GSK-3β Protein Phosphorylates and Stabilizes HLXB9 Protein in Insulinoma Cells to Form a Targetable Mechanism of Controlling Insulinoma Cell Proliferation

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Background: Germ line heterozygous loss of the MEN1 tumor suppressor gene causes tissue-specific tumors such as insulinomas.

Results: GSK-3β and GSK-3β-mediated phosphorylation of HLXB9, a β-cell differentiation factor, is elevated in insulinomas, and GSK-3β inhibition blocks insulinoma cell proliferation.

Conclusion: GSK-3β and phospho-HLXB9 form a targetable mechanism of insulinoma pathogenesis.

Significance: Reactivation of a tissue-specific differentiation factor accounts for tumor tissue specificity.

Insulinomas (pancreatic islet β cell tumors) are the most common type of functioning pancreatic neuroendocrine tumors that occur sporadically or as a part of the MEN1 syndrome that is caused by germ line mutations in MEN1. Tissue-specific tumor predisposition from germ line mutations in ubiquitously expressed genes such as MEN1 could occur because of functional consequences on tissue-specific factors. We previously reported the proapoptotic β cell differentiation factor HLXB9 as a downstream target of menin (encoded by MEN1). Here we show that GSK-3β inactivates the proapoptotic activity of HLXB9 by phosphorylating HLXB9 at Ser-78/ Ser-80 (pHLXB9). Although HLXB9 is found in the nucleus and cytoplasm, pHLXB9 is predominantly nuclear. Both pHXB9 and active GSK-3β are elevated in β cells with menin knockdown, in MEN1-associated β cell tumors (insulinomas), and also in human sporadic insulinomas. Pharmacologic inhibition of GSK-3β blocked cell proliferation in three different rodent insulinoma cell lines by arresting the cells in G2/M phase and caused apoptosis. Taken together, these data suggest that the combination of GSK-3β and pHLXB9 forms a therapeutically targetable mechanism of insulinoma pathogenesis. Our results reveal that GSK-3β and pHLXB9 can serve as novel targets for insulinoma treatment and have implications for understanding the pathways associated with β cell proliferation.

Precise control of the expression and activity of differentiation factors is essential during embryonic development for the proper proliferation and differentiation of target cells and, later, for the maintenance of the differentiated state. It is important to understand how this control could go awry in differentiated cells, leading to re-entry into the cell cycle and increased cell proliferation. Tissue-specific tumor predisposition from germ line mutations in ubiquitously expressed genes could be attributed to the dysregulation of factors that specify the target tissues. Such tissue-specific endocrine tumors are manifested in the multiple endocrine neoplasia type 1 (MEN1)3 syndrome and recapitulated in the mouse model of this syndrome (1, 2). In both humans and in the mouse model, germ line heterozygous loss of the MEN1 tumor suppressor gene (encoding the protein menin) predisposes to simultaneous occurrence of tumors in three main endocrine organs: the parathyroids, the anterior pituitary, and the pancreas (3).

In mouse models, homozygous loss of Men1 in the insulin-secreting pancreatic islet β cells or in the whole pancreas leads to only tumors of the β cells (insulinoma) (4, 5). Interestingly, Men1 loss in the islet α cells causes insulinomas rather than glucagonomas because of trans-differentiation of menin-null α cells into β cells (6, 7). Also, in a mouse model, Men1 loss in the liver did not cause tumors in the liver (8). These observations suggest the potential involvement of tissue-specific factors and differentiation factors in the pathogenesis of insulinomas. Furthermore, 40–50% of sporadic pancreatic neuroendocrine tumors, including insulinomas, have somatic inactivation of at least one copy of MEN1 (9, 10). Thus, the MEN1-encoded protein menin is critical for maintaining normal β cell mass. Given that there are human tumors with MEN1 mutation and without 11q13 LOH (location of the gene), it is possible that menin could be haploinsufficient in certain tissues. For example, prior to the loss of the wild-type allele at 12 months, abnormal hyperplastic islets are observed in the conventional germ line Men1 heterozygous mouse model. Whether the effect on cell proliferation and function is due to menin haploinsufficiency together with other additional genetic or functional lesions is not known. Therefore, investigating downstream targets of menin could not only reveal the pathologic pathways associated

3 The abbreviations used are: MEN1, multiple endocrine neoplasia 1; WCE, whole cell extract; IHC, immunohistochemistry.
with menin loss in MEN1 syndrome, but it could also provide insights into the cause of sporadic tumors that lack MEN1 mutations.

Kinases from the two major proliferation pathways, MAPK/ERK and PI3K/AKT/mammalian target of rapamycin, have been investigated for targeted therapy of insulinomas (11). The serine/threonine kinase glycogen synthase kinase 3β (GSK-3β) regulates a variety of physiological functions, including proliferation, differentiation, cell cycle progression, motility, and apoptosis (12). Interestingly, in ex vivo mouse model studies, GSK-3β inhibition suppressed the growth of medullary thyroid cancer, a type of neuroendocrine tumor (13). However, whether GSK-3β is important in insulinoma, a tumor of neuroendocrine cells of the pancreatic islet β cells, has not been explored.

