2016-11-01

Overexpression of HDAC1 Induces Functional β-cell Mass

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

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Type 2 diabetes is a metabolic disorder that results in β-cell dysfunction and ultimate destruction, and leads to impaired glucose homeostasis. High rates of proliferation and differentiation of pancreatic β-cells occurs mostly during neonatal development. However, research shows these mechanisms remain intact as β-cell proliferation has been observed during pregnancy and obesity. We have shown that overexpression of the β-cell transcription factor Nkx6.1 is sufficient to induce β-cell proliferation. Exploration of the transcriptional targets of Nkx6.1 has identified histone deacetylase 1 (HDAC1) as a down-stream target of Nkx6.1. Here we demonstrate that HDAC1 overexpression is sufficient to induce β-cell proliferation, enhance β-cell survival upon exposure to apoptotic stimuli and maintains glucose stimulated insulin secretion (GSIS). Our data suggests overexpression of HDAC1 leads to p15/INK4b suppression, a cell cycle inhibitor, potentially explaining the mechanism behind these observed effects. These data demonstrate that HDAC1 overexpression is sufficient to induce β-cell proliferation and enhance cell survival while maintaining glucose stimulated insulin secretion.

Keyword: diabetes, β-cell, proliferation, HDAC1, p15/INK4b

ACKNOWLEDGEMENTS

I would like to thank first and foremost my mentor Jeff Tessem. Jeff has supported me throughout my undergraduate and graduate career. He allowed me to join his lab as an undergraduate with no experience and encouraged me to apply to graduate school; thank you for pushing me and opening my eyes to the many opportunities in science. Jeff has spent countless hours in the lab with me coaching me through this project and the many other projects I have worked on. I have learned a great deal from him and he has helped me grow as a student, teacher and leader. Without Jeff as my mentor I would not be where I am today. I would also like to thank my committee members: Ben Bikman and Chad Hancock. Thank you for mentoring me throughout this process, your guidance and expertise helped make this experience a success.

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Introduction

Type 1 (T1D) and Type 2 diabetes (T2D) affect more than 9% of the American population and the incidences of diabetes continue to increase at a startling rate. Decreased β-cell function is common with both T1D and T2D. Decreased function impedes insulin secretion and causes unstable blood glucose levels. Recent studies demonstrate that β-cell proliferation occurs most rapidly during embryogenesis and replication significantly decreases with age [1-3]. However, studies have shown that these mechanisms remain intact, as β-cell proliferation has been observed during pregnancy and obesity[4-6]. Modifying this tightly regulated proliferative mechanism could lead to potential treatments for diabetes.

We, and others, have shown that the homeobox β-cell transcription factor Nkx6.1 is sufficient to induce β-cell proliferation [7, 8]. Developmental studies have shown that the rapid period of embryonic β-cell replication is dependent on Nkx6.1 [9]. Furthermore, studies have shown that Nkx6.1 inactivation causes rapid-onset diabetes, demonstrating the importance of Nkx6.1 on β-cell proliferation and stability [10]. Our studies have shown that overexpression of Nkx6.1 in primary rat and human β-cells results in a significant induction of β-cell proliferation. Interestingly, while Nkx6.1 protein levels are observed as early as 24 hours after transduction with AdCMV-Nkx6.1, there is a 72-hour delay in β-cell proliferation. This suggests that early Nkx6.1 targets may be necessary to permit β-cells to reenter the cell cycle and proceed through cellular replication.

HDAC1 is a member of the histone deacetylation enzyme family. HDAC1 plays an essential role in the regulation of gene transcription, cell growth, and survival [11]. HDACs function by removing acetyl groups from histones, which results in decreased gene expression. HDACs have been shown to downregulate expression of cell cycle inhibitors through post translational

modifications. Recent studies suggest that HDAC1 plays an important role in cell cycle regulatory pathways, making HDACs an ideal target for inducing β-cell proliferation [12]. HDACs have also been shown to have anti-inflammatory activity and play a role in insulin signaling, thus potentially allowing the HDACs to enhance cell survival while maintaining insulin secretion [13-15].

HDACs have been shown to modulate specific cell cycle regulators. Researchers show inhibition of HDAC specifically upregulates the cell cycle regulator p15/INK4b leading to decreased cell growth [16]. Consistently, our data suggests over expression of HDAC1 leads to suppression of p15/INK4b. P15/INK4b is a specific inhibitor of the cyclin D-dependent kinases and impacts cells in the G1 phase of the cell cycle. We predict overexpression of HDAC1 leads to β-cell proliferation through p15/INK4b suppression.

In this study we demonstrate that HDAC1 is induced by transcription factor Nkx6.1. We show that HDAC1 is sufficient to induce β-cell proliferation, and is necessary for Nkx6.1 mediated proliferation. We demonstrate that HDAC1 overexpression in β-cells maintains glucose stimulated insulin secretion (GSIS) and increases β-cell survival when exposed to apoptotic stimuli. Furthermore, we demonstrate that overexpression of HDAC1 results in increased expression of cell cycle regulators and down-regulation of the cell cycle inhibitor p15/INK4b. Our data demonstrate that HDAC1 is necessary for Nkx6.1 mediated β-cell proliferation, and suggests that HDAC1 activation could be used to increase β-cell mass *ex vivo* for transplantation or *in vivo* to increase a patient's residual β-cell mass.

