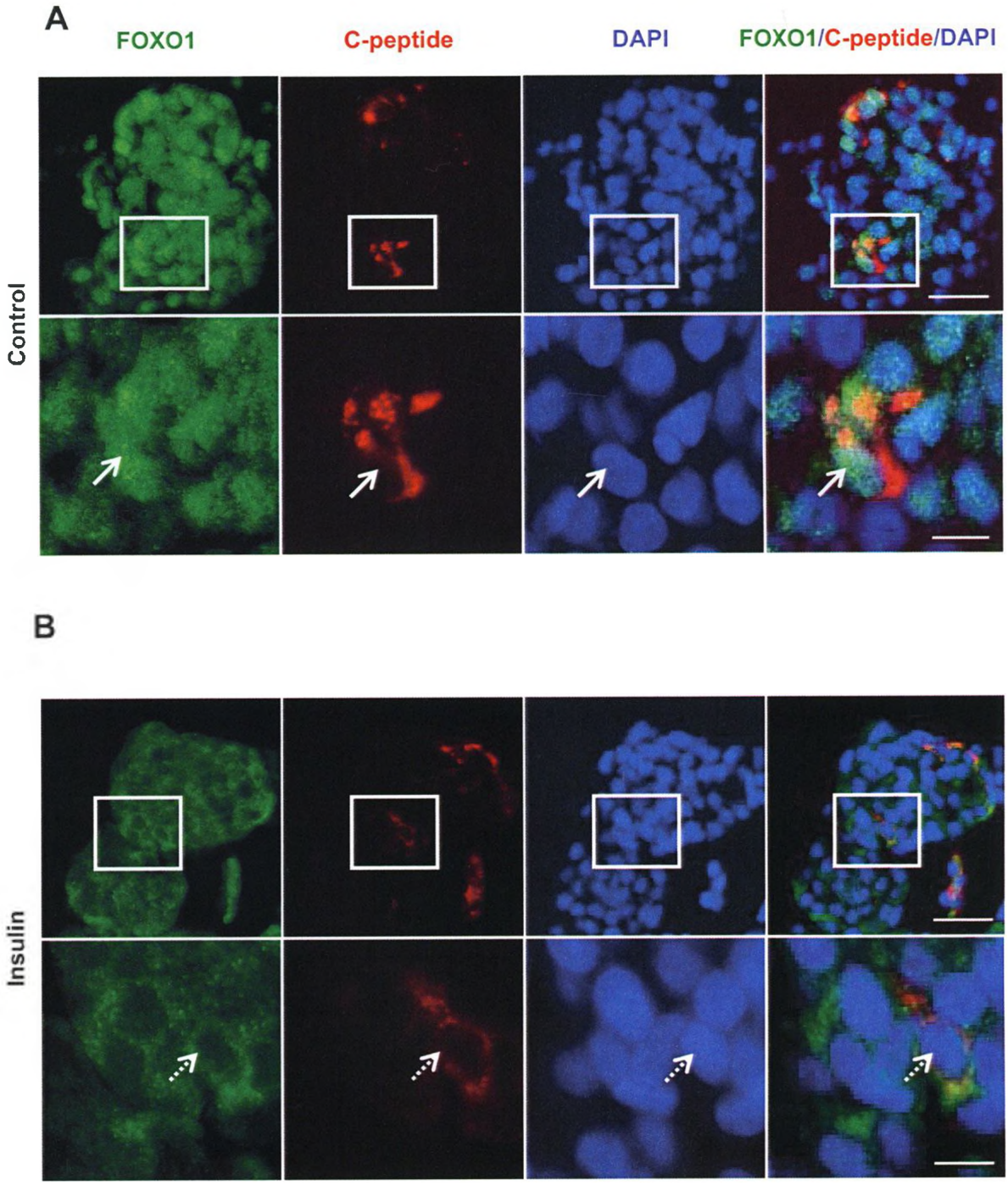

A
FOXO1
C-peptide
DAPI
FOXO1/C-peptide/DAPI
Control
B
Insulin

Figure 3.10

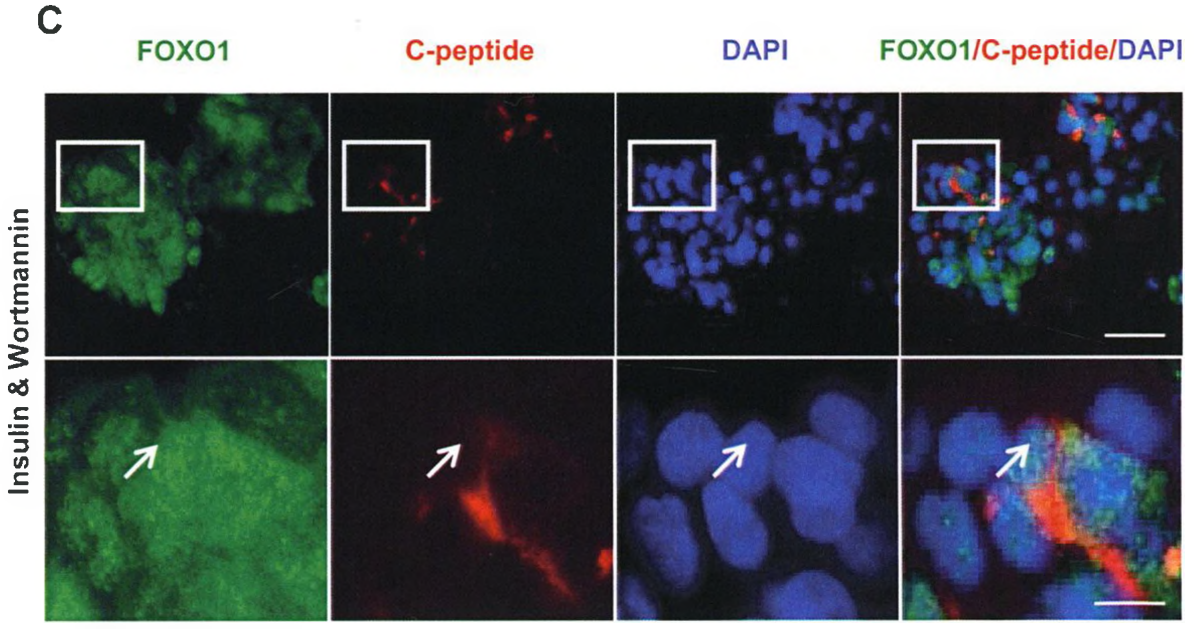
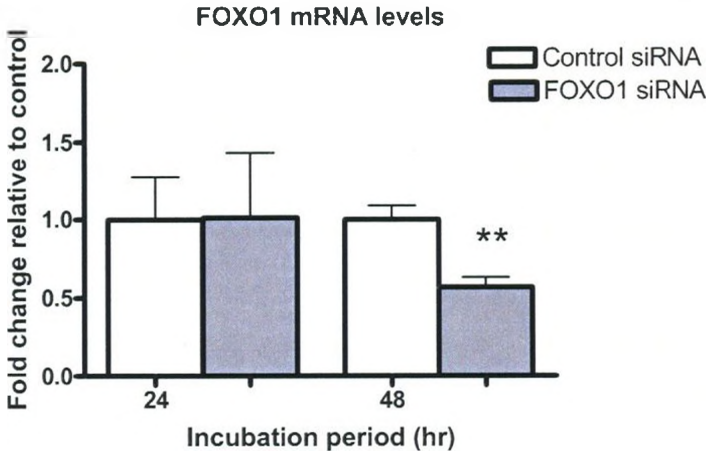

C
FOXO1
C-peptide
DAPI
FOXO1/C-peptide/DAPI
Insulin & Wortmannin

Figure 3.10

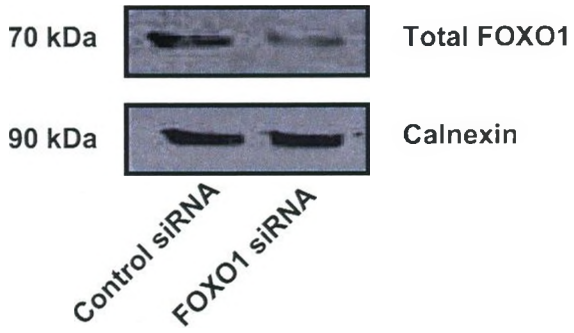
Figure 3.11. Effect of *FOXO1* siRNA on Total *FOXO1* mRNA and FOXO1 Protein Levels in Islet-Epithelial Cell Clusters.

(A) qRT-PCR analysis of total *FOXO1* mRNA expression in islet-epithelial cell clusters (18-21 wks) transfected with either control or *FOXO1* siRNA for 24 or 48 hr. Data were normalized to 18S rRNA and are expressed as means ± SEM (n = 6 per experimental group). Asterisks indicate $^{**}p<0.01$ vs. corresponding 48 hr control siRNA group by Student's t-test (paired). **(B)** Western blot analysis of total FOXO1 (molecular weight of bands is 70 kDa) and total calnexin (molecular weight of bands is 90 kDa) protein levels in islet-epithelial cell clusters treated with either control or *FOXO1* siRNA for 48 hr. Densitometric quantifications were performed. Data were normalized to total calnexin and are expressed as means ± SEM. A representative blot of n = 3 per experimental group is displayed.

A



B



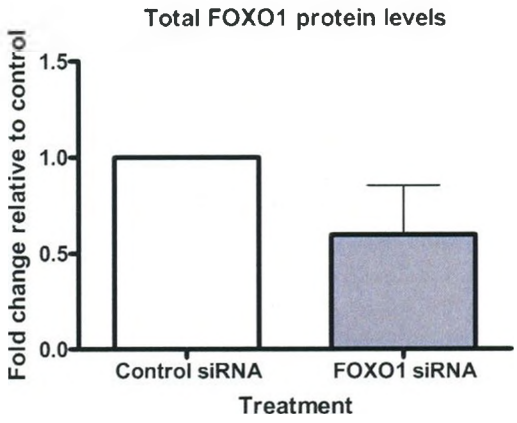


Figure 3.11

Figure 3.12. Effect of *FOXO1* siRNA on nFOXO1 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters isolated from human fetal pancreata (18-21 wks) were transfected with either control or *FOXO1* siRNA for 24 or 48 hr. Cells were then harvested and stained (paraffin cell sections) with anti-FOXO1 antibody, which was visualized with FITC-conjugated anti-rabbit IgG (green). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows immunostaining for FOXO1, DAPI, and an overlay of the images as stated. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Arrows indicate a $nFOXO1^{+}$ cell, and dashed arrows indicate a $nFOXO1^{-}$ cell. Scale bar corresponds to 10 μm.