We have previously investigated a pancreatic β-cell differentiation factor, HLXB9 (HB9, MNXI, or MNR2) in the pathogenesis of insulinomas caused by menin loss (14, 15). HLXB9 is a homeobox-containing transcription factor that acts early during embryonic β cell development and differentiation and, later, in mature β cells for the maintenance of the β cell characteristic (16–18). Also, it is involved in hematopoiesis and in the development of motor neurons (19, 20). In the pancreas, HLXB9 is only expressed in β cells (16).

We have shown that, similar to its function in motor neurons, HLXB9 overexpression caused apoptosis in β cells (MIN6 cells). However, upon menin knockdown, HLXB9 could not cause apoptosis in β cells (14). In this investigation, we found that HLXB9 was phosphorylated by GSK-3β and that this phosphorylation was increased upon menin knockdown, suggesting that the proapoptotic function of HLXB9 was inactivated by phosphorylation. Furthermore, both active GSK-3β and pHLXB9 were elevated under the following conditions: insulinoma cell line with menin knockdown, insulinomas from the mouse model of MEN1, and human sporadic insulinomas. Also, inhibition of GSK-3β in multiple insulinoma cell lines caused reduced cell viability, decreased proliferation, and induced apoptosis, implying GSK-3β and pHLXB9 as potential targets to control cell proliferation in insulinoma.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Antibodies, and Primers**—The human menin (pcDNA3.1-mh-menin) and mouse HLXB9 (pcDNA3.1-mh-HB9-wt and pcDNA3.1-mh-HB9-AA (Ser-78 and Ser-80 to alanine) plasmids) have been described previously (14, 21). The HA-tagged GSK-3β plasmids (HA-GSK-3β-WT and HA-GSK-3β-S9A in pcDNA3) were purchased from Addgene (22). For menin knockdown, pSuperpuro-Men1-shRNA was used (14), which is specific for mouse Men1 (23). For the FLAG-Frat1 plasmid, the mouse Frat1 coding region was PCR-amplified from MIN6 cDNA and cloned into the EcoRI and BamHI sites of pCMV-FLAG (Sigma). Frat1 primers were as follows: mouse-Frat1, GCCCGAATTCGACGACCTGCTCGCCGACAGAGGT (forward) and GCCGGATCCCTGCAAGCAGACAGAGGT (reverse). All antibodies used in this study are listed in [supplemental Table 1](#). The specificity of the two GSK-3β antibodies, GSK-3β-pSer9 (inactive GSK-3β) and pTyr216 (active GSK-3β), was validated by Western blot analysis of MIN6 cells transfected with HA-tagged GSK-3β ([supplemental Fig. 1](#)).

**Mammalian Cell Culture and Transfection**—The insulinoma cell lines MIN6 (mouse) (24), β-TC3 (mouse) (ATCC), and RINm5F (rat) (ATCC) were cultured in low-glucose DMEM (Invitrogen) supplemented with 15% fetal calf serum (Gemini, West Sacramento, CA) and antibiotic/antimycotic (Invitrogen). For protein overexpression, plasmids were transfected using Lipofectamine 2000 (Invitrogen) or nucleofection (AMAXA/Lonza, Walkersville, MD), and the cells were processed for RNA and protein isolation 48h post-transfection. For protein knockdown, cells were transfected with a control shRNA or Men1 shRNA plasmid by nucleofection (AMAXA/Lonza), and the cells were processed for RNA and protein isolation 72 or 96 h post-transfection.

**RNA Isolation and Quantitative Real-time-PCR**—Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) and treated with DNase I (Ambion, Grand Island, NY). Oligo(dT)-primed first-strand cDNA was used for regular PCR or SYBR Green quantitative real-time PCR (Agilent, Santa Clara, CA). GAPDH was used as the internal control. The primers used were as follows: human Men1, GTACCTGGCTGCG-TACCATGTGTC (forward) and CCGGCTCTCCAGCAGACC-ACGG (reverse); mouse Men1, GTACATGGCTCGACC-GTAAG (forward) and TCACTGCTGATTAGGCTTTAG (reverse); mouse Hlx9b, CTACATGCTCACCAAGACTCA (forward) and TCACGTCTCTTCCTCCTTCTCT (reverse); and mouse Gapdh, ACTACGGCACCACAGGAC (forward) and CAACCTGTGCTCTAGTCTAG (reverse). All primers used in this study were synthesized at Integrated DNA Technologies (Corvalle, IA).

**Western Blot Analysis**—Whole cell extracts (WCE) were processed for Western blot analysis with primary antibodies ([supplemental Table 1](#)), followed by HRP-conjugated secondary antibody and ECL (Millipore, Billerica, MA).

**Cell Culture Treatments**—All reagents were purchased from Sigma. For protein stability assays, 48 h post-transfection, cells were cultured with or without 20 µM cycloheximide for 1–4 h, 10 µM MG132 for 4 h, or 20 or 40 mM NH4Cl for 5 h. For kinase inhibitor assays, cells transfected with mh-HB9-wt 48 h post-transfection were cultured with 40 µM kenpaullone, 40 µM SB216763, 20 µM bisindolylmaleimide, 20 µM CR8, or 40 mM LiCl for 1 h. DMSO and NaCl were used for control treatments.