Methods

Cell Culture

832/13 rat insulinoma β-cells were cultured as previously described [17]. For chemical inhibition of HDAC1, cells were cultured with 200 nM trichostatin A (TSA) in DMSO for 24 hours.

Adenoviral Cloning and Preparation

Recombinant HDAC1 adenovirus was generated and purified as previously described [18]. HDAC1 overexpression was verified using RT-PCR. Adenoviruses expressing Nkx6.1 and GFP have been described elsewhere [7, 19].

Islet Isolation and Culture

Islet isolation from Wistar rats (Harlan, Indianapolis, IN, USA) was completed as described [17]. All animal studies were approved and performed in accordance with Brigham Young University's IACUC guidelines. Islet isolation, culture, and transduction were completed as described [17, 20, 21].

[^{3}H]-Thymidine Incorporation

DNA synthesis rates were measured by [methyl-3 H]-thymidine incorporation and were completed as described [17, 20, 21].

Cell Viability Assays

The 832/13 cells were transduced with AdCMV-GFP, AdCMV-HDAC1, or left untreated (No Virus-NV). After transduction, cells were treated with apoptotic stimulants (9.14uM etoposide, 0.31uM thapsigargin, and 2.01uM camptothecin) and viability was determined as described [22].

EdU Incorporation

Islet EdU (Invitrogen, Waltham, MA, USA) incorporation, with DAPI and insulin (DAKO, Carpinteria, CA, USA) counterstaining, was completed as described [21]. Three sections containing ≥400 nuclei were evaluated for EdU signals using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA) for each condition.

Glucose-Stimulated Insulin Secretion

Glucose-stimulated insulin secretion assays from rat islets or 832/13 cells were completed as described [20, 23]. Secreted insulin and total insulin were measured using a rat insulin RIA kit (MP Biomedicals, Santa Ana, CA, USA) [17].

RT-PCR

RNA isolation, cDNA synthesis, and RT-PCR were performed as described [17, 22] using Taq-man assays on demand probes and primers for GFP, Nkx6.1, HDAC1, Cdet1, Cdc2a, Cdc6, Cdk2, Cyclin A2, Cyclin B1, Cyclin D2, Cyclin E1, E2F1, FoxM1, AURKA, HDAC7, Nr4a1, Nr4a3, p15, p21, p27, p57, p16, p15, p18, p19 and PPIA (Life Technologies). Primer sequences are available upon request.

Statistical Analysis

Data was analyzed using two-tailed Student t test or two-way ANOVA where appropriate (Prism Software). Statistical significance was defined as $P< 0.05$.

Results

Nkx6.1 Induces HDAC1 Expression and is Necessary for Proliferation

We have shown that AdCMV-Nkx6.1 transduced primary rat and human β-cells proliferate after 72 hours [7] suggesting down-stream targets are needed for Nkx6.1 mediated proliferation. Analysis of previously published microarray data demonstrate that HDAC1 is induced within 48 hours of Nkx6.1 overexpression [21]. We measured HDAC1 mRNA levels in primary rat islets transduced with AdCMV-Nkx6.1 at 24, 48, 72 and 96 hours after transduction. Islets transduced with AdCMV-Nkx6.1 were compared to AdCMV-GFP transduced and untreated islets. HDAC1 mRNA levels were significantly increased 48 hours after Nkx6.1 overexpression, and remained elevated throughout the 96 hour time course (Fig 1A).

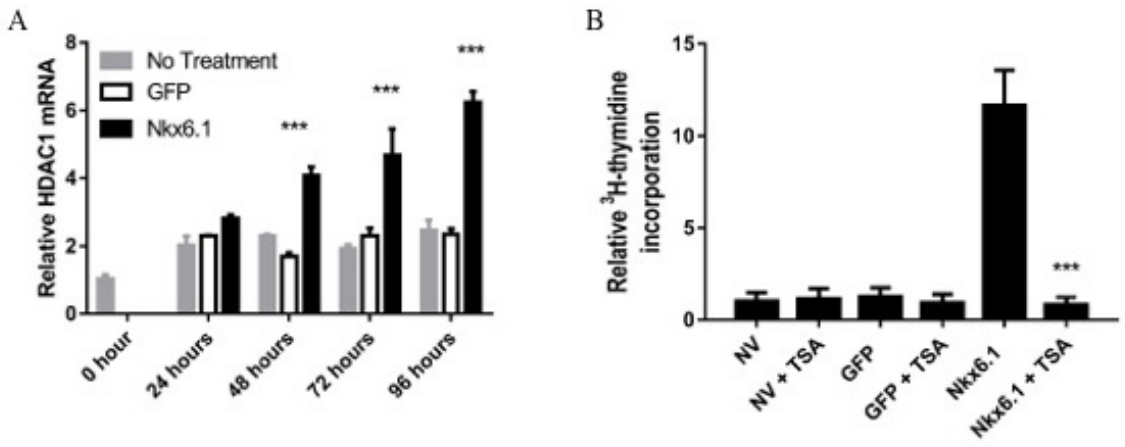

Figure 1. HDAC1 is induced by Nkx6.1 in β-cells, and HDAC activity is necessary for Nkx6.1 mediated proliferation.