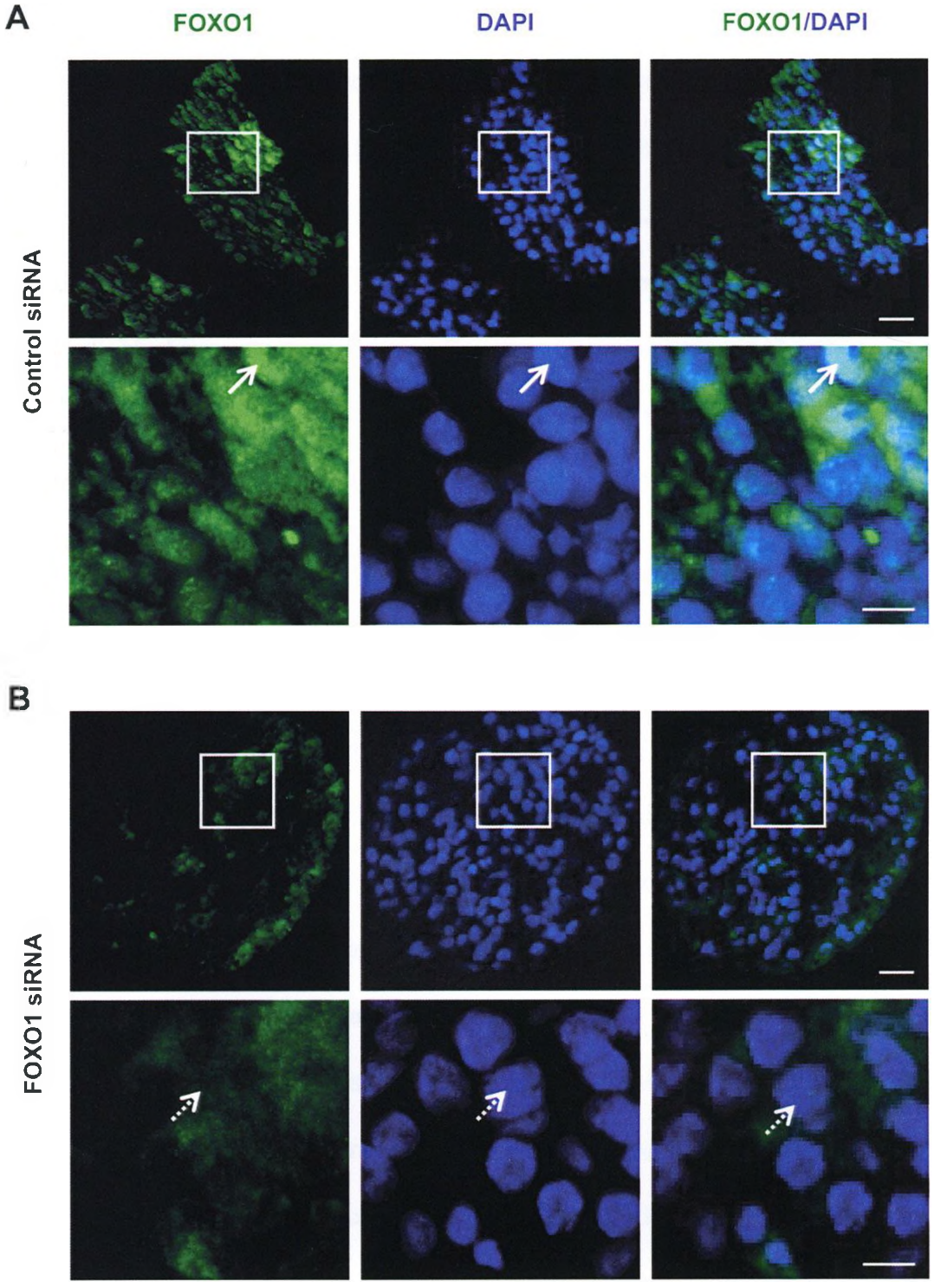


Figure 3.12

blot analysis further confirmed the decrease in FOXO1 protein levels in cell clusters transfected with *FOXO1* siRNA for 48 hr (**Figure 3.11B**). Interestingly, knockdown of *FOXO1* gene expression induced a significant 1.5-fold increase in the number of C-peptide$^+$ cells ($p<0.05$ vs. control) as determined by double immunostaining and morphometric analyses (**Figure 3.13BC**), as well as *insulin* mRNA levels ($p<0.001$ vs. control) as determined by qRT-PCR analysis (**Figure 3.13D**), in comparison to the control siRNA group (**Figure 3.13A**). However, the proliferative capacity of islet-epithelial cell clusters as determined by morphometric analysis of the Ki-67 labeling index, displayed no change (Figure 3.13E), with no co-staining observed between C-peptide$^+$ cells and Ki-67$^+$ in both treatment groups (**Figure 3.13AB**).

To analyze the influence of down-regulating FOXO1 on the levels of transcription factors that are critical for β-cell development, I examined the mRNA levels of key transcription factors associated with endocrine cell differentiation, following 48 hr of *FOXO1* and control siRNA transfections. Interestingly, I observed that the level of *NGN3* mRNA increased by approximately 2-fold as determined by qRT-PCR analysis in *FOXO1* siRNA transfected group in comparison to the control siRNA group (**Figure 3.14A**). This was associated with a significant 8-fold increase in the number of NGN3$^+$ cells as determined by immunostaining (**Figure 3.15AB**) and morphometric analyses ($p<0.01$, **Figure 3.14C**). An increase in the level of *NKX6-1* mRNA (**Figure 3.14B**) was also observed in *FOXO1* siRNA transfected cell clusters, that was accompanied with a significant 3-fold increase in the number of NKX6-1$^+$ cells as determined by immunostaining (**Figure 3.16AB**) and morphometric analysis ($p<0.05$, **Figure 3.14D**) in comparison to the control siRNA group. Furthermore, immunostaining and qRT-PCR

Figure 3.13. Effect of *FOXO1* siRNA on β-cell Differentiation and Proliferation in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters isolated from human fetal pancreata (18-21 wks) were transfected with either control or *FOXO1* siRNA for 48 hr. Cells were then harvested and co-stained (paraffin cell sections) with anti-Ki-67 and anti-C-peptide antibodies. The anti-Ki-67 antibody was visualized with FITC-conjugated anti-rabbit IgG (green), and the anti-C-peptide antibody with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA, or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows double immunostaining for FOXO1, Ki-67, DAPI, and an overlay of the images as stated. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Arrows indicate a C-peptide$^+$ cell, and dashed arrows indicate a Ki-67$^+$ cell. No co-staining is observed. Scale bar corresponds to 10 μm. Quantitative analysis of **(C)** C-peptide$^+$ cells, and **(E)** Ki-67$^+$ cells relative to the total number of C-peptide$^+$ or Ki-67$^+$ cells counted within islet-epithelial cell clusters transfected with either control or *FOXO1* siRNA for 48 hr. Data were determined by morphometric analysis and are expressed as means ± SEM (n = 4-5 per experimental group). Asterisk indicates *p< 0.05 vs. control siRNA group by the Student's t-test (paired). **(D)** qRT-PCR analysis of total *insulin* mRNA levels in islet-epithelial cell clusters transfected with either control or *FOXO1* siRNA for 48 hr. Data were normalized to 18S rRNA and are expressed as means ± SEM (n = 5 per experimental group). Asterisks indicate ***p< 0.001 vs. control siRNA group by the Student's t-test (paired).

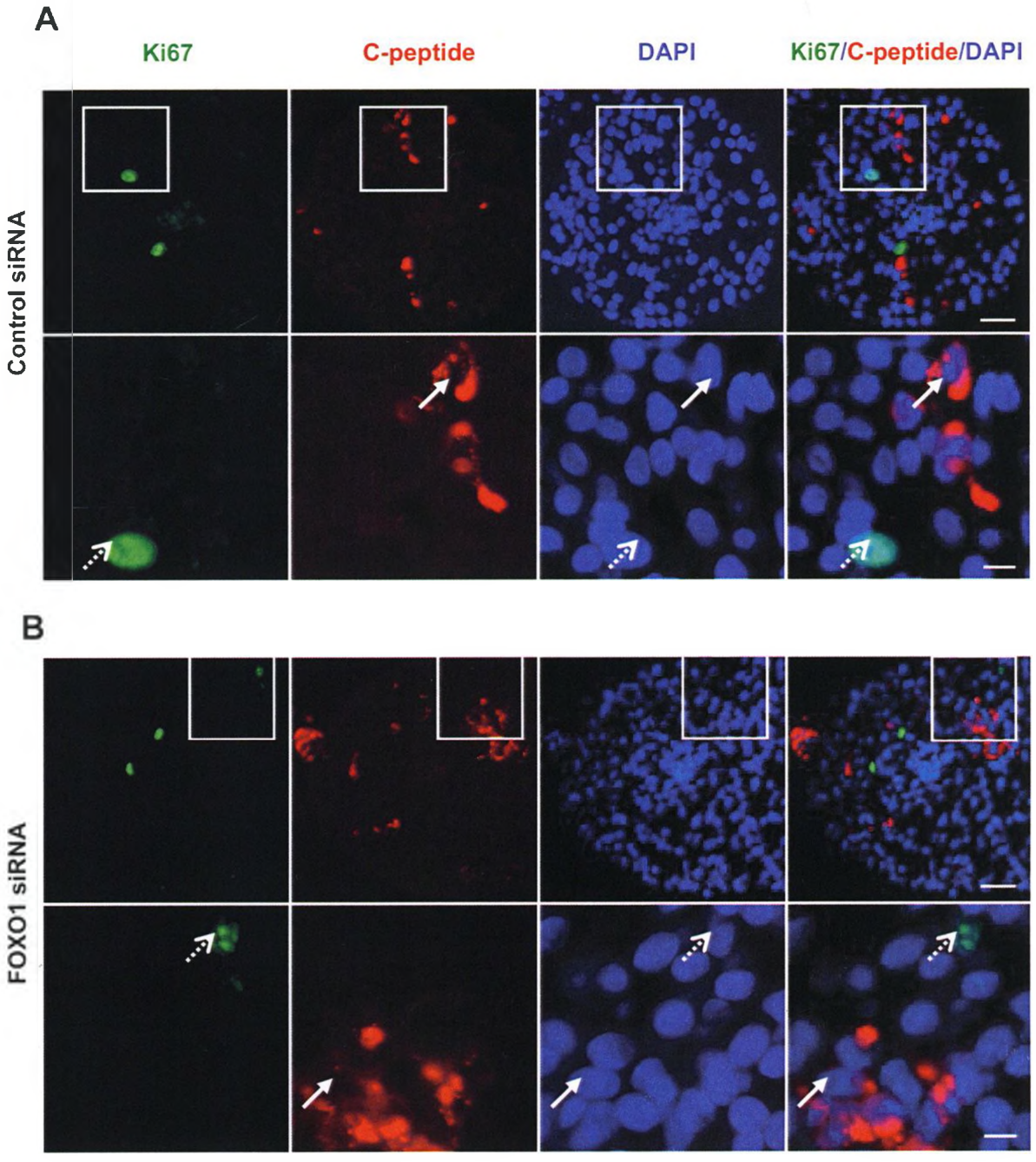

A
Ki67
C-peptide
DAPI
Ki67/C-peptide/DAPI
Control siRNA
B
FOXO1 siRNA

Figure 3.13

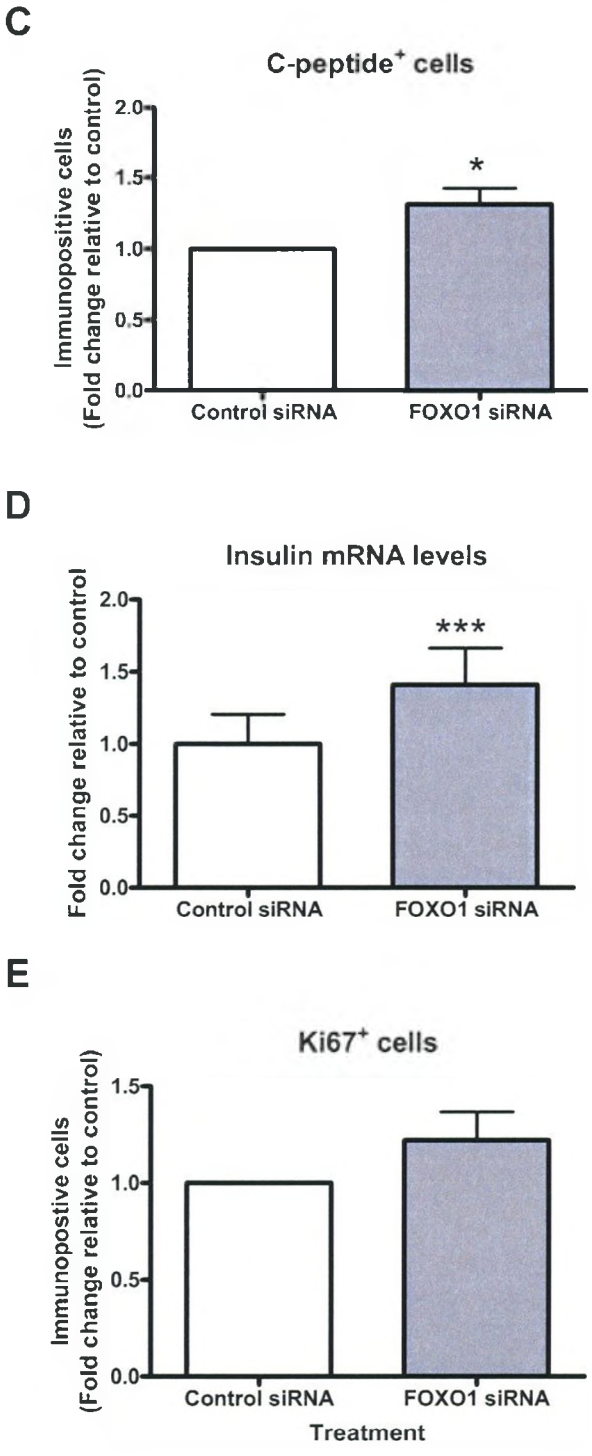

C
C-peptide+ cells
Immunopositive cells
(Fold change relative to control)
2.0
1.5
1.0
0.5
0.0
*
Control siRNA
FOXO1 siRNA
D
Insulin mRNA levels
Fold change relative to control
2.0
1.5
1.0
0.5
0.0
***
Control siRNA
FOXO1 siRNA
E
Ki67+ cells
Immunopostive cells
(Fold change relative to control)
1.5
1.0
0.5
0.0
Control siRNA
FOXO1 siRNA
Treatment

Figure 3.13

Figure 3.14. Effect of *FOXO1* siRNA on the Cellular Distribution and Total mRNA Levels of NGN3 and NKX6-1 is Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters isolated from human fetal pancreata (18-21 wks) were transfected with either control or *FOXO1* siRNA for 48 hr. qRT-PCR analysis of total **(A)** *NGN3* or **(B)** *NKX6-1* gene expression in islet-epithelial cell clusters transfected with either control or *FOXO1* siRNA for 48 hr. Data were normalized to 18S rRNA and are expressed as means ± SEM (n = 4 per experimental group). Quantitative analysis of **(C)** $NGN3^+$ cells or **(D)** $NKX6\text{-}1^+$ cells relative to the total number of $NGN3^+$ cells or $NKX6\text{-}1^+$ cells counted in islet-epithelial cell clusters transfected with either control or *FOXO1* siRNA for 48 hr. Data were determined by morphometric analysis and are expressed as means ± SEM (n = 5 per experimental group). Asterisks indicate*$p< 0.05$, **$p< 0.01$ vs. control siRNA group by the Student's t-test (paired).