**Kinase Assay**—GST, GST-HB9-wt, or GST-HB9-AA expressed in the Escherichia coli BL-21 CodonPlus-RIL strain (Agilent, Santa Clara, CA) and purified on glutathione-Sepharose beads (14) was used for in vitro kinase assays with active recombinant GSK-3β or CDK5 (Signal-Chem, Richmond, Canada). Purified GST-HB9-wt and GST-HB9-AA showed a prominent band at 64 kDa for full-length protein together with multiple lower molecular weight bands that also contained GST-HB9, as assessed by Western blot analysis with anti-Hlx9b (data not shown). GST-HB9 expression and purification from bacterial cells was prone to possible premature truncations or protein clipping.

**Isolation of Mouse Islets**—All mouse experiments were done under approved animal study protocols according to the guide-
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lines of the National Institutes of Health Animal Care and Use Committee. Mouse islets were isolated by intraductal collagenase perfusion following a standard protocol (25).

Immunofluorescence Staining—WT or Men1<sup>+/+</sup> mouse islets (from 10-week-old mice) or MIN6 cells in chamber slides were fixed and processed for immunofluorescence (26).

Mouse and Human Tissues and Immunohistochemistry (IHC)—Formalin-fixed, paraffin-embedded tissue sections from WT and Men1<sup>+/+</sup> mice (15 months old) and sporadic human insulinomas and normal human pancreas were obtained as follows. Mouse pancreas sections were cut from paraffin blocks provided by Dr. Peter Scacheri (Case Western Reserve University, Cleveland, OH). Human insulinomas (<i>n</i> = 26) were obtained from patients under National Institutes of Health Institutional Review Board-approved protocols (NCT01005654) after written informed consent was obtained. The diagnosis of insulinoma was made on the basis of supervised fasting, with all patients having serum glucose levels of less than 45 mg/dl within 48 h of fasting, together with elevated plasma insulin, proinsulin, and C-peptide levels. All patients had their tumor within 48 h of fasting, together with elevated plasma insulin, proinsulin, and C-peptide levels. All patients had their tumor localized and removed without any recurrence during follow-up. Biochemical testing for MEN1 was performed to confirm that patients had sporadic insulinoma. The <i>MEN1</i> mutation status of these insulinomas is not known. Normal human pancreas (<i>n</i> = 6) sections were obtained from different sources (Dr. Michael Emmert-Buck, NCI, National Institutes of Health; Oriogene, Rockville, MD; ProSci, Poway, CA; Abcam, Cambridge, MA; Zyagen, San Diego, CA; and US Biomax, Rockville, MD).

IHC was performed using an EnVision Flex high pH kit (Dako, Carpinteria, CA). Microscopy and imaging was performed on a Keyence BZ900 microscope (BIOREVO series, Keyence, Itasca, IL) or Leica DMR microscope (Leica Microsystems, Buffalo Grove, IL).

GSK-3β Inhibition and Cell Proliferation Assays—Cells seeded in 12-well plates were treated with different GSK-3β inhibitors (Sigma), LiCl, SB415286, or penkapluone, for 3–4 days with a daily change of the inhibitor-supplemented medium. Cell growth and morphology were observed and imaged (Zeiss, Axiovert 40 CFL, Thornwood, NJ). Cell viability was determined by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega, Madison, WI).

GSK-3β Inhibition in Isolated Normal Mouse Islets—Handpicked islets seeded in 12-well plates (about 20 islets/well) were revived overnight in RPMI medium and treated with 1000-parts per million camptothecin or 20 mM LiCl for 24–48 h, followed by TUNEL staining (Promega). Immunofluorescence with anti-cleaved caspase 3 was also done, but the antibody was not very specific (data not shown).

Flow Cytometry and Cell Cycle Analysis—Cells seeded in 12-well plates (10<sup>5</sup> cells/well) were treated with LiCl (0–20 mM) and incubated for 5 days (two cell divisions) with a change of LiCl-supplemented medium every other day. On day 5, cells were detached and incubated in Vindelov’s propidium iodide buffer for 1 h on ice in darkness. Cell cycle histograms were generated by FACS (FACSCalibur, BD Biosciences). Each sample was analyzed in triplicate for 10,000 events each. The raw data were subjected to ModFit analysis to determine the percentage of cells in G<sub>0</sub>/G<sub>1</sub>, S, and G<sub>2</sub>/M phase.

Statistical Analysis—Data from at least three independent experiments were considered and presented as mean ± S.E. Differences between groups were compared by Student’s <i>t</i> test. <i>p</i> < 0.05 (*) or <i>p</i> < 0.005 (**) were considered significant.