(A) Rat islets were transduced with AdCMV-Nkx6.1, AdCMV-GFP or left untreated and HDAC1 mRNA levels were measured at 24, 48, 72 and 96 hours after transduction using RT-PCR. (B) Cells were transduced with AdCMV-Nkx6.1, Ad-CMV-GFP or left untreated and exposed to 200 nmol/ml TSA in order to chemically inhibit HDAC. Islet proliferation was measured by [^{3}H]-thymidine incorporation. For each experiment n=3-5 replicates per condition. ***p<0.001.

Furthermore, to determine if HDAC1 is necessary for Nkx6.1 mediated proliferation, primary rat islets were transduced with AdCMV-Nkx6.1 and compared to AdCMV-GFP or untreated islets and cultured for 96 hours. At 72-hours of culture the cells were treated with either HDAC inhibitor TSA or vehicle control (DMSO) and labeled with ^{3}H-thymidine. While Nkx6.1 is able to induce β-cells proliferation when cultured with DMSO, addition of TSA significantly inhibits Nkx6.1 mediated β-cell proliferation (Fig 1B). These data demonstrate that Nkx6.1 induces expression of HDAC1 and that HDAC1 activity is necessary for Nkx6.1 mediated proliferation.

Overexpression of HDAC1 Induces β-cells Proliferation

To determine if HDAC1 is sufficient to induce β-cell proliferation, 832/13 cells were transduced with AdCMV-GFP or AdCMV-HDAC1 and compared to untreated cells. HDAC1 overexpression increased 832/13 cellular proliferation as compared to untreated cells or cells expressing GFP (Fig 2A). In addition, we validated our results by transducing primary rat islets with AdCMV-GFP or AdCMV-HDAC1 and comparing to untreated islets. The primary islets were labeled with ^{3}H-thymidine and incorporation was measured at 24, 48, 72 and 96 hours to determine if HDAC1 induced proliferation of primary islets. Supporting our studies in the 832/13 β-cells, HDAC1 overexpression resulted in a significant increase in islet proliferation as compared to untreated islets or islets expressing GFP (Fig 2B).

To determine if HDAC1 is necessary for basal 832/13 β-cell proliferation, we transfected β-cells with siRNA against HDAC1, HDAC2, HDAC7, or HDAC10. ^{3}H-thymidine labeling was used to determine proliferation 96 hours after knockdown. Comparing untransfected and cells transfected with a scrambled control (siCTRL) demonstrated that siRNA mediated HDAC1 knockdown resulted in decreased ^{3}H-thymidine while knockdown of the other HDACs had no significant effect on cellular proliferation (Fig 2C).

Our data demonstrate that HDAC1 is sufficient and necessary for proliferation in primary rat islets. To determine if β-cells specifically replicate in response to HDAC1 overexpression, primary rat islets left untreated or transduced with AdCMV-GFP or AdCMV-HDAC1 were cultured with the thymidine analog Edu for 96 hours. Islets were dispersed and the percentage of insulin positive and Edu positive and insulin negative and EdU positive cells were calculated. Our findings demonstrate that untransduced islets and islets transduced with AdCMV-GRP had less than 1% of cells positive for EdU in the insulin positive and insulin