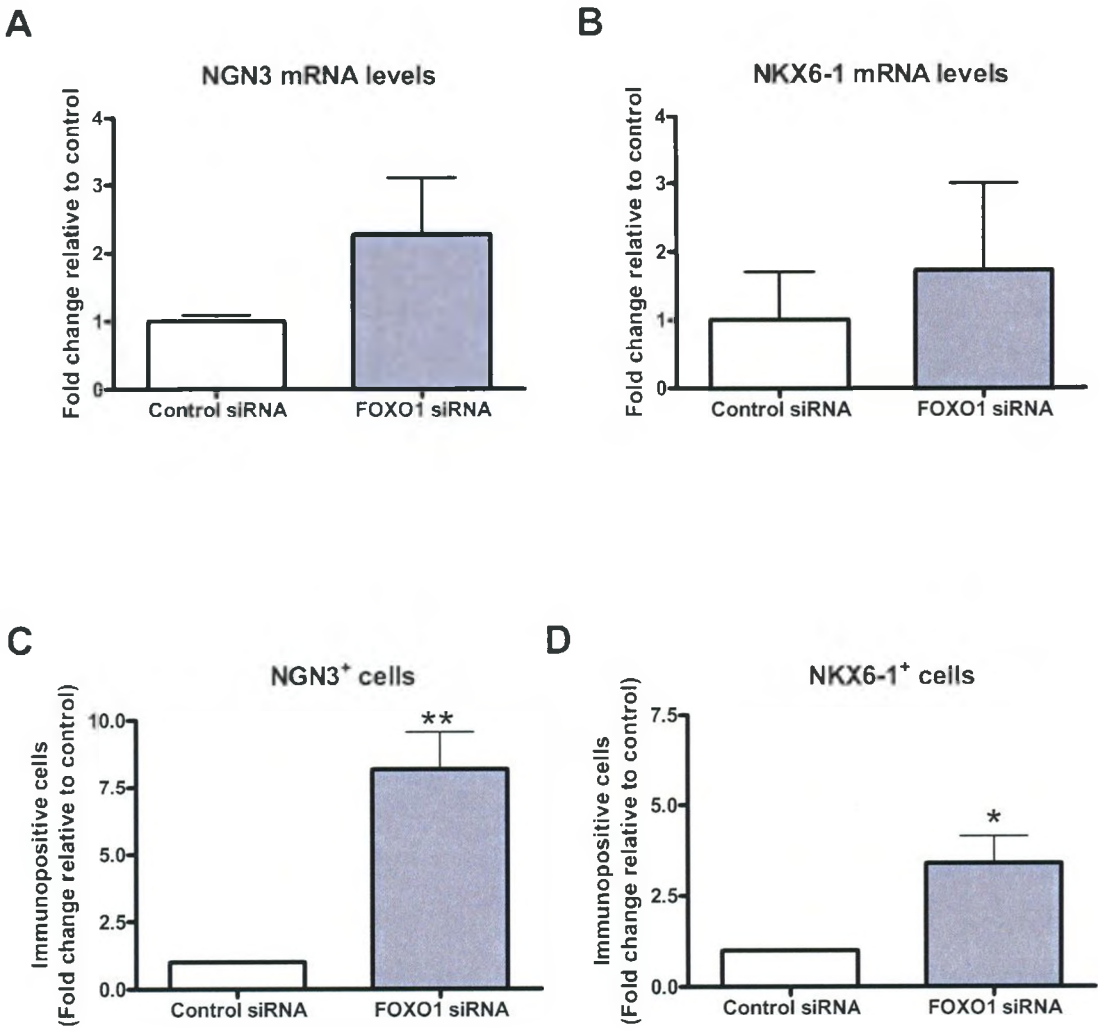

A
NGN3 mRNA levels
Fold change relative to control
0
1
2
3
4
Control siRNA
FOXO1 siRNA
B
NKX6-1 mRNA levels
Fold change relative to control
0
1
2
3
4
Control siRNA
FOXO1 siRNA
C
NGN3+ cells
Immunopositive cells
(Fold change relative to control)
0.0
2.5
5.0
7.5
10.0
**
Control siRNA
FOXO1 siRNA
D
NKX6-1+ cells
Immunopositive cells
(Fold change relative to control)
0.0
2.5
5.0
7.5
*
Control siRNA
FOXO1 siRNA

Figure 3.14

Figure 3.15. Effect of *FOXO1* siRNA on the Cellular Distribution of NGN3 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters (18-21 wks) were transfected with either *FOXO1* or control siRNA for 48 hr. Cells were then harvested and stained (paraffin cell sections) with anti-NGN3 antibody. The anti-NGN3 antibody was visualized with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows immunostaining for NGN3, DAPI, and an overlay of the images as stated. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Arrows indicate a $NGN3^+$ cell. Scale bar corresponds to 10 μm.

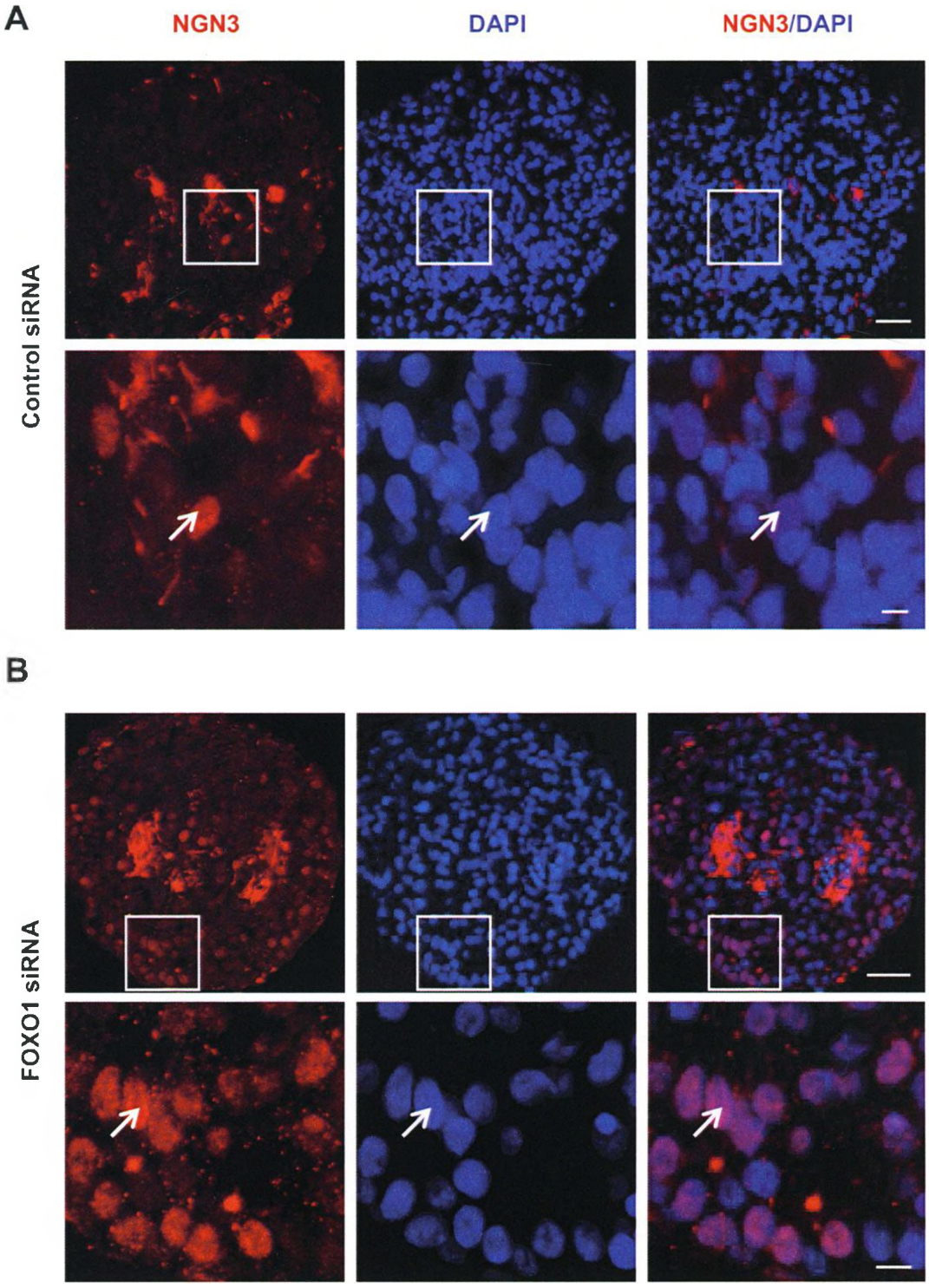


Figure 3.15

Figure 3.16. Effect of *FOXO1* siRNA on the Cellular Distribution of NKX6-1 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters (18-21 wks) were transfected with either *FOXO1* or control siRNA for 48 hr. Cells were then harvested and stained (paraffin cell sections) with anti-NKX6-1 antibody. The anti-NKX6-1 antibody was visualized with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA, or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows immunostaining for NKX6-1, DAPI, and an overlay of the images. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a NKX6-1^{+} cell.

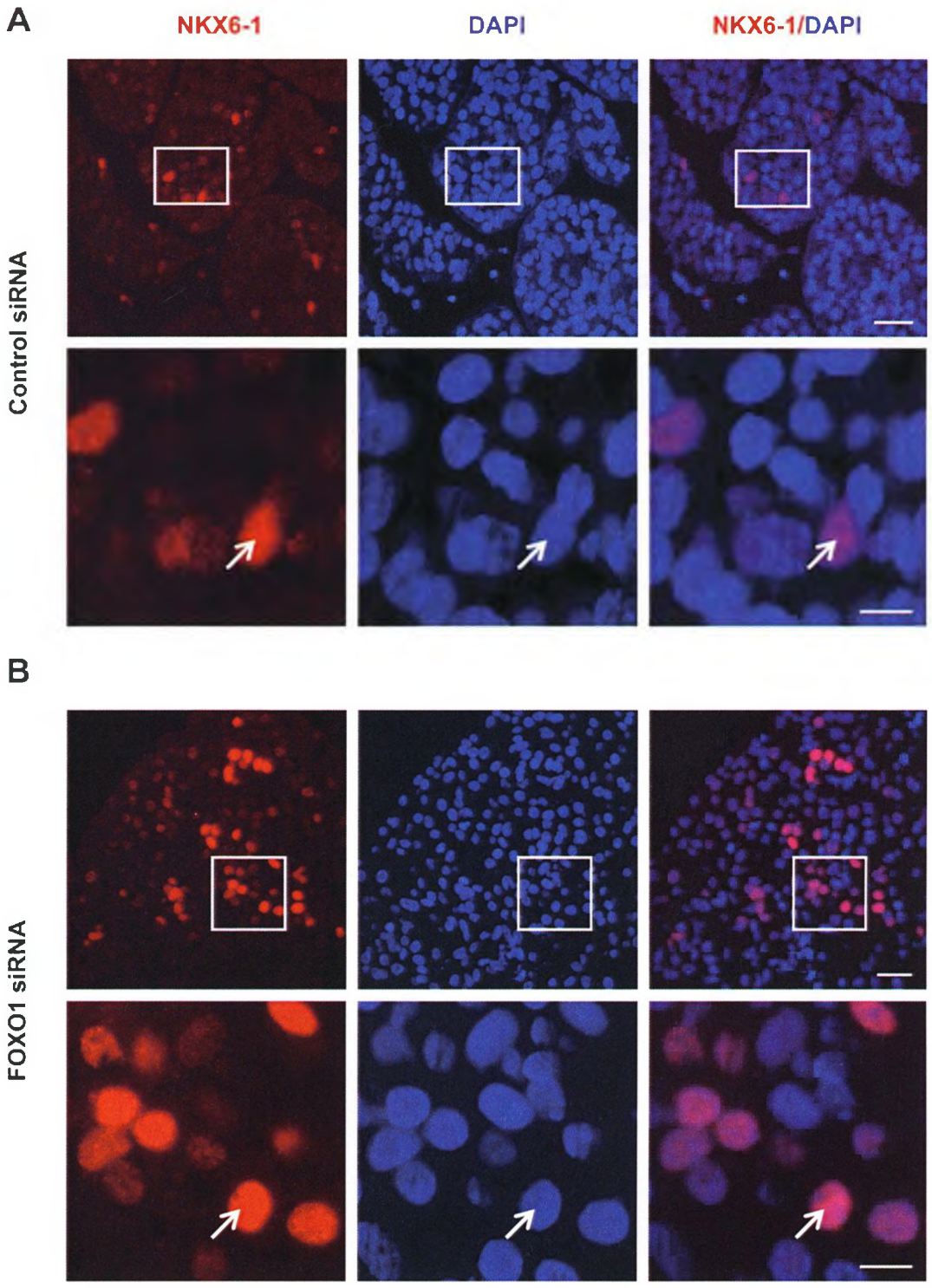


Figure 3.16

analyses of several transcription factors up-stream and down-stream of NGN3 were performed. No changes were detected in the cellular distribution of the transcription factors ISL1 (**Figure 3.17AB**), PAX6 (**Figure 3.18AB**), and NKX2-2 (**Figure 3.19AB**), or the mRNA levels of *ISL1* (**Figure 3.20A**), *PAX6* (**Figure 3.20B**), and *NKX2-2* (**Figure 3.20C**) in the *FOXO1* siRNA transfected group. Analysis of transcription factors up-stream of NGN3 showed no differences in the cellular distribution of PDX-1 (**Figure 3.21AB**) or the mRNA levels of *PDX-1* (**Figure 3.23A**). Interestingly, immunostaining showed, that several cell clusters exposed to *FOXO1* siRNA stained negative for nHES1 (**Figure 3.22B**) in comparison to cell clusters exposed to control siRNA, the majority of which stained positive for nHES1 (**Figure 3.22A**). Furthermore, I observed a 2-fold decrease in mRNA levels of the transcription factor *HES1* as detected by qRT-PCR analysis (**Figure 3.23B).**