RESULTS

HLXB9 Phosphorylated at Ser-78/Ser-80 (pHLXB9) Is Predominantly Nuclear in β Cells—Transfected myc-tagged HLXB9 (mh-HB9-wt) in MIN6 cells was detected as a doublet band on Western blot analyses probed with an anti-Hlb9 and anti-Myc-tag. Anti-P84 was used as the protein loading control. <i>B</i>, mouse and human HLXB9 amino acid sequence alignment by the multiple alignment tool Clustal Omega. Amino acids in boldface type in the mouse sequence indicate the peptide sequence (unphosphorylated or phosphorylated at Ser-78 and Ser-80) that was used for anti-HB9 and anti-HB9-PO<sub>4</sub>. Western blot analyses of WCE of WT and Men1<sup>−/−</sup> mouse islets transfected with mh-V (vector), mh-HB9-wt, or mh-HB9-AA (Ser-78/Ser-80 substituted with Ala-78/Ala-80) were detected using anti-Hlb9 and anti-Myc-tag. Anti-P84 was used as the protein loading control. <i>B</i>, mouse and human HLXB9 amino acid sequence alignment by the multiple alignment tool Clustal Omega. Amino acids in boldface type in the mouse sequence indicate the peptide sequence (unphosphorylated or phosphorylated at Ser-78 and Ser-80) that was used for anti-HB9 and anti-HB9-PO<sub>4</sub>. C, Western blot analyses of WCE of WT and Men1<sup>−/−</sup> mouse islets transfected with mh-V, mh-HB9-wt, or mh-HB9-AA probed with anti-HB9-PO<sub>4</sub> without peptide competition (left panel), competition with unphosphorylated peptide (center panel), or competition with a phosphorylated peptide (right panel). The peptide sequence is shown in <i>B</i>. <i>D</i>, immunofluorescence analysis of MIN6 cells transfected with mh-V, mh-HB9-wt, or mh-HB9-AA using an anti-Myc tag and anti-HB9-PO<sub>4</sub>. DAPI was used as nuclear counterstain. Magnification is ×100.
78/Ser-80 (mh-HB9-AA) showed a single band on Western blot analyses probed with an anti-Myc tag (corresponding to the lower band of the doublet) (Fig. 1A). Our Western blot analysis of mh-HB9-wt and mh-HB9-AA and the phospho-peptide sequencing data reported previously indicate that HLXB9 could be phosphorylated at Ser-78/Ser-80. Therefore, antibodies against a non-phosphorylated or phosphorylated peptide in the Ser-78/Ser-80 region of HLXB9 were generated (anti-HB9 and anti-HB9-PO4) (Fig. 1B).

On Western blot analyses, anti-HB9-PO4 detected no band for mh-HB9-AA, and a single band was detected for mh-HB9-wt that could be competed with the cognate phosphopeptide but not with the non-phosphorylated peptide (Fig. 1C). These observations demonstrate that the HB9-PO4 antibody could specifically detect the phosphorylated isoform of HLXB9 (pHLXB9) and that the phosphorylation was located at Ser-78/Ser-80.

HLXB9 is found both in the nucleus and in the cytoplasm (14) Immunofluorescence analysis of transfected MIN6 cells with the Myc tag antibody detected mh-HB9-wt in the nucleus and cytoplasm (Fig. 1D, panel 2), whereas mh-HB9-AA was detected only in the cytoplasm (Fig. 1D, panels 6 and 7). However, with the HB9-PO4 antibody, staining was observed predominantly in the nucleus of mh-HB9-wt-transfected cells (Fig. 1D, panel 3), whereas mh-HB9-AA-transfected cells did not show any staining (data not shown). Taken together, these results show that, in β cells, HLXB9 could be phosphorylated at Ser-78/Ser-80 and that pHLXB9 was localized predominantly in the nucleus.

Decreased HLXB9 and pHLXB9 upon Treatment with Proteasomal or Lysosomal Inhibitors—During the characterization of HB9 antibodies, we observed that the phospho-defective form of HLXB9 (mh-HB9-AA) showed slightly reduced expression (Fig. 1A). RT-PCR analysis showed no significant differences at the mRNA level (data not shown), indicating that the decrease in expression was post-transcriptional, possibly because of lack of phosphorylation. A cycloheximide (Chx) treatment assay showed that the overall protein turnover was similar for both phospho- and phospho-defective HLXB9 (Fig. 2A). Another possible reason for reduced expression of the mh-HB9-AA could be proteasome- or lysosome-mediated degradation. Unexpectedly, MG132 treatment (proteasome inhibitor) or NH4Cl treatment (lysosome inhibitor), rather than increasing the protein level, caused a decrease of both phospho- and phospho-defective HLXB9 (Fig. 2, B and C). These data indicate that inhibition of the proteasome or lysosome might stabilize another protein that could degrade HLXB9, irrespective of its phosphorylation state.

HLXB9 is Phosphorylated at Ser-78/Ser-80 by GSK-3β in Vitro and in Vivo—To identify and characterize the kinase that could phosphorylate HLXB9, we first analyzed the kinases GSK-3β and CDK5 that were predicted by NetPhosK (Uniprot) for the consensus amino acid sequence near Ser-78/Ser-80. An in vitro kinase assay showed that GST-HB9-wt (Fig. 3A) was phosphorylated in the presence of GSK-3β (Fig. 3, B and C). The amount of phosphorylated GST-HB9 was low, possibly because of the absence of a priming kinase in the in vitro kinase assays. Usually, for GSK-3β to phosphorylate, a priming kinase phosphorylates a nearby amino acid (28). CDK5 could, in vitro, phosphorylate GST-HB9-wt and GST-HB9-AA almost equally. However, the overall phosphorylation was reduced in the presence of GSK-3β (Fig. 3, B and D), possibly because of competition between the two kinases.