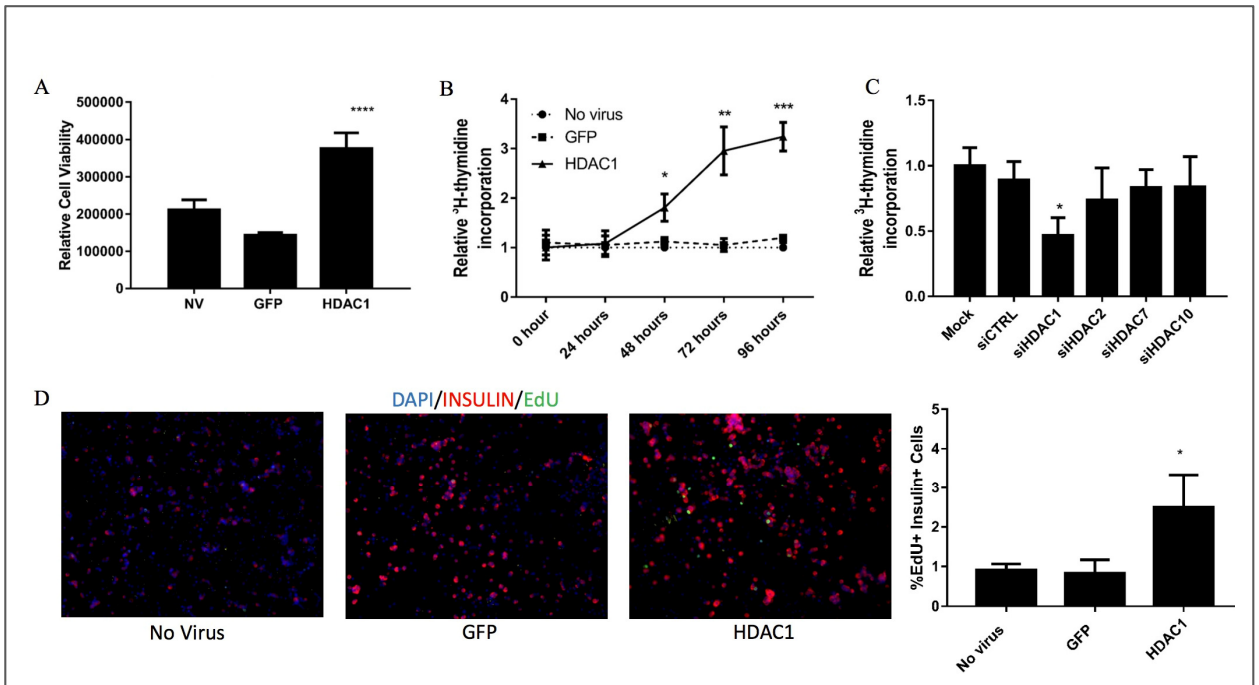

Figure 2. HDAC1 overexpression induces β-cell proliferation.

(A) 832/13 cells were transduced with AdCMV-GFP, AdCMV-HDAC1 or left untreated. Cell proliferation was determined 96 hours after transduction by cell counts. (B) Rat islets were transduced with AdCMV-GFP, AdCMV-HDAC1 or left untreated and 3H-thymidine incorporation was measured at 24, 48, 72 and 96 hours to determine proliferation. (C) 832/13 cells were transfected with siRNA against HDAC1, HDAC2, HDAC7, HDAC10. 3H-thymidine labeling was used to determine proliferation 96 hours after knockdown. For each experiment n=3-5 replicates per condition. (D) Rat islets were transduced with AdCMV-GFP, AdCMV-HDAC1 or left untreated and cultured with the thymidine analog EdU for 96 hours. Islets were dispersed and the percentage of Insulin+Edu+ and Insulin−EdU+ cells were calculated. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

negative population (Fig 2D). Comparatively, islets transduced with AdCMV-HDAC1 had significantly greater amounts of insulin positive Edu positive cells (Fig 2D). These data demonstrate that overexpression of HDAC1 induces β-cell specific proliferation.

HDAC1 Overexpression Maintains Glucose Stimulated Insulin Secretion

Our previous data demonstrated that Nkx6.1 overexpression, in addition to inducing β-cell proliferation, enhanced glucose stimulated insulin secretion (GSIS). Therefore, we sought to determine the effect of HDAC1 overexpression on GSIS. 832/13 β-cells were transduced with AdCMV-GFP, AdCMV-HDAC or cells were left untreated. Insulin secretion was measured 96 hours after transduction. No significant changes in insulin content were observed in any of our experimental conditions (Fig 3A). Furthermore, no differences were observed in insulin secretion at a non-stimulatory (2.5 mM glucose) or stimulatory (16.7mM glucose) environment (Fig 3B).

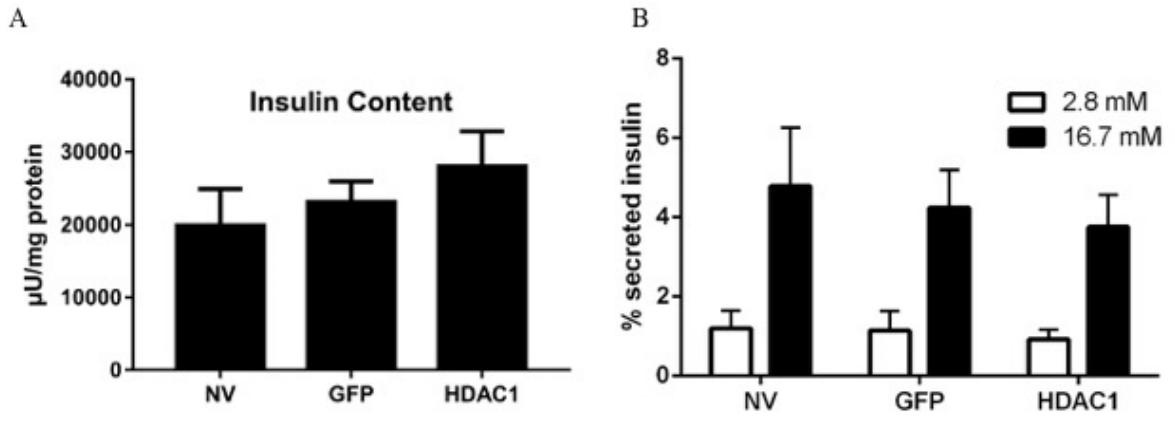

Figure 3. Overexpression of HDAC1 maintains glucose stimulated insulin secretion.