Figure 3.17. Effect of *FOXO1* siRNA on the Cellular Distribution of ISL1 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters (18-21 wks) were transfected with either *FOXO1* or control siRNA for 48 hr. Cells were then harvested and stained with anti-ISL1 antibody. The anti-ISL1 antibody was visualized with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows immunostaining for ISL1, DAPI, and an overlay of the images as stated. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a $ISL1^+$ cell.

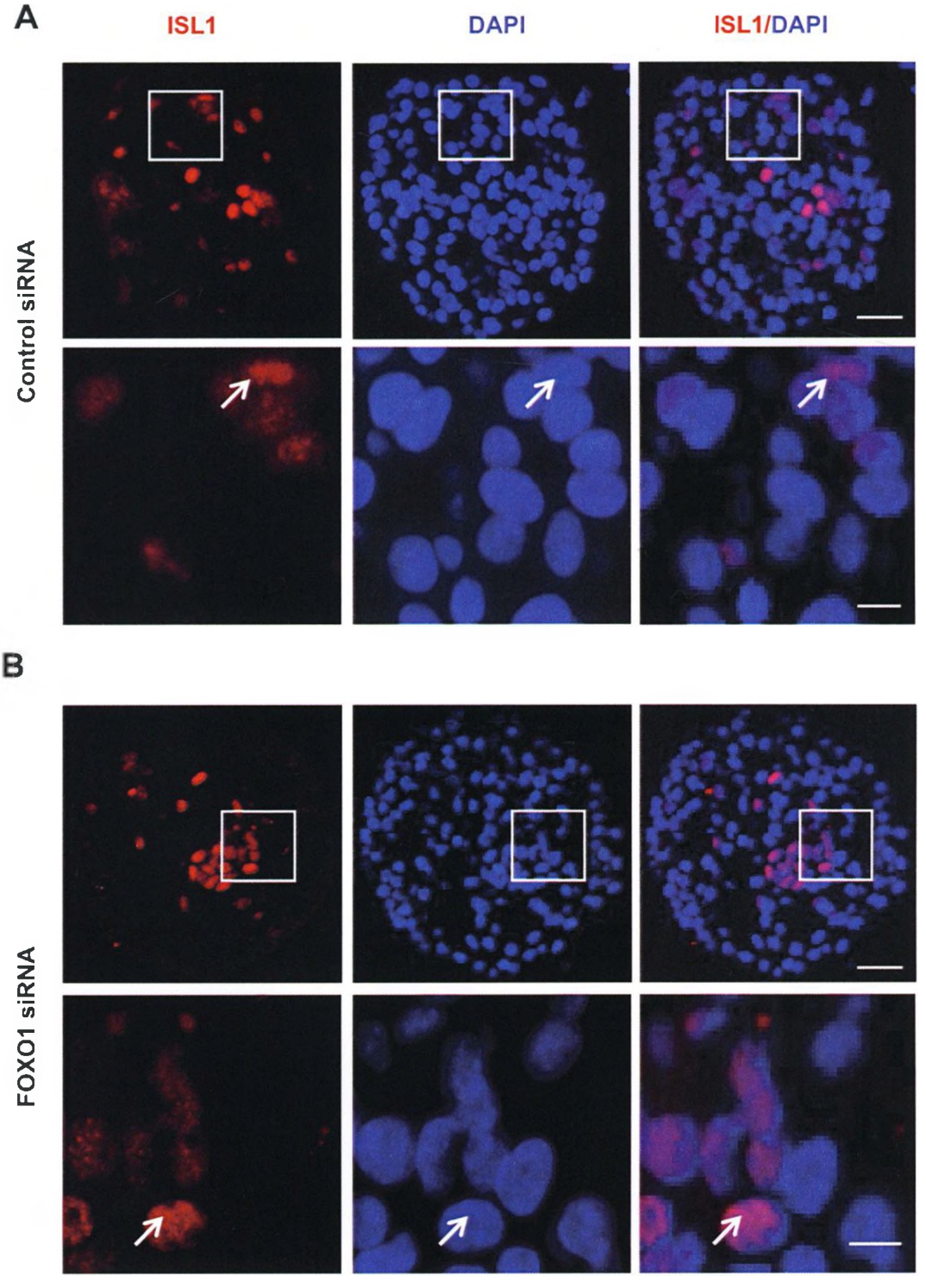


Figure 3.17

Figure 3.18. Effect of *FOXO1* siRNA on the Cellular Distribution of PAX6 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters (18-21 wks) were transfected with either *FOXO1* or control siRNA for 48 hr. Cells were then harvested and stained with anti-PAX6 antibody. The anti-PAX6 antibody was visualized with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows immunostaining for PAX6, DAPI, and an overlay of the images as stated. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a $PAX6^+$ cell.

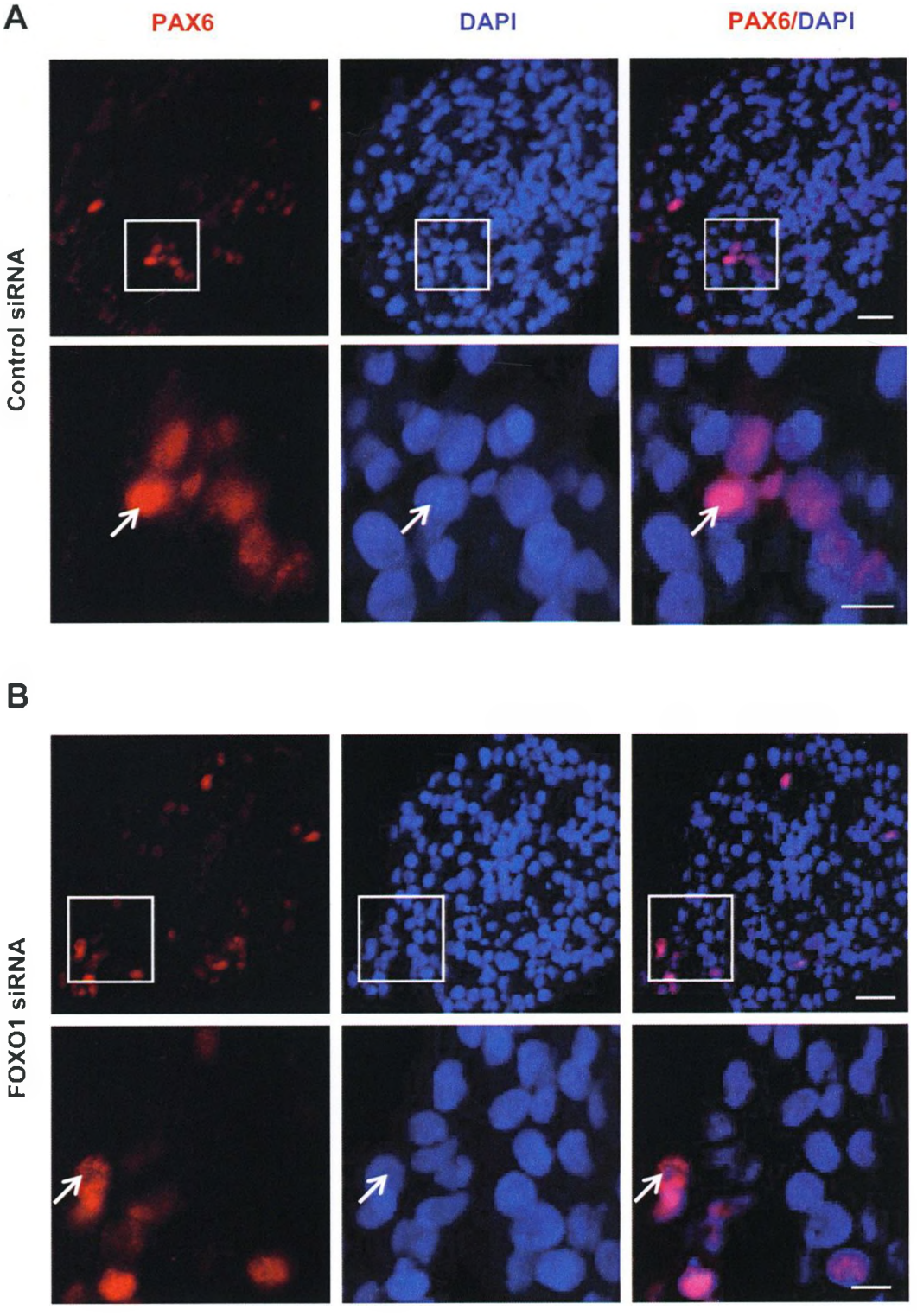


Figure 3.18

Figure 3.19. Effect of *FOXO1* siRNA on the Cellular Distribution of NKX2-2 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters (18-21 wks) were transfected with either *FOXO1* or control siRNA for 48 hr. Cells were then harvested and stained (paraffin cell sections) with anti-NKX2-2 antibody. The anti-NKX2-2 antibody was visualized with TRITC-conjugated anti-mouse IgG (red). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows immunostaining for NKX2-2, DAPI, and an overlay of the images as stated. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a NKX2-2^{+} cell.