To study the in vivo phosphorylation of HLXB9, MIN6 cells transfected with mh-HB9-wt were treated with various kinase inhibitors: bisindolylmaleimide (protein kinase C inhibitor), CR8 (CDK5 inhibitor), and GSK-3β inhibitors (kenpaullone, SB216763, and LiCl). Compared with the protein kinase C inhibitor or CDK5 inhibitor, the GSK-3β inhibitors decreased pHLXB9 (Fig. 3, E–G). Further analysis of MIN6 cells with Frat1 expression (known to degrade GSK-3β) (29) showed decreased phosphorylation of mh-HB9-wt (Fig. 3H). The GSK-3β inhibitor LiCl efficiently reduced pHLXB9 (top band of the doublet). However, this did not cause an increase in the intensity of the lower band of the doublet (un-phosphorylated HLXB9), indicating that perhaps the unphosphorylated HLXB9 is not stable or is short-lived.

Immunofluorescence analysis of MIN6 cells transiently transfected with constitutively active GSK-3β (HA-GSK-3β-S9A) together with mh-HB9-wt showed that pHLXB9 increased in the presence of GSK-3β (supplemental Fig. 2A, panels 2 and 6). Interestingly, in mh-HB9-wt and GSK-3β coexpressing cells, GSK-3β was localized in the nucleus with pHLXB9 (supplemental Fig. 2A, panels and 7). However, in mh-HB9-AA and...
FIGURE 3. GSK-3β phosphorylates HLXB9 in vitro and in mouse β cells, in vivo. A. GST, GST-HB9-wt (wt), or GST-HB9-AA (AA) was expressed in E.coli BL21-PRIL, purified on glutathione-Sepharose beads, separated on SDS-PAGE, and visualized by Coomassie Blue staining. The position of full-length GST-HLXB9 is marked with an arrow. B–D. in vitro kinase assay was performed by incubating [γ-32P]ATP with GST, GST-HB9-wt, or GST-HB9-AA with or without active GSK-3β and/or active CDK5/p25. The arrow in B indicates the position of the full-length GST-pHLXB9 obtained in the in vitro kinase assay. The asterisks in B indicate lanes with GSK-3β-mediated phosphorylation of GST-HB9-wt and GST-HB9-AA. The relative intensities of bands corresponding to phosphorylated GST-HLXB9 (pHLXB9) from B are shown for GSK-3β-catalyzed (C), CDK5-catalyzed (D), or GSK-3β plus CDK5-catalyzed (D) phosphorylation. E–G, representative Western blot analysis of WCE from MIN6 cells transfected with mh-HB9-wt and treated 48h post-transfection with the indicated kinase inhibitors: Bisindolylmaleimide (BIMI), CR8, kenpaullone (Kenp), SB216763 (SB21), or lithium chloride (LiCl). Unt, cells treated with NaCl or dimethyl sulfoxide (DMSO) as controls. Blots were probed with anti-HB9-PO4, an anti-Myc tag, and loading control anti-P84. Relative amount of HLXB9 protein (percent) detected by the HB9-PO4 antibody was normalized to the untreated cells (100%) (F). The bars marked with an asterisk show a significant decrease in pHLXB9 (F). The relative amount of HLXB9 protein (percent) detected by the Myc tag antibody in E is shown in G. The top band (pHLXB9) was normalized to the corresponding band intensity in the untreated cells (100%). Bars marked with an asterisk show significantly decreased pHLXB9 upon treatment with the GSK-3β inhibitors. *, p < 0.05. (Also see supplemental Fig. 2, A and B). H, Western blot analysis of WCE from MIN6 cells transfected with pFLAG-Frat1 (a physiological inhibitor of GSK-3β) together with mh-HB9-wt or mh-HB9-AA, probed with an anti-Myc tag, an anti-FLAG tag, and loading control anti-P84.
GSK-3β coexpressing cells, GSK-3β was not detected in the nucleus (supplemental Fig. 2A, panel 11), suggesting that GSK-3β phosphorylates nuclear HLXB9. Additionally, LiCl decreased pHLXB9 in MIN6 cells (supplemental Fig. 2B, panels 6, 9, and 12). These observations suggested that LiCl treatment inhibited GSK-3β, which subsequently decreased the phosphorylation of HLXB9.