(A) Rat islets were transduction with AdCMV-GFP, AdCMV-HDAC1 or left untreated, and glucose stimulated insulin secretion was measured 96 hours later by insulin RIA. (B) Total insulin content was measured in 832/13 cells 96 hours after transduction with AdCMV-GFP, AdCMV-HDAC1 or left untreated. For each experiment n=3 replicates per condition.

These data demonstrate that HDAC1 overexpression, which results in enhanced β-cell proliferation, maintains insulin secretion and produces functional β-cells.

HDAC1 Overexpression Protects Against Apoptotic Stimuli

To determine the effects of HDAC1 overexpression on cell survival, 832/13 cells were treated with AdCMV-GFP, AdCMV-HDAC1 or left untreated. Twenty-four hours after adenoviral transduction, cells were cultured with apoptotic stimuli for 18 hours using camptothecin, thapsigargin, etoposide, or the vehicle control. Untransduced cells or cells transduced with AdCMV-GFP demonstrated between 40% and 50% viability after cultured with the respective apoptotic stimuli. Interestingly, cells overexpressing HDAC1 demonstrated a significant increase in cell viability when treated with etoposide or thapsigargin (Fig 4 A,B) but not with camptothecin (Fig 4A). These data demonstrate that HDAC1 overexpression protects 832/13 β-cells from etoposide or thapsigargin induced apoptosis.

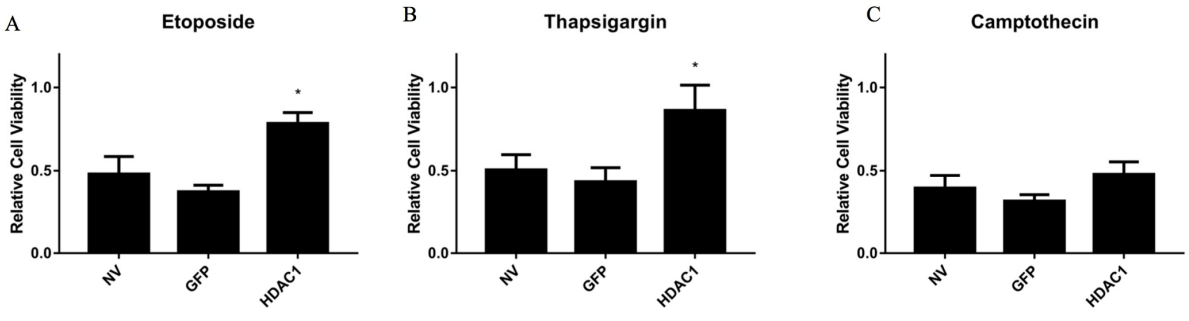

Figure 4. HDAC1 overexpression protects β-cells from drug induced apoptosis.

832/13 cells were transduced with AdCMV-GFP, AdCMV-HDAC1 or left untreated. Twenty-four hours after adenoviral transduction, cells were cultured for 18 hours in the presence of (A) camptothecin, (B) thapsigargin, (C) or etoposide. Cell counts were completed 18 hours after drug treatment and cell viability was determined by comparing the untreated population to the treated population. For each experiment n=3-5 per condition. *$p<0.05$.

HDAC1 Overexpression Induces Changes in Cell Cycle Gene Expression

To determine if HDAC1 modifies specific cell cycle genes, primary rat islets were transduced with AdCMV-GFP, AdCMV-HDAC1 or left untreated. Islets were harvested 96 hours after adenoviral transduction, and mRNA expression of cell cycle genes were measured using RT-PCR. RT-PCR were performed as described [17, 22] using Taq-man assays on demand probes and primers for GFP, Nkx6.1, HDAC1, Cdet1, Cdc2a, Cdc6, Cdk2, Cyclin A2, Cyclin B1, Cyclin D2, Cyclin E1, E2F1, FoxM1, AURKA, HDAC7, Nr4a1, Nr4a3 and PPIA (Life Technologies). These data demonstrate HDAC1 overexpression promotes S to G2 cell cycle progression (Fig 5A).