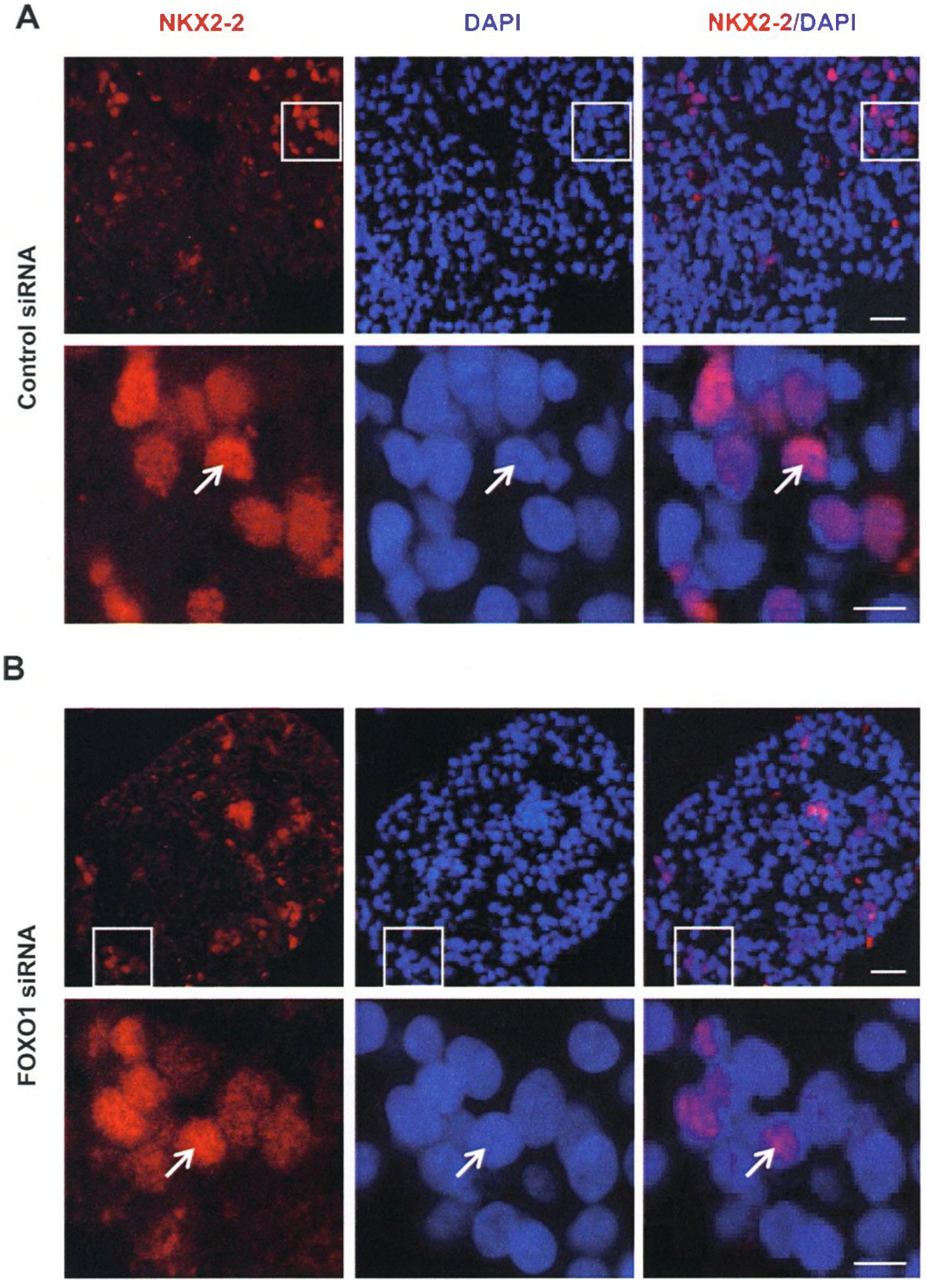


Figure 3.19

Figure 3.20. Effect of *FOXO1* siRNA on the Total mRNA Levels of *ISL1*, *PAX6*, and *NKX2-2* in Islet-Epithelial Cell Clusters.

qRT-PCR analysis of total mRNA levels of **(A)** *ISL1*, **(B)** *PAX6*, or **(C)** *NKX 2-2* in islet-epithelial cell clusters (18-21 wks) transfected with either control siRNA or *FOXO1* siRNA for 48 hr. Data were normalized to 18S rRNA and are expressed as means ± SEM (n = 6 per experimental group).

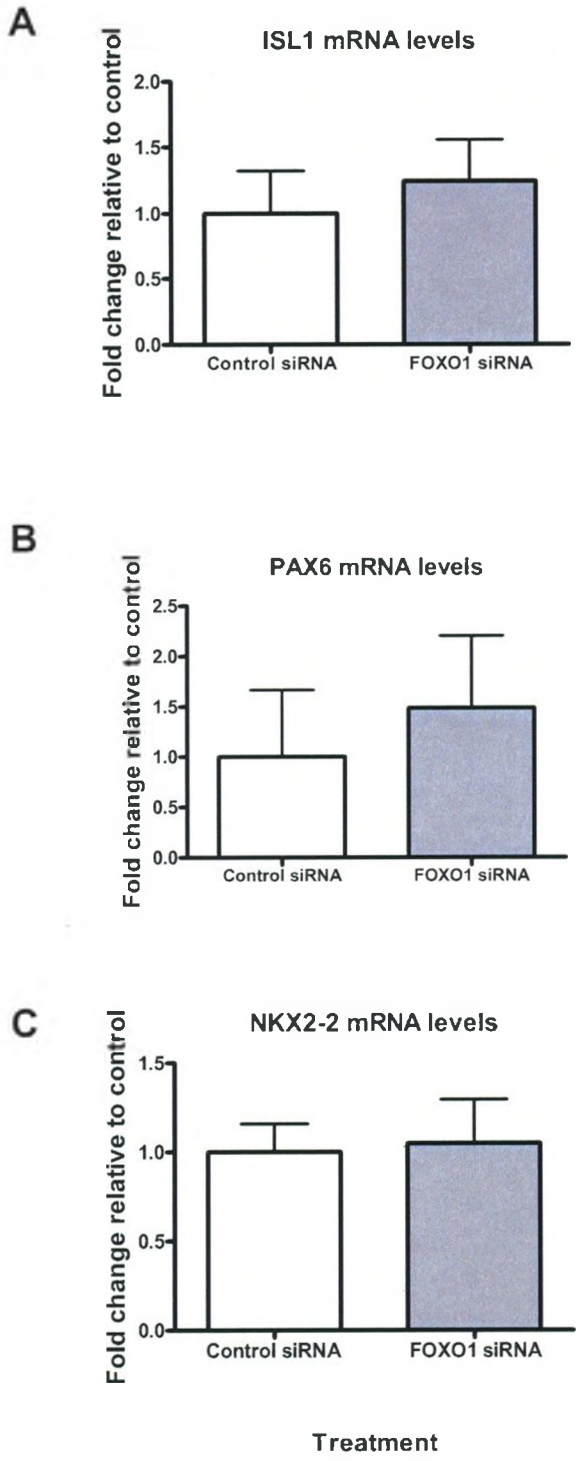

A
ISL1 mRNA levels
Fold change relative to control
2.0
1.5
1.0
0.5
0.0
Control siRNA
FOXO1 siRNA
B
PAX6 mRNA levels
Fold change relative to control
2.5
2.0
1.5
1.0
0.5
0.0
Control siRNA
FOXO1 siRNA
C
NKX2-2 mRNA levels
Fold change relative to control
1.5
1.0
0.5
0.0
Control siRNA
FOXO1 siRNA
Treatment

Figure 3.20

Figure 3.21. Effect of *FOXO1* siRNA on the Cellular Distribution of PDX-1 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters (18-21 wks) were transfected with either *FOXO1* or control siRNA for 48 hr. Cells were then harvested and stained (paraffin cell sections) with anti-PDX-1 antibody. The anti-PDX-1 antibody was visualized with TRITC-conjugated anti-rabbit IgG (red). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows immunostaining for PDX-1, DAPI, and an overlay of the images as stated. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a PDX-1^+ cell.

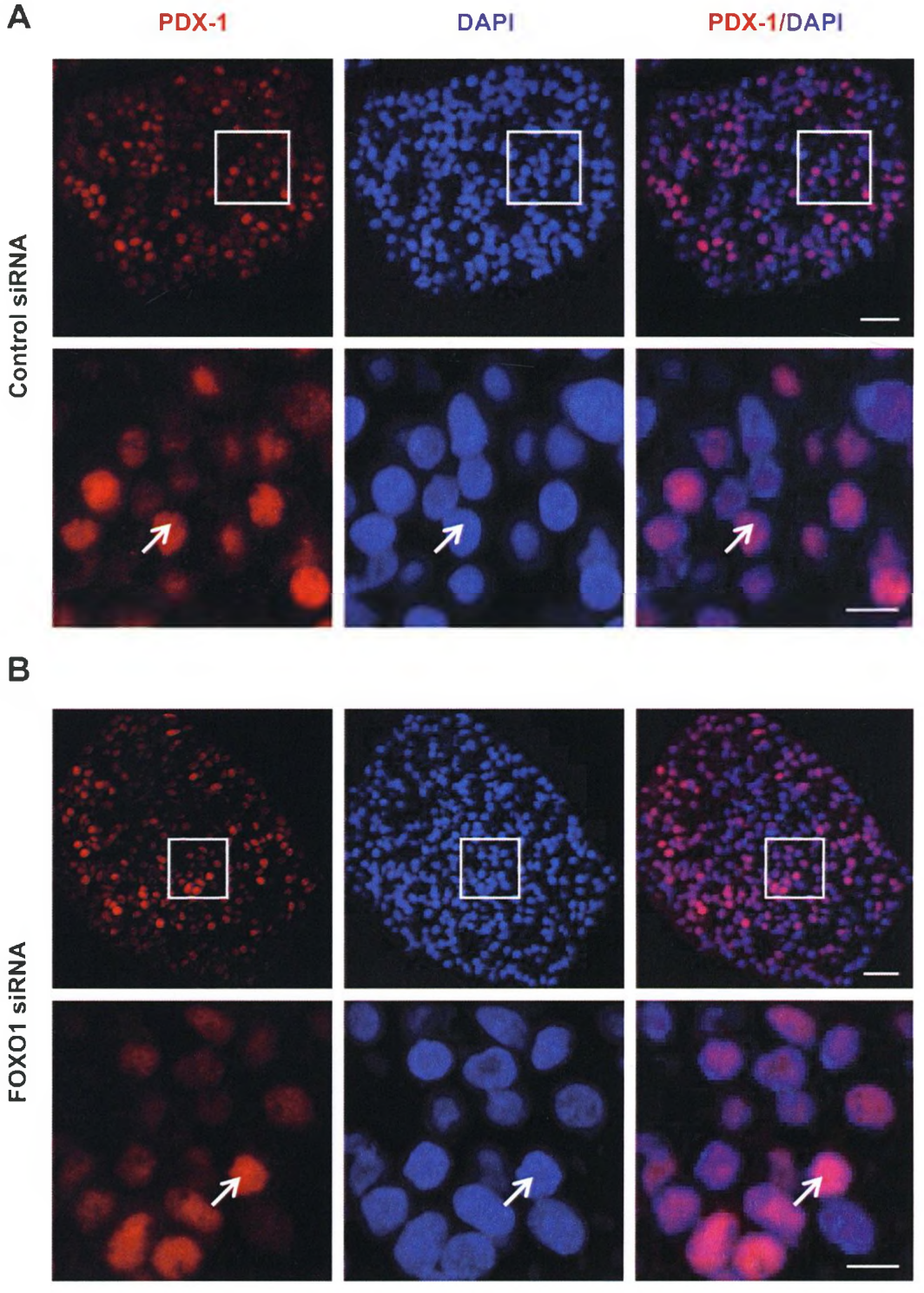


Figure 3.21

Figure 3.22. Effect of *FOXO1* siRNA on the Cellular Distribution of HES1 in Islet-Epithelial Cell Clusters.

Islet-epithelial cell clusters (18-21 wks) were transfected with either *FOXO1* or control siRNA for 48 hr. Cells were then harvested and stained (paraffin cell sections) with anti-HES1 antibody. The anti-HES1 antibody was visualized with FITC-conjugated anti-rabbit IgG (green). Nuclei were stained with DAPI (blue). A representative section of cell clusters transfected with **(A)** control siRNA or **(B)** *FOXO1* siRNA for 48 hr is shown. For each set the top panel of images shows immunostaining for HES1, DAPI, and an overlay of the images as stated. Scale bar corresponds to 100 μm. Bottom panel shows magnified images each corresponding to the section within the square. Scale bar corresponds to 10 μm. Arrows indicate a $HES1^+$ cell and dashed arrows indicate a $HES1^-$ cell.