Together, these results demonstrate that GSK-3β could phosphorylate HLXB9 in vitro and in vivo. However, it is possible that other kinases, such as CDK5, could also phosphorylate HLXB9 at sites other than Ser-78/Ser-80.

pHLXB9 and GSK-3β Are Increased under Reduced Menin Conditions—We have demonstrated previously that the protein level of HLXB9 increased upon menin knockdown in β cells (14). Also, the proapoptotic effect of HLXB9 was reduced upon menin knockdown (14). Therefore, we investigated whether the phosphorylation state of HLXB9 was responsible for the reduction in HLXB9-induced apoptosis upon menin knockdown. In MIN6 cells, menin knockdown followed by overexpression of mh-HB9-wt showed that pHLXB9 was increased 24 h post-transfection (Fig. 4, A and B). Similar results were obtained under another menin knockdown condition,
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islets from young Men1+/− mice (10 weeks) that retain one copy of Men1 (30). Elevated levels of pHLXB9 were seen in the Men1+/− islets compared with WT islets, and the staining for pHLXB9 was predominantly nuclear (Fig. 4C, panels 3 and 7). A Western blot analysis of MIN6 cells showed that reduced menin expression also led to increased GSK-3β and that menin overexpression led to decreased GSK-3β (Fig. 4D). However, GSK-3β mRNA levels were unaffected (Fig. 4, E and F). Analysis of posttranscriptional mechanisms of GSK-3β regulation revealed that overexpression or underexpression of menin did not affect the protein turnover or protein stability of GSK-3β (data not shown). Therefore, the precise mechanism by which menin regulates GSK-3β is not known. However, the above data show that, under reduced menin conditions, the protein level of GSK-3β, HLXB9, and Ser78/Ser80 phosphorylation of HLXB9 was increased.

pHLXB9 and GSK-3β Are Elevated in MEN1-associated Mouse β Cell Tumors (Insulinomas)—The mouse model of MEN1 recapitulates the human MEN1 syndrome (30). To determine whether pHLXB9 and GSK-3β were altered in insulinomas in MEN1 syndrome, pancreatic sections of 15-month-old WT (n = 2) and Men1+/− mice (n = 4) were analyzed by IHC. Both Men1+/− and WT islets stained positive for insulin, and the Men1+/− pancreas showed very large islets, indicating the development of insulinomas (Fig. 5A). In both WT islets and insulinomas, anti-HB9 stained the nucleus and cytoplasm, whereas anti-HB9-PO4 detected pHLXB9 specifically in the nucleus (Fig. 5A, panels 3 and 7 and panels 4 and 8). Compared with WT islets, all insulinomas showed an elevated level of both HLXB9 and pHLXB9 (Fig. 5, A–C). Increased GSK-3β staining was observed in the insulinomas of all four Men1+/− mice analyzed compared with the WT islets (Fig. 5D). Interestingly, IHC showed that in all insulinomas, GSK-3β-pTyr-216 (active) was elevated and GSK-3β-pSer-9 (inactive) was very low or absent (Fig. 5, D–F). These data provide a mechanism for the elevated level of pHLXB9 because of the simultaneous presence of elevated GSK-3β in the menin-null insulinoma cells.

pHLXB9 and GSK-3β Are Elevated in Human Sporadic Insulinomas—Pathways affected in tumors of familial tumor syndromes (such as MEN1) could also be affected in the sporadic counterpart tumors. We explored this hypothesis in the context of human insulinomas. The levels of HLXB9, pHLXB9, and GSK-3β and its active and inactive forms were determined in normal pancreas sections (n = 6) and in human sporadic insulinomas (n = 26 total, n = 19 for HLXB9 and n = 22 for GSK-3β; n = 15 for both HLXB9 and GSK-3β). Compared with the normal islets, in the insulinomas HLXB9, pHLXB9, and GSK-3β and its active form were elevated significantly (p < 0.05), and the inactive form of GSK-3β was absent or very low (Fig. 6, A–C).

Of the 19 human sporadic insulinomas analyzed, 14 showed elevated nuclear pHLXB9 (74%) (Fig. 6, A and C). GSK-3β staining was mostly cytoplasmic, and GSK-3β-pTyr-216 was both nuclear and cytoplasmic in all sections analyzed (normal and tumor) (Fig. 6C). In 12 of 15 tumors analyzed (80%), elevated nuclear staining was observed for pHLXB9 and active GSK-3β simultaneously. Elevated pHLXB9 and active GSK-3β in insulinomas of the mouse model of MEN1 and in human sporadic insulinomas further underscores the importance of GSK-3β-mediated HLXB9 phosphorylation in the genesis of β cell tumors.

Inhibition of GSK-3β in Rodent Insulinoma Cells Decreases Cell Viability and Cell Proliferation by Inducing Apoptosis and Cell Cycle Arrest—To elucidate the role of GSK-3β in insulinoma cell proliferation, we investigated whether GSK-3β inhibition would affect the proliferation of insulinoma cell lines. First, the effect of a commonly used GSK-3β inhibitor, LiCl, was examined in a mouse insulinoma cell line (MIN6—4N) (for derivation of MIN6—4N, see supplemental Fig. 3). LiCl (20 mM) was lethal to the cells (Fig. 7A), causing significantly reduced cell number and cell viability (Fig. 7B). Increased inactive GSK-3β-pSer-9 confirmed GSK-3β inhibition upon LiCl treatment (Fig. 7C). Similar results were obtained with two other GSK-3β inhibitors, SB415286 and kenpaullone (Fig. 7D). Treatment with 20 mM LiCl also led to decreased viability and proliferation in other insulinoma cell lines that express GSK-3β: MIN6 (mix), β–TC3 (mouse cell line generated by SV-40 T-antigen expression), and Rinm5F (rat cell line generated by UV irradiation) (Fig. 7, E–G).