Furthermore, to determine the specific mechanism by which HDAC1 upregulates cell cycle genes, primary rat islets were transduced with AdCMV-GFP, AdCMV-HDAC1 or left untreated. Islets were harvested after 96 hours and mRNA expression of cell cycle regulators

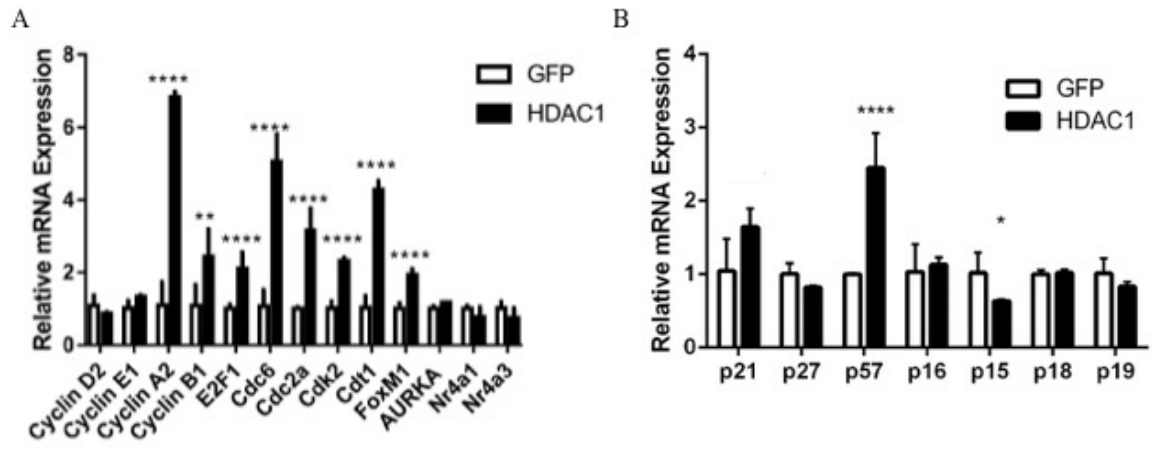

Figure 5. HDAC1 overexpression induces changes in cell cycle gene expression. Primary rat islets were transduced with AdCMV-GFP or AdCMV-HDAC1. Cells were harvested 96 hours after transduction and mRNA expression for (A) a panel of cell cycle activators and (B) cell cycle inhibitors was measured using RT-PCR. For each experiment n=3-5 per condition. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

were measured using RT-PCR. RT-PCR were performed as described [17, 22] using Taq-man assays on demand probes and primers for p15, p21, p27, p57, p16, p15, p18, p19 and PPIA (Life Technologies). Islets overexpressing HDAC1 showed a significant increase in p57 but more importantly a decrease in p15/INK4b expression, a known cell cycle inhibitor (Fig 5B). Together, these data suggest that HDAC1 overexpression may result in suppression of p15/INK4b expression, thus allowing greater S to G2 cell cycle progression.

P15/INK4b Expression is Suppressed with HDAC1 Overexpression

Finally, to verify the previous p15/INK4b suppression, siCTRL and sip15/INK4b treated 832/13 β-cells were transduced with AdCMV-GFP, AdCMV-HDAC1 or left untreated. As previously shown, HDAC1 overexpression leads to greater proliferation when compared to untreated cells or cells expressing GFP. Moreover, cells deficient for p15/INK4b had enhanced proliferation, and cells deficient for p15/INK4b that overexpressed HDAC1 had the greatest

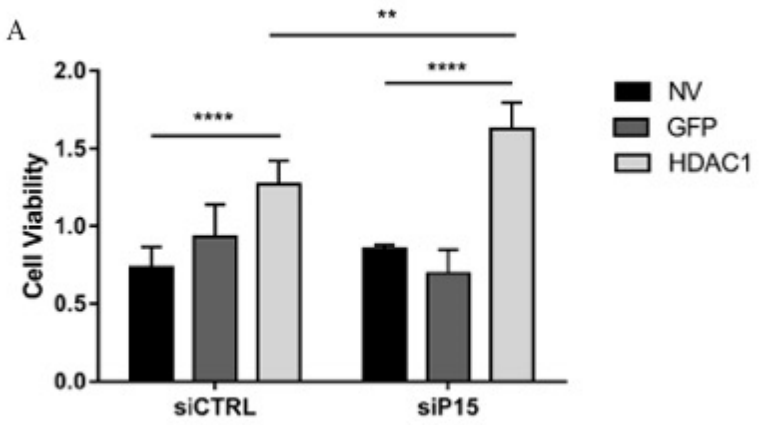

Figure 6. p15/INK4b knockdown enhances HDAC1 mediated β-cells proliferation.

(A) siCTRL and sip15/INK4b treated 832/13 cells were transduced with AdCMV-GFP, AdCMV-HDAC1 or left untreated. Cell counts were completed 96 hours after transduction to determine cell viability. For each experiment n=3-5 per condition. **p<0.01, ****p<0.0001.

level of proliferation (Fig 6A). These data demonstrate that p15/INK4b inhibits β-cell proliferation, and that the loss of p15/INK4b results in improved HDAC1 mediated proliferation (presumably through stimulation of the cell cycle activators previously demonstrated).

Discussion

All forms of diabetes ultimately result in β-cell destruction. While most β-cell proliferation is limited to embryogenesis and the neonatal period [1], the process can be activated during circumstances such as pregnancy and obesity, demonstrating that the process is fundamentally intact [4, 5]. Thus, with proper stimulation, this tightly regulated process could be activated to increase functional β-cell mass as a treatment modality for diabetic individuals through *ex vivo* expansion of β-cells as sources for islet transplantation or *in vivo* expansion of endogenous β-cells.