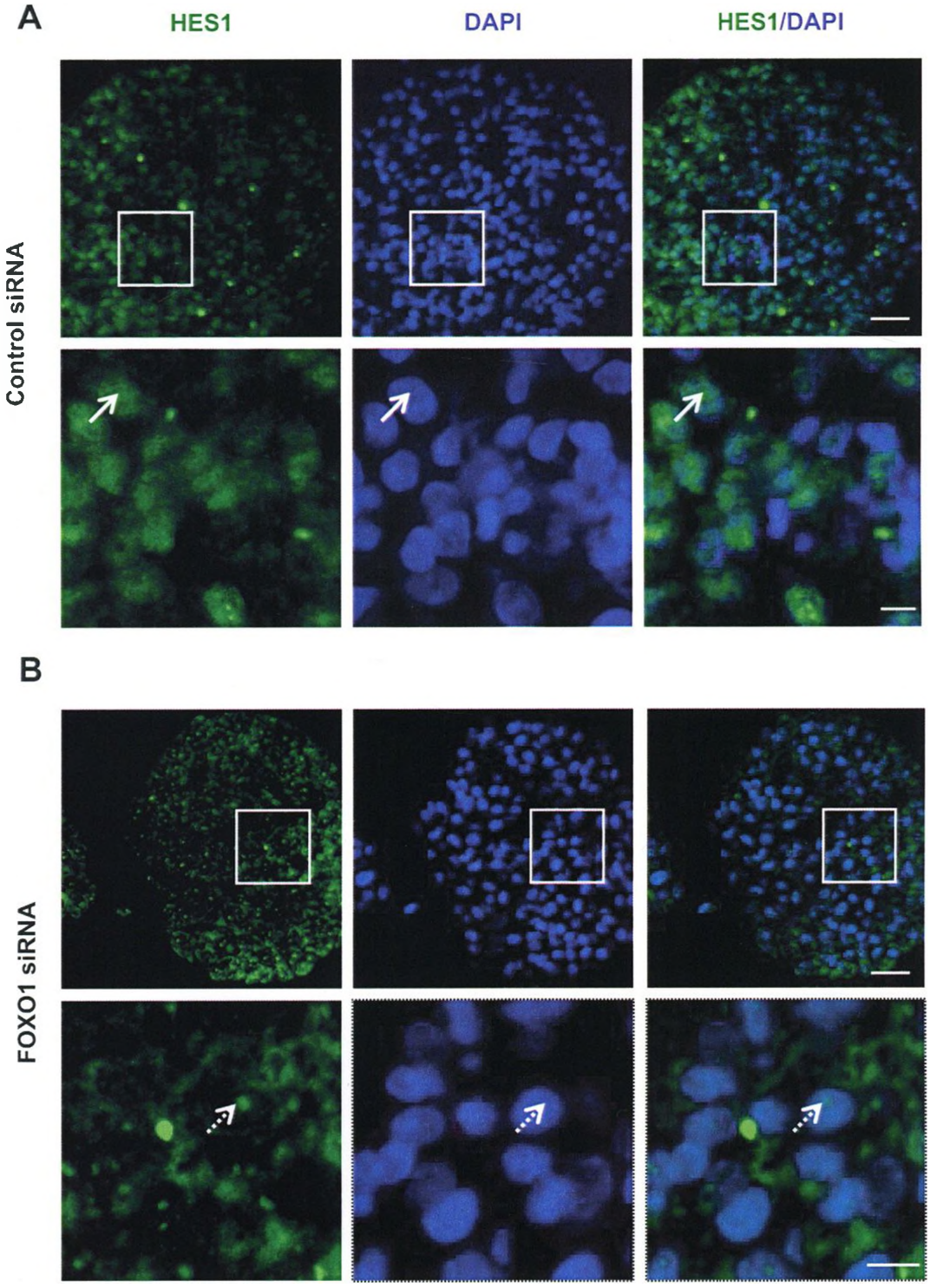


Figure 3.22

Figure 3.23. Effect of *FOXO1* siRNA on the Total mRNA Levels of *PDX-1* and *HES1* in Islet-Epithelial Cell Clusters.

qRT-PCR analysis of the total mRNA levels of **(A)** *PDX-1* or **(B)** *HES1* in islet-epithelial cell clusters (18-21 wks) transfected with either control or *FOXO1* siRNA for 48 hr. Data were normalized to 18S rRNA and are expressed as means ± SEM (n = 6 per experimental group).

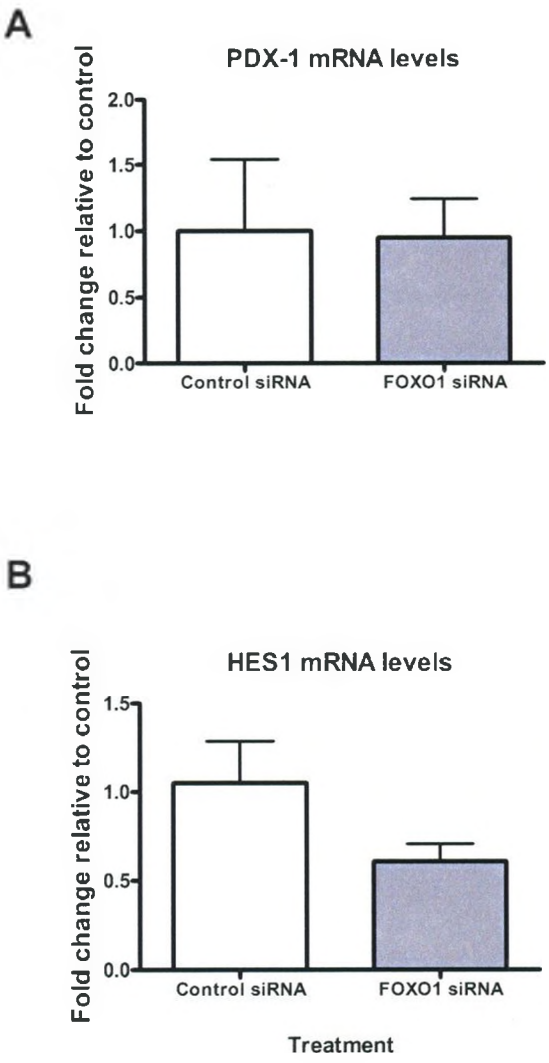

A
PDX-1 mRNA levels
Fold change relative to control
2.0
1.5
1.0
0.5
0.0
Control siRNA
FOXO1 siRNA
B
HES1 mRNA levels
Fold change relative to control
1.5
1.0
0.5
0.0
Control siRNA
FOXO1 siRNA
Treatment

Figure 3.23

Chapter 4

Discussion

Chapter 4 – **Discussion**

The objective of this study was to investigate the role of FOXO1 in regulating human fetal islet cell development, with a specific focus on the role it plays during β-cell development. I hypothesized that FOXO1 directs the differentiation of β-cells through the regulation of endocrine cell specific transcription factors. By employing *ex vivo* experimental procedures, my research has demonstrated constant distribution patterns of FOXO1 protein throughout the period of human pancreatic development during which major developmental changes in islet cell differentiation occur (8 to 21 wks) (Lyttle *et al.*, 2008). Use of a *FOXO1* siRNA specific knockdown *in vitro* enabled me to highlight a significant influence for FOXO1 on the differentiation of β-cells through the regulation of the pro-endocrine cell marker, NGN3. Addressing the five major aims of this study, which centered on identifying the pattern of distribution and functional role of FOXO1 transcription factor, sheds insight on the contribution of this forkhead box family member during human pancreatic organogenesis.

4.1. **Is FOXO1 Restricted to a Specific Spatiao-Temporal mRNA/Protein Distribution During Islet Cell Development?**

Defining the spatio-temporal distribution patterns of FOXO1 transcription factor contributes to the on-going investigation into the roles that this factor may play in mediating human islet cell development and differentiation. In this study, I have quantitatively examined and compared the relative total levels of *FOXO1* mRNA and protein in the pancreas during the 1st and 2nd trimester of human development. Quantitative temporal examination of the relative total *FOXO1* mRNA transcript showed

an increase during the late stage (20 to 21 wks) in comparison to the early (8 to12 wks) and middle stages (14 to 16 wks) of development. However, FOXO1 protein abundance displayed a consistent pattern throughout 8 to 21 weeks of pancreatic development. The qRT-PCR data suggests that protein levels may increase in developmental stages past 21 weeks, a change I was unable to confirm due to limitations in sample access. However, the results obtained are by no means reflective of the *FOXO1* mRNA and FOXO1 protein levels of individual cell populations that make up the developing fetal pancreas. *FOXO1* mRNA and FOXO1 protein levels per cell population may vary dramatically if they are assessed individually. In addition, FOXO1 protein abundance in the cytoplasm versus the nucleus may also vary at different stages, even if the total amount remains constant. Such future analysis heavily relies on advanced cell isolation strategies and nuclear/cytoplasm extractions to provide highly purified cell populations for various comparative analyses.

Analysis of the relative spatial pattern focused on the localization of FOXO1 protein with ductal and endocrine cells, specifically β- and α-cells, using double immunostaining and morphometric analyses. Nuclear FOXO1 assessment, which in most cases is indicative of biological activity, showed co-localization with both ductal and endocrine cell-specific markers (Kitamura *et al.*, 2005). Cytoplasmic FOXO1 assessment, although necessary, was not taken into consideration in the current study. Such future analysis would be best conducted through the use of cell sorting strategies in combination with nuclear/cytoplasmic extractions. This will allow for the precise assessment and comparison of FOXO1 protein abundance and distribution not only within purified cell populations, but also within separate cellular compartments.

Nevertheless, based on the above results, the protein distribution of FOXO1 during pancreatic organogenesis appears to contrast with the protein distribution observed in the adult islet, where FOXO1 is spatially restricted to the cytoplasm or nucleus of β-cells only (Kitamura *et al.*, 2002; Buteau and Accili, 2007). In fact, the FOXO1 protein distribution pattern observed in the current study is consistent with the expression pattern observed in developing mouse embryos, where FOXO1 is widely expressed at e14.5, a period corresponding to the human developmental window examined in this study (Kitamura *et al.*, 2008). This however, is followed by a global decline whereby the distribution of FOXO1 protein becomes restricted to endocrine progenitors at e17.5, until finally it is limited to β-cells post-natally (Kitamura *et al.*, 2008; Kitamura and Ido Kitamura, 2007). I was unable to verify this change in the distribution of FOXO1 protein due to a limitation in the access of human fetal pancreata past the age of 21 weeks. Nevertheless, the distribution of nFOXO1 throughout the examined time period is cell-type independent, which led me to propose that FOXO1 regulates a global cellular function or pathway common to most cell types found in the pancreas.

The differences in the mRNA pattern and protein distribution are suggestive of a different role for FOXO1 factor during early cellular development than in terminally differentiated adult β-cells. This pattern of distribution is frequently displayed by several transcription factors recognized to be critical regulators of pancreatic development such as PDX-1 (Habener *et al.*, 2005). Early in the developing pancreas, PDX-1 expression is widespread and highly maintained to provide both spatial and temporal contributions to the commitment of the endoderm to a pancreatic phenotype. However during later stages of islet development, PDX-1 becomes primarily restricted to the mature β-cells of the

islet, where it is mainly required for regulating β-cell function (Habener *et al.*, 2005; Kitamura and Ido Kitamura, 2007).

4.2. **Does FOXO1 Influence the Subcellular Localization/Expression of PDX-1 in the Human Fetal Pancreas?**

The most prominent role for FOXO1 in β-cells of adult islets is the transcriptional regulation of PDX-1. This observation is based on the ability of a *FOXO1* haploinsufficiency to partially rescue the diabetic phenotype of *Irs2*$^{-/-}$ mice by restoring PDX-1 expression and consequently β-cell proliferation (Kitamura *et al.*, 2002). The proposed mechanism is a direct or a recently proposed indirect, negative regulation of *PDX-1* transcription by FOXO1 (Gupta *et al.*, 2008). This mechanism is reversed by AKT-induced FOXO1 phosphorylation and nuclear export, thereby allowing FOXA2-induced *PDX-1* expression (Kitamura *et al.*, 2002; Lantz and Kaestner, 2005). Given that PDX-1 is an important regulator of pancreatic development and also given its role as a pancreatic progenitor cell marker, the prediction of a transcription factor regulating its expression made it imperative to investigate whether FOXO1 and PDX-1 co-localize during pancreatic development (8 to 21 wks), and whether the abundance of FOXO1 influences the mRNA levels and subcellular localization of PDX-1.

Unlike the definite exclusive subcellular localization observed for both factors in adult β-cells, were FOXO1 is localized to the cytoplasm and PDX-1 is localized to the nucleus (Kitamura *et al.*, 2002; Buteau and Accili, 2007), FOXO1 and PDX-1 both showed clear nuclear localization throughout the examined human pancreatic developmental period (8 to 21 wks). Furthermore, knockdown of *FOXO1* mRNA in islet-

epithelial cell clusters had no effect on either the total mRNA levels or the nuclear localization of PDX-1.

Although immunostaining analysis allowed me to assess the subcellualr localization of PDX-1, any possible changes in protein levels were not quantified. Furthermore, PDX-1 is expressed in two cellular populations, early pancreatic progenitors and terminally differentiated β-cells (Lyttle *et al.*, 2008). Therefore, it is necessary to assess mRNA and protein levels independently for each cellular population through the use of cell sorting strategies in conjunction with qRT-PCR and western blot analyses. Nevertheless, the above data suggests that FOXO1 may influence PDX-1 differently during pancreatic development than in the adult β-cell, or the role it plays is independent of PDX-1 regulation.

4.3. Does FOXO1 Influence Cellular Proliferation in the Human Fetal Pancreas?

Quantification of cells expressing the Ki-67 nuclear proliferation antigen was the method used to assess both the localization of FOXO1 with proliferating cells during pancreatic development (8 to 21 wks), and the effect of *FOXO1* siRNA on cell proliferation in human fetal islet-epithelial cell clusters. The Ki-67 nuclear proliferation antigen is expressed during all phases of the cell cycle except G0, and provides an estimate of the tissue growth fraction (Scholzen and Gerdes, 2000). Ki-67 is tightly associated with chromatin, and therefore correlates with the proliferative status of tissues under a variety of physiological conditions (Scholzen and Gerdes, 2000).

According to the results, down-regulation of *FOXO1* mRNA levels in human fetal islet-epithelial cell clusters did not affect the relative proliferation of cells. This is an

unexpected result given that several studies have shown the repressive influence of FOXO1 factor on cell cycle progression in a variety of cell types (Greer and Brunet, 2005; Huang and Tindall, 2007; Tran *et al.*, 2003). Furthermore, knockdown of *FOXO1* mRNA did not promote the proliferation of existing β-cells given that Ki-67 did not co-stain with the existing β-cells. However, the effect of *FOXO1* siRNA is very short lived, therefore it is possible that knockdown of *FOXO1* mRNA levels requires more than 48 hr to influence cellular proliferation, or a higher knockdown effect. On the other hand, the *in vitro* data are consistent with the *ex vivo* data that showed a progressive decrease in the localization of nFOXO1 with proliferating cells during pancreatic development (8 to 21 wks), which could indicate that FOXO1 transcription factor is not required for this cellular process.