In 20 mM LiCl-treated cell lysates of MIN6 cells, cleaved caspase 3 was detected, indicating that GSK-3β inhibition caused apoptosis (Fig. 8, A and B). We also examined whether GSK-3β inhibition in normal β cells would cause apoptosis. Normal β cell lines are not available. Therefore, the effect of 20 mM LiCl treatment was analyzed on normal mouse islets, which did not cause apoptosis, as assessed by TUNEL staining (Fig. 8C). Together, these results suggest that GSK-3β inhibition specifically reduces cell viability and proliferation in actively dividing insulinoma cell lines by inducing apoptosis.

We next investigated the mechanism by which GSK-3β inhibition reduces insulinoma cell proliferation. MIN6 cells treated with 20 mM LiCl showed a 16% increase in the number of cells in G2/M phase at the expense of cells in S phase of the cell cycle (Fig. 8, D and E). Thus, cells accumulated in G2/M phase might ultimately fail to enter the next cell cycle and die. These data indicate that GSK-3β modulates the cell cycle to control insulinoma cell proliferation.

DISCUSSION

Dysregulation of tissue differentiation factors could be potentially associated with aberrant cell proliferation and function in neoplasia of the target cell types. In this study, we investigated the modulation of a pancreatic β cell differentiation factor, HLXB9, and its role in the insulin-secreting β cell tumors (insulinomas) found in MEN1 syndrome, which are also the most common class of clinically significant and functional sporadic pancreatic neuroendocrine tumors. Identification of critical factors associated with insulinoma tumorigenesis is essential for the successful design of diagnostic and treatment modalities. Such investigations can also provide valuable insights into pathways that could be targeted for β cell mass expansion during conditions of β cell loss or increased insulin demand. We demonstrate that HLXB9 can be regulated by GSK-3β-mediated phosphorylation at Ser78/Ser80, and we
describe the importance of GSK-3β and pHLXB9 in MEN1-associated and sporadic insulinomas and in the proliferation of insulinoma cell lines.

GSK-3β-mediated Phosphorylation of HLXB9—The mouse and human HLXB9 protein consists of 404 and 401 amino acids, respectively, with a predicted molecular weight of 41 kDa.

However, on Western blot analyses, the endogenous and transfected myc-his-tagged HLXB9 migrate at a higher molecular weight, 55 and 64 kDa, respectively. The slow migration of HLXB9 predicts the presence of secondary modifications such as phosphorylation. The following evidence shows that HLXB9 undergoes GSK-3β-mediated phosphorylation at

![Image](https://example.com/image.png)
Ser-78/Ser-80 in vitro and in vivo. Phosphorylation at Ser-78/Ser-80 in HLXB9 was observed by phosphopeptide sequencing (27). On Western blot analysis, mh-HLXB9-wt is detected as a doublet band, whereas alanine substitution at Ser-78/Ser-80 (mh-HB9-AA) abolishes the top band of the doublet. The pHLXB9-specific antibody reacts with the top band of the doublet but does not detect the lower band or mh-HB9-AA. Colocalization of pHLXB9 with active GSK-3β in the nucleus of β-cells, recombinant pure GSK-3β phosphorylated GST-HLXB9 by in vitro kinase assay, and GSK-3β inhibitor treatment or Frat-1 expression in MIN6 cells reduced the level of pHLXB9.

Consistent with our observation of reduced expression of phospho-defective HLXB9 in β-cells, in human fetal neural stem cells inhibition of PI3K/AKT or overexpression of active GSK-3β increased HLXB9 mRNA and protein expression (31), implicating that GSK-3β-mediated phosphorylation could promote pHLXB9 stability. Also, decreased HLXB9 expression correlated with the presence of the inactive form of GSK-3β (31). Whether HLXB9 is phosphorylated on Ser-78/Ser-80 in neural stem cells or in other cell types remains to be determined.

Phosphorylation at Ser-78/Ser-80 Inactivates the Proapoptotic Function of HLXB9—we have shown previously that, similar to the function of HLXB9 in motor neurons (20), overexpression of HLXB9 in β cells (MIN6 cells) caused apoptosis (14). However, upon menin knockdown, overexpression of HLXB9 could not cause apoptosis (14). In this study, we found that, upon menin knockdown, the levels of pHLXB9 and GSK-3β were increased. These data indicate that phosphorylation of HLXB9 inactivates its proapoptotic function. Evasion of apoptosis is one of the hallmarks of cancer (32). The elevated levels of GSK-3β and pHLXB9 could drive the proliferation and accumulation of defective cells that evade apoptosis, leading to tumor formation.

Role of GSK-3β and pHLXB9 in MEN1-associated and Sporadic Insulinomas—In this study, we identified the elevated expression of GSK-3β and its active form and pHLXB9 as a common event in MEN1-associated mouse insulinomas and in sporadic human insulinomas. About 40–50% of sporadic insulinomas show somatic mutation or deletion of at least one copy of the MEN1 gene (9, 10, 33). Therefore, we expected to find elevated pHLXB9 and GSK-3β in at least 50% of the insulinomas. However, our data show that the majority of the insulinomas examined had significantly elevated GSK-3β (73%), and
pHLXB9 (74%) levels, supporting a tumor promoter role of the combined expression of a tissue differentiation factor (HLXB9) and its kinase (GSK-3β) in insulinomas. Thus, investigations that study familial tumor syndromes have the potential to unravel the mechanisms of tumorigenesis in their sporadic counterpart tumors.