The β-cell transcription factor Nkx6.1 is sufficient to induce β-cell proliferation [7, 21]. Nkx6.1 mediated proliferation is observed 72 hours after overexpression in primary β-cells, suggesting early Nkx6.1 targets may be necessary to permit β-cells to reenter the cell cycle and proceed through cellular replication. Previously published microarray data demonstrate that HDAC1 is induced within 48 hours of Nkx6.1 overexpression [21]. In this study, we show that HDAC1 is induced through Nkx6.1 overexpression in β-cells. We demonstrate that HDAC1 activity is necessary for Nkx6.1 mediated β-cell proliferation, and that HDAC1 knockdown significantly decreases β-cell proliferation. Our data demonstrates that HDAC1 overexpression in primary rat β-cells or the INS-1 832/13 β-cell line is sufficient to induce proliferation, while maintaining insulin secretion and insulin content. We demonstrate that HDAC1 overexpression is sufficient to protect INS-1 832/13 β-cells from etoposide and thapsigargin induced cell death. Concomitant with the observed β-cell proliferation, we demonstrate that HDAC1 overexpression

enhances expression of key cell cycle genes, as well as decreases expression of the cell cycle inhibitor p15/INK4b. Finally, we demonstrate that p15/INK4b knockdown in the INS-1 832/13 β-cell line is sufficient to enhance HDAC1 mediated proliferation. Together, our data demonstrates that HDAC1 is able to induce β-cell proliferation by decreasing expression of the cell cycle inhibitor p15/INK4b.

Interestingly, all HDACs (1-11) are expressed in INS-1 cells and rat islets, suggesting their conceivable function in β-cell growth [24]. Studies propose HDACs play a vital role in development of pancreatic endocrine tissue. Specifically, one study demonstrates HDAC1 expression is observed in pancreatic tissue during development and expression decreases with aging [25]. These results mimic β-cell proliferation with increased rates during neonatal development and β-cell proliferation absent during maturity. Other studies showed that deletion of HDAC enzymes had deleterious effects on animals and completely blocked early embryonic β-cell development, demonstrating the importance of HDAC's in β-cell development [26-28]. In mature β-cells, deletion of specific HDACs in mice and cell models decreases pancreatic insulin content and increased susceptibility to developing type 2 diabetes [29]. Our data is supported by these findings and demonstrate a correlation between HDAC1 expression and β-cell proliferation. Taken together, these data describe a critical role for HDAC1 in β-cell development.

The results demonstrating HDAC1 overexpression provided apoptotic protection are somewhat controversial. A recent study indicated that HDAC1 knock down significantly protected INS-1 cells from cytokine induced apoptosis [30], and other studies demonstrated that HDAC inhibition can prevent inflammation and reduce apoptosis [30-33]. While these studies have focused on the use of HDAC inhibitors, we have not found any studies which focus on the

protective effects of HDAC1 overexpression. In addition, in comparing our findings and these previously published findings there are differences in the type of apoptotic stimuli, cell lines, and methods of HDAC inhibition. Our data is supported by other reports that demonstrate in β-cells that other HDACs act as pro-apoptotic but concluded HDAC1 concomitantly did not add to the apoptotic phenotype and may in fact be counterproductive [34]. In addition, other reports looking at HDAC3 and HDAC1 inhibition in islets showed similar results and concluded knockdown of HDAC1 did not provide apoptotic protection [35, 36]. Taken together, these data do support a role for HDAC1 in providing apoptotic protection; however, at the moment it is unclear how HDAC1 activity stimulates this protection in varying cell lines.

Class I HDACs primarily function in the nucleus and are responsible for removing acetyl groups from histone lysine residues [27]. Histones are responsible for packaging the DNA into chromatin complexes [37] and posttranslational modification of these chromatin complexes regulates gene transcription and other cellular functions. HDACs removal of acetyl groups generally results in a more compact chromatin structure resulting in decreased gene expression [38]. Our findings and others demonstrate that HDAC1 directly regulates cell cycle genes and modifies p15/INK4b expression [16]. p15/INK4b is a known cell cycle inhibitor and studies show upregulation leads to cell cycle arrest [39, 40]. With HDAC1 overexpression we also see an induction of p57/KIP2. However, these results have been observed with various β-cell proliferative genes, and should only counteract proliferative actions [21, 41]. Our findings of HDAC1 cell cycle modifications are supported by other findings that demonstrate a correlation between HDAC1 and suppression of cell cycle inhibitors [42, 43]. Taken together, these data describe a potential pathway by which HDAC1 overexpression mediates β-cell proliferation.

Conclusion

In summary, we have demonstrated that HDAC1 acts as a down-stream Nkx6.1 target that is necessary and sufficient for β-cell proliferation. The increased proliferation occurs with maintenance of GSIS and increased protection from apoptotic stimuli. Our data demonstrate that HDAC1 overexpression results in enhanced expression of cell cycle genes and decreased expression of the cell cycle inhibitor p15/INK4b. Our data suggests that induction of proliferation is an effect of enhanced HDAC activity resulting in decreased p15/INK4b expression, presumably through decreased acetylation of the p15/INK4b. These findings and others directly link decreased cell cycle regulators to increased functional β-cell mass [17, 21, 22]. These results suggest that inhibition of the cell cycle inhibitor, specifically here p15/INK4b, may be a critical step for increasing β-cell proliferation as a treatment modality for individuals with diabetes.

Future Direction

Our goal is to determine the mechanism by which HDAC1 and p15/INK4b interact in order to induce β-cell proliferation. We will use chromatin immunoprecipitation as previously described [7, 17, 21] to quantify the amount of acetylation at the p15/INK4b promoter site. With overexpression of HDAC1, we expect to measure decreased acetylation at the promoter site. With chemical inhibition of HDAC1 through TSA, we expect the opposite effect of increased acetylation to be observed. We also hypothesize that overexpression of HDAC1 will result in an increase of HDAC1 at this site, as well as a reduced effect when TSA is present. Together, these experiments will help us understand if a direct binding relationship exists between HDAC1 and the p15/INK4b promoter.

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APPENDIX A

Problem Statement

Decreased β-cell function is a common characteristic with Type 1 and Type 2 diabetes. Decreased β-cell function impedes insulin secretion and causes unstable glucose levels in the body. Increasing β-cell mass and function could be used as a treatment for diabetes. β-cell proliferation occurs most rapidly during embryogenesis and replication decreases immensely with age [1-3]. However, studies have shown that these mechanisms remain intact, as β-cell proliferation has been observed during pregnancy and obesity [4-6]. Modifying this tightly regulated proliferative mechanism could lead to potential treatments for diabetes.

During these unique periods of mature β-cell replication, expression of the β-cell transcription factor Nkx6.1 increases and is necessary for the development of insulin-producing β-cells [7, 8]. Furthermore, Nkx6.1 inactivation causes rapid-onset diabetes, demonstrating the importance of Nkx6.1 on β-cell proliferation and stability [10]. Our studies have shown that overexpression of Nkx6.1 in primary rat and human β-cells results in a significant induction of β-cell proliferation after 72-hours of viral transduction. This delay suggests that early Nkx6.1 targets may be necessary to permit β-cells to reenter the cell cycle and proceed through cellular replication.

We identified HDAC1 as a gene upregulated as early as 48 hours after introduction of Nkx6.1 expression. HDAC1 is a member of the histone deacetylation enzyme family and plays an essential role in the regulation of gene transcription, cell growth, and cell survival [11]. Recent studies suggest that HDAC1 plays an important role in cell cycle regulatory pathways, making HDACs an ideal target for inducing β-cell proliferation [12].

Increasing proliferation and function of β-cells through HDAC1 overexpression could potentially be used as a cure for diabetes by increasing β-cell mass for ex vivo transplantation or in vivo proliferation of residual β-cells. With the incidences of diabetes increasing at a starting rate, a treatment for diabetes is an essential need for the diabetic community.

Purpose

The purpose of this research is to determine the following:

1. Does overexpression of HDAC1 induce β-cell proliferation in INS1 832/13 cells and primary rat islets?
2. Can overexpression of HDAC1 enhance glucose stimulated insulin secretion (GSIS)?
3. Does overexpression of HDAC1 exhibit protective properties and increases β-cell survival when exposed to apoptotic stimuli?

APPENDIX B

Literature Review

Type 1 and Type 2 diabetes are a disease of improper glucose homeostasis due to β-cell dysfunction and loss. According to the American Diabetes Association, an estimated 29.1 million Americans suffer from diabetes and on average 1.4 million Americans are diagnosed each year. As diabetes incidences continue to increase at a startling rate, treatment options and potentially a cure are essential for these inflicted individuals. Currently, minimal research is available regarding the β-cell proliferation pathway during embryogenesis and even less is known about inducing ex vivo mature β-cell proliferation. However, from multiple study it is known β-cell proliferation predominately occurs during adolescence and little proliferation occurs after the first three years of life [1-3]. Nevertheless, studies show that the proliferation pathway involved in β-cell propagation remains intact throughout maturity [4-6]. Understanding the mechanism behind β-cell proliferation is an important step to modifying the pathway to increase β-cell mass in diabetic patients, potentiating a cure for the disease.

A specific homeobox transcription factor known in the β-cell pathway is Nkx6.1. This specific homeobox transcription factor is upregulated during the β-cell proliferation pathway and expression of this specific gene is necessary for β-cell growth [7, 8]. Our research showed a down-stream target induced during Nkx6.1 mediated β-cell proliferation was the enzyme histone deacetylase 1 (HDAC1). HDAC1 is involved in posttranslational modification of DNA and is responsible for monitoring the activity of histone acetyltransferases. Studies showed that deletion of HDAC