It is possible that the localization pattern of nFOXO1 with Ki-67 could vary amongst different cell populations and is different than the overall observed pattern. However, antibody availability and technical restrictions prevented me from applying triple antibody immunostaining analysis in an attempt to identify and classify the type of proliferating cells localizing with nFOXO1. In future experiments other techniques such as viability assays or bromodeoxyuridine (BrdU) cell labeling could be employed for assessing relative states of cell proliferation. In addition, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) could be utilized for detecting apoptotic cells and their localization with nFOXO1 to provide information on any possible role for FOXO1 during apoptosis.

4.4. Does Insulin or Glucose Influence the Subcellular Localization of FOXO1 in the Human Fetal Pancreas?

The effects of altering the nutritional status on the β-cell are associated with changes in FOXO1 transcriptional activity (Martinez *et al.*, 2006). These changes are predominantly mediated through glucose-stimulated insulin secretion acting through its own receptor, to stimulate the Irs1/2/PI3-Kinase/AKT branch of insulin signaling, which is largely responsible for negatively regulating FOXO1 function by phosphorylation and nuclear export (Martinez *et al.*, 2006). Furthermore, FOXO1 is an effector of insulin action in pancreatic β-cells, and is a key part of a model where FOXO1 links insulin signaling to the regulation of β-cell mass (Kitamura *et al.*, 2002; Nakae *et al.*, 2002). Given that this regulatory mechanism is suggested to be conserved, I investigated whether this model also applies to cells of the developing human pancreas.

In this study, I tested the ability of both exogenously added and endogenously released insulin, through glucose stimulation, to regulate the activity of FOXO1 by nuclear export in islet-epithelial cell clusters. As expected, the addition of insulin at high concentrations that exceed both the normal maternal and fetal insulin levels (Lyttle *et al.*, 2008), stimulated a reduction in the nuclear localization of FOXO1 which under normal conditions localized mostly to the nucleus of cells in our *in vitro* model. On the other hand, exogenous glucose stimulation had no obvious effect on the subcellular localization of FOXO1. This result contradicts the increase in the nuclear export of FOXO1 protein observed in adult mouse β-cells exposed to a high glucose concentration as part of an anti-oxidative stress response mechanism (Kitamura *et al.*, 2005). This may indicate that at the developmental stages examined (18 to 21 wks) glucose/insulin signaling is either

not yet fully developed in the immature human fetal pancreatic cells, or is not sufficient to bring about any effect on FOXO1 subcellular localization. Data regarding the nuclear localization of FOXO1 under the insulin or glucose treatment were obtained by immunostaining and morphometric analyses. Although using these methods allowed me to quantify to some extent the nuclear localization of FOXO1, these methods do not provide exact quantifications to indicate whether the FOXO1 protein was being transported to the cytoplasm. In the future, further investigations could be conducted by monitoring the cytoplasmic transport of FOXO1 protein from the nucleus using a time-lapse video microscopy setup (Haraguchi, 2002).

As for the combined insulin/wortmannin treatment experiment, it tested if the observed reduction in the nuclear localization FOXO1 within human fetal β-cells under the insulin treatment is dependent on the PI3-Kinase/AKT pathway (Powis *et al.*, 1994). Although the results obtained by immunostaining and morphometric analyses showed that the total number of cells staining positive for nFOXO1 under the combined treatment and the control is similar in both conditions, exact protein levels most likely vary. Consistent with previous results, further analysis is required to quantify the precise abundance of FOXO1 protein in the cytoplasm and nucleus to verify the results obtained. Finally, western blot analysis showed that wortmannin was effective in blocking phosphorylated AKT formation, but it is necessary in future experiments to examine the effects of wortmannin alone. Nevertheless, my results indicate that the effect of insulin on FOXO1 localization is similar to its effect in other cell models, and is likely to be mediated at least, in part, through the PI3-Kinase/AKT signaling pathway.

4.5. Does FOXO1 Influence the Expression of Pro-endocrine Cell Markers Important for Islet Cell Growth and Differentiation in the Human Fetal Pancreas?

The siRNA silencing system is an effective method for specifying a functional role for FOXO1 in mature β-cells. However, most *FOXO1* siRNA studies have been carried out in cell lines, with no information on the effects of *FOXO1* gene silencing in primary cells (Kawamori *et al.*, 2006; Kitamura *et al.*, 2005). This study, examined the effect of *FOXO1* siRNA transfections on β-cell development in human fetal islet-epithelial cell clusters for the first time. Knockdown of *FOXO1* mRNA levels led to a notable increase in the β-cell population, as well as *insulin* mRNA levels.

My assessment of the β-cell population was dependent on immunosatining and morphometric analyses of C-peptide positive cells. Although the results obtained showed an increase in the β-cell population, the value could be much higher given that not all β-cells are fully mature and functional at the developmental stage examined (18 to 21 wks). Nevertheless, these findings agree with a recent study that reported a selective increase in juxta-ductal β-cells following a conditional *FOXO1* knockdown in pancreatic progenitors of *PDX-1*-cre mice (Kitamura *et al.*, 2008). Therefore, my results indicate a negative influence of FOXO1 transcription factor on the differentiation of β-cells. The observed increase in β-cell numbers is most probably due to an increase in the levels of pro-endocrine cell markers, and according to my results is not accounted for by a general increase in cellular proliferation.

The formation and maturation of β-cells is a complex process involving multiple gene products, all of which contribute to an integrated sequence of events (Habener *et al.*,

2005). Consistent with an important role of FOXO1 transcription factor in these processes, this study identified *NGN3* as a potential FOXO1 target that was up-regulated in *FOXO1* silenced islet-epithelial cell clusters. Concurrent with the role of FOXO1 in regulating *NGN3* gene expression and the subsequent initiation of the endocrine program, a cluster of binding sites for forkhead factors, including FOXA2, was recently identified in the distal region of the *NGN3* promoter (Lee *et al.*, 2001). During development FOXA2 plays an important role in patterning the early gut endoderm. What is interesting is that in the adult islet FOXA2 competes with FOXO1 for the same binding site within the *PDX-1* promoter to regulate the expression of this gene (Kitamura *et al.*, 2002). Although possible, whether or not the control of *NGN3* transcription during development is a forkhead factor dependant mechanism, similar to that regulating *PDX-1* gene expression in the adult islet, remains to be determined.

NGN3 expression depends on positional cues that limit it to specific cells within the gut and pancreatic endoderm. The Notch signaling pathway, in a manner parallel to its role in lateral inhibition, prevents *NGN3* expression in all but a few scattered cells within the ducts of the developing pancreas (Murtaugh *et al.*, 2003). As studies show, disruption of Notch signaling or down-stream mediators, including HES1, causes widespread expression of NGN3 in the pancreas and accelerated endocrine differentiation (Apelqvist *et al.*, 1999; Jensen *et al.*, 2000). Although the effect of *FOXO1* knockdown on Notch signaling was not examined in this study, analysis revealed a decrease in *HES1* mRNA levels in *FOXO1* siRNA transfected islet-epithelial cell clusters. A plausible explanation for this regulation could lie in a recently proposed mechanism that describes a functional interaction between FOXO1 and Notch, required for the regulation of

progenitor cell maintenance and differentiation (Kitamura *et al.*, 2007). Upon ligand-induced cleavage, the intracellular domain of the Notch receptor translocates to the nucleus where it interacts with the DNA binding protein CSL (Chisel), thereby transforming it into a transcriptional activator of target genes (Lai, 2002). Using muscle differentiation as a model system, FOXO1 promotes Notch signaling independent of its transcriptional function (Kitamura *et al.*, 2007). This model suggests that FOXO1 binds to the CSL element within the *HES1* promoter, stabilizes the Notch/CSL complex, and promotes MAML1 (mastermind-like protein-1) recruitment, therefore allowing co-repressor clearance from CSL and activating HES1 (Kitamura *et al.*, 2007). It remains to be determined whether FOXO1 and Notch interact in a similar manner within the developing pancreas, and the subsequent effects that such an interaction may have on HES1 expression and lateral inhibition.

Up-regulation of NKX6-1 cellular protein distribution was also associated with *FOXO1* gene knockdown in islet-epithelial cell clusters. Although we saw a significant increase in NKX6-1 cellular protein distribution, no statistically significant increase was observed in the mRNA levels, perhaps because the level of and restricted pattern of the *NKX6-1* gene is precisely regulated by several homeodomain transcription factors including NKX6-1 itself (Iype *et al.*, 2004). On the other hand, other transcription factors reported to act downstream of NGN3 during rodent pancreatic islet cell differentiation (Gu *et al.*, 2004; Habener *et al.*, 2005), including ISL1, NKX2-2 and PAX6, showed no changes in their mRNA levels or cellular distribution, which does not support the pro-endocrine phenotype of the FOXO1 silenced islet-epithelial cell clusters. Interestingly, it has been reported that NKX6-1 was expressed only in β-cells during human fetal

pancreatic development, indicating that it may be exclusively involved in human β-cell development (Lyttle *et al.*, 2008). That contrasts with knockout studies in mice that have reported NKX6-1 involvement in both α and β-cell differentiation (Henseleit *et al.*, 2005). However, this is a plausible explanation for why β-cells seem to be affected by the surge in NGN3 levels, regardless of the other transcription factors, keeping in mind however that other transcription factors are yet to be investigated.

Finally, islet-epithelial cell clusters could not be maintained in culture past 48 hr, which would have been useful to allow for more time for differentiation of NGN3/NKX6-1^{+} cells to occur, or for further transcription factor analysis. In addition, the effect of *FOXO1* siRNA is very short lived which does not allow for analyses of factors or processes that require a period that exceeds 48 hr to display a change in response to the treatment. Furthermore, the effect of *FOXO1* siRNA varied slightly amongst samples. Although the mean of all samples showed a 50% reduction in mRNA levels of *FOXO1*, knockdown efficiency varied between individual samples, which inevitably influenced the outcome of the data for the parameters examined.

4.6. **Limitations of the Study & Methodology**

What makes this study so unique is that it was performed using human fetal pancreatic tissues rather than a cell line, which at the same time is the primary source of limitations of my study. Given that endocrine cells represent a very small portion of the total cell population in the developing human fetal pancreas (Li *et al.*, 2006), restricts the range of analytical methods that can be used to investigate specific cell populations. Therefore, prospective cell isolation strategies, such as direct fluorescence-activated cell

sorting, coupled with developmental assays would help maximize use of an inherently limited tissue and would provide ideal conditions for studies of human pancreas development (Sugiyama and Kim, 2008). Efficient isolation and culture of β-cells would exclude any interference by other pancreatic cell types. Systematic and quantitative assessment of transcription factors that regulate growth dynamics and survival of human fetal β-cells could then be performed using western blot for quantitative analysis, in parallel with nuclear extraction for subcellular analysis. Both techniques could not be applied in the current study and were substituted by immunostaining, morphometric, and qRT-PCR analyses.

Although conventional microscopy was used in the current study to quantify and examine the subcellular localization of the protein of interest, it is not the favored technique. Confocal microscopy offers several advantages over conventional wide field optical microscopy, including the ability to control depth of field, and more importantly reduction of background noise away from the focal plane, making it the best method to visualize precisely the intracellular localization of proteins in intact cells (Paddock, 2000). Furthermore, although qRT-PCR analysis is reliable and sensitive for the quantitative measurement of low abundant mRNA expression, the great sample variation that comes with the use of human tissue had a large influence on the outcome and consistency of the data obtained.

Finally, although the use of siRNA as a loss-of-function genetic method provides a technically easy transfection with minimized nonspecific effects for many subsequent studies, a number of aspects of the technology require further development (Summerton, 2007). Recent studies have suggested that there are situations where mismatches between

the siRNA and target sequence can occur (Summerton, 2007). Furthermore, its short-term transient knock down effect makes it ineffective for long term studies. Alternatively, morpholinos are virtually free of off-target effects in large part because they achieve high sequence specificity since they must bind at least 14 to 15 adjacent bases to block a gene transcript, making them the most effective of all gene knockdown applications (Summerton, 2007). These types of limitations are important to consider, however, I was successful in knocking down mRNA levels of *FOXO1* in the current study.

4.7. **Conclusion and Future Direction**

In summary, this study supports the on-going efforts to understand the role of FOXO1 in the human fetal pancreas following the discovery of the significant role it plays in the β-cell of adult islets. Demonstrating that FOXO1 is a potential regulator of *NGN3* gene expression provides an explanation for one of its protein functions as a transcription factor during islet cell development. Given that the signals that regulate NGN3 expression ultimately control islet cell formation, the most interesting question emerging from the current study involves the main mechanism of FOXO1 mediated repression on NGN3 expression. To investigate this functional interaction, we first need to determine whether it involves a direct mechanism that requires the binding of FOXO1 to a regulatory sequence within the *NGN3* promoter, or if it is an indirect mechanism that involves the regulation of factors that control *NGN3* expression. However, given the widespread protein distribution of FOXO1 during pancreatic development, investigations of its roles that may be different should not be limited to the development of the endocrine portion of the pancreas and should be expanded to include exocrine cell

development as well. Finally, although FOXO1 is the most predominantly expressed and studied forkhead factor in the adult pancreas, it is still important to analyze the expression patterns and function of other FOXO transcription factors, such as FOXO3, reported to be expressed in mouse islets, in order to shed light on the possible roles they may play in pancreatic cell development and later on in pancreatic cell maintenance (Kitamura *et al.*, 2002).

Chapter 5

References

Chapter 5 - **References**

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Appendix I

Western

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Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R. Wang
Review Number: 10060 **Revision Number:** 5
Review Date: April 09, 2008 **Review Level:** Expedited
Protocol Title: Development of Human Fetal Pancreas
Department and Institution: Physiology, London Health Sciences Centre
Sponsor:
Ethics Approval Date: May 12, 2008 **Expiry Date:** August 31, 2009
›cuments Reviewed and Approved: revised study methodology, revised Letter of Information and Consent
cuments Received for Information:

is is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human bjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research 'olving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws l regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval e noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 :he Food and Drug Regulations.

: ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the REB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time ı must request it using the UWO Updated Approval Request Form.

ring the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior tten approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve y logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor nge(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

estigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

ıese changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the /ly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

mbers of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in :ussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. John W. McDonald

Ethics Officer to Contact for Further Information			
Janice Sutherland	☐	☐ Grace Kelly	☐ Denise Grafton

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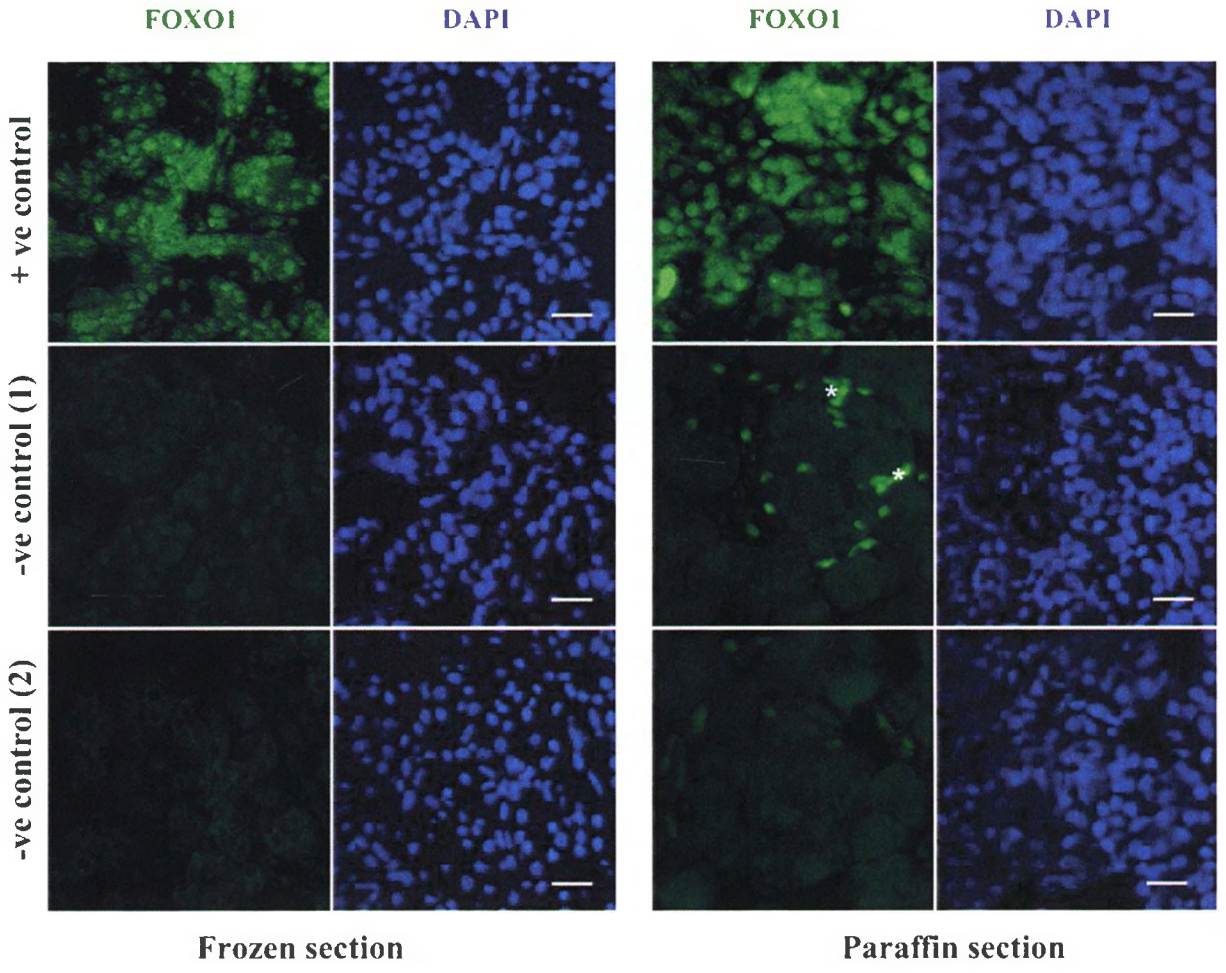
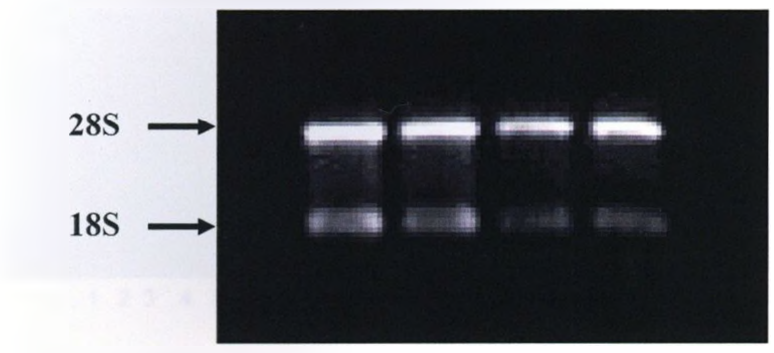
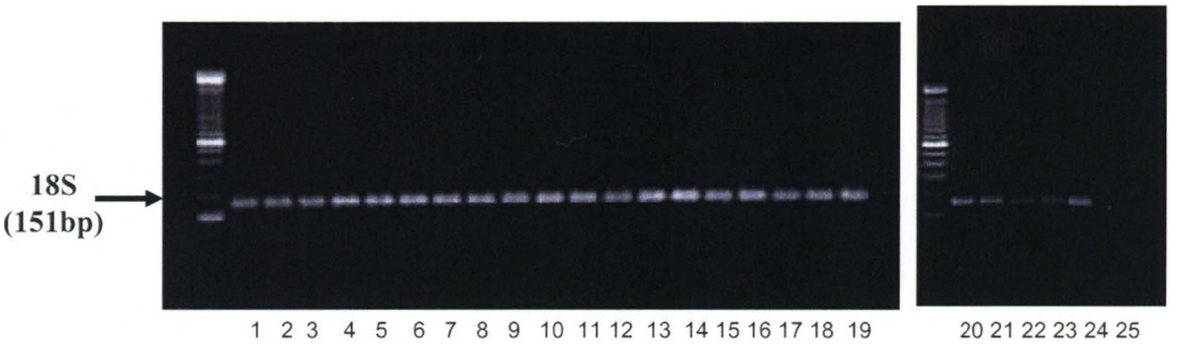
Appendix II



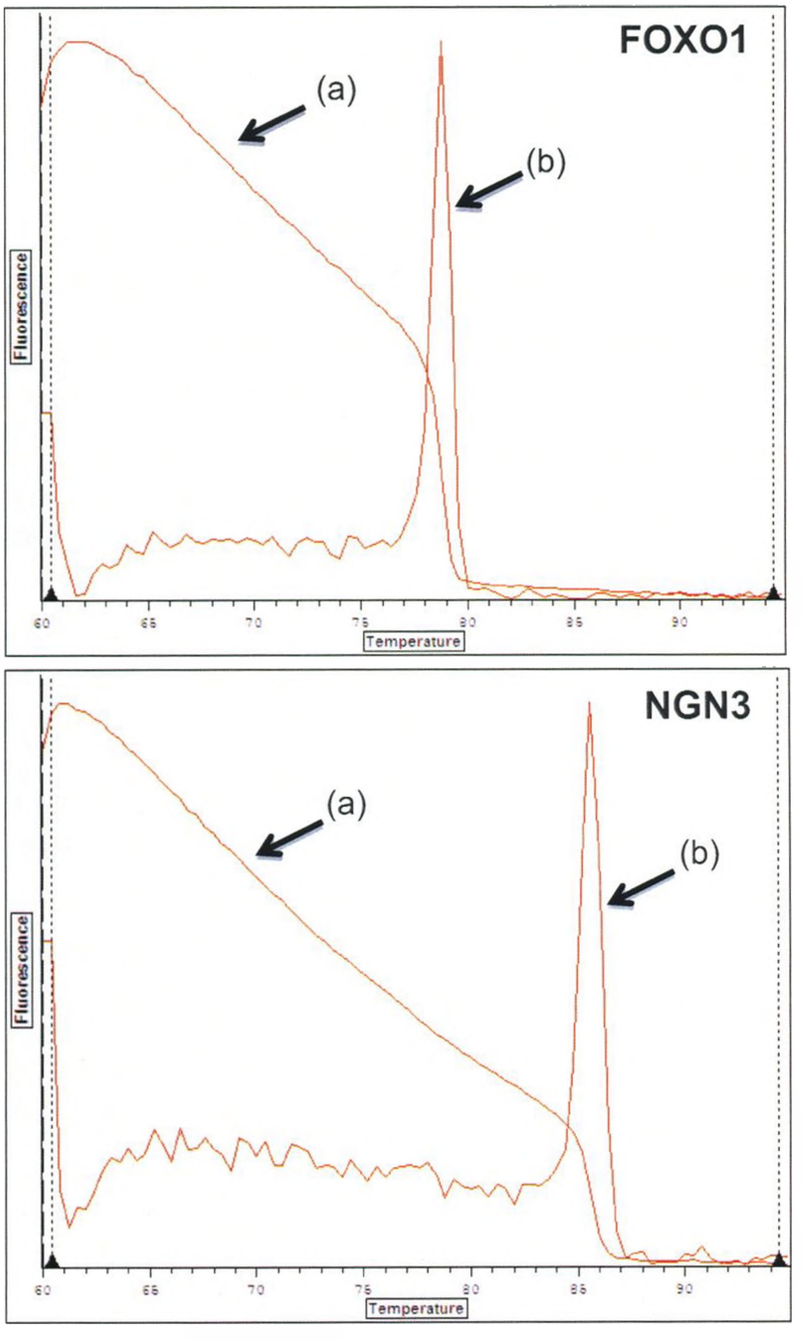
Supplementary Figure 1. **Controls for FOXO1 Immunostaining.** Human fetal pancreatic frozen sections (1st column) or paraffin sections (2nd column) were stained with: (1) anti-FOXO1 antibody and FITC-conjugated anti-rabbit IgG (+ ve control); (2) FITC-conjugated anti-rabbit IgG only (-ve control 1); (3) anti-FOXO1 antibody only (-ve control 2). Nuclei were stained with DAPI. Scale bar corresponds to 100 μm. Asterisks indicate non-specific staining of blood cells .



Supplementary Figure 2. **Agarose Gel Electrophoresis to Check RNA Quality.** Total RNA extracted from islet-epithelial cell clusters (1 µl) was run on a 1% agarose gel stained with ethidium bromide, and showed a sharp, clear 28S and 18S rRNA bands.



Supplementary Figure 3. **Agarose Gel Electrophoresis Analysis of RT-PCR Products.** RT-PCR products (18S rRNA) were run on a 1% agarose gel stained with ethidium bromide to check for quality. Lanes 1 to 24 represent the set of 18S samples extracted from FOXO1 or control siRNA transfected islet-epithelial cell clusters; lane 25 is a negative control (RT-).



Supplementary Figure 4. **The Melting Curves and Derivative Melting Curves of qRT-PCR Products.** The melting curves (a) and derivative melting curves (b) of FOXO1 and NGN3 show that qRT-PCR assays have amplified the target sequences with excellent specificity.