The homeobox protein PDX1, which, like HLXB9, is essential in early pancreatic development, is stabilized by GSK-3β-mediated phosphorylation (34). The oncogenic activity of MafA, another β cell differentiation factor, was enhanced by GSK-3β-mediated phosphorylation (35). Therefore, in addition to HLXB9, GSK-3β targets such as PDX-1 and MafA may also activate oncogenic pathways during β cell tumorigenesis. Identification of the affected pathways will provide insights into β cell selective tumorigenesis in the pancreas with or without menin loss. Also, the elucidation of the precise mechanism by which menin regulates GSK-3β levels could provide insights into pathways that could regulate

**FIGURE 7.** GSK-3β inhibition reduces the viability and proliferation of three different rodent insulinoma cell lines. A, bright field microscopy images of MIN6 cells upon treatment with increasing concentrations of LiCl (0–20 mM). B, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for the viability and proliferation of MIN6 cells treated with LiCl (0–20 mM). **, p < 0.005. C, representative Western blot analysis of WCEs of MIN6 cells (from A) treated with 20 mM LiCl probed for the presence of GSK-3β and inactive GSK-3β (GSK-3β-pSer-9). P84 was used as a protein loading control. D, bright field microscopy images of MIN6–4N cells treated with the indicated GSK-3β inhibitors showing decreased cell viability and cell number. Dimethyl sulfoxide (DMSO) was used as a control. E, bright field microscopy images of the rodent insulinoma cell lines MIN6 (mix), β-TC3, and RINm5F treated with 20 mM LiCl. F, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for the viability and proliferation of MIN6 (mix), β-TC3, and RINm5F cells treated with 20 mM LiCl. *, p < 0.05; **, p < 0.005. G, Western blot analyses showing the expression levels of GSK-3β in MIN6 (mix), β-TC3, and RINm5F cells.
GSK-3β to understand menin loss-dependent and menin loss-independent tumorigenesis.

**GSK-3β Inhibition Reduces Tumor Cell Proliferation**—The tumor-promoter role of GSK-3β has been shown in various cancer cells: ovarian, colon, neuroblastoma, and non-endocrine pancreas and in neuroendocrine cell lines such as medullary thyroid cancer, BON1, gastrointestinal carcinoid, and H727 pulmonary carcinoid (36, 37). Treatment of these cell types with the GSK-3β inhibitor LiCl caused apoptosis because of cell cycle arrest in G2/M, and decreased proliferation and tumor growth (13). GSK-3β inhibition studies in rodent pancreatic islet-tumor cell lines (InR1G9 and TGP-61) have been reported in the context of the role of menin in the Wnt signaling pathway, where LiCl reduced cell proliferation (38). We show that LiCl is toxic to 3 different rodent insulinoma cell lines (MIN6, β-TC3 and RINm5F), and in MIN6 cells LiCl reduced proliferation and increased apoptosis because of cell cycle arrest in G2/M. Therefore, GSK-3β inhibition can block cell proliferation via similar mechanisms in neuroendocrine tumor cells.

The importance of Wnt signaling has been investigated in the context of β cell loss and diabetes where, in rodent cells, small molecule inhibitors of GSK-3β could improve β cell function and β cell mass (39). However, we found that, in actively dividing insulinoma cells, GSK-3β inhibition with LiCl could reduce cell proliferation and induce apoptosis, whereas LiCl did not cause apoptosis in normal mouse islets. It remains to be determined which signals up-regulate GSK-3β in β cell tumors (other than menin loss) and how GSK-3β inhibition would affect the growth of usually slow-growing β cell tumors.

**Therapeutic Potential of Inhibiting GSK-3β and pHLXB9 in Insulinomas**—Although we have not demonstrated a direct oncogenic effect of GSK-3β or pHLXB9, our data implicate a tumor-promoting role of GSK-3β and pHLXB9 in insulinomas that can be targeted by GSK-3β inhibitors such as LiCl to inhibit insulinoma cell proliferation. LiCl and other inhibitors of GSK-3β have been successfully used with no or minimal side effect in bipolar disorders for many years (40). The antimitotic effect of LiCl has been tested in mouse models (*in vivo* and *ex vivo*) of different cancers (13). Similarly, the mouse model of MEN1 could serve as an excellent preclinical model to test the efficacy of LiCl treatment in the context of insulinoma and other MEN1-associated endocrine tumors.

Our study establishes a targetable mechanism whereby the activity of a tissue differentiation factor (HLXB9) could be modulated by GSK-3β-mediated phosphorylation to explain the tissue specificity of tumors in the β cells of the pancreas. Whether similar mechanisms of tissue differentiation factor and kinase combination are active in other MEN1-associated tumors and their sporadic counterpart tumors remains to be determined.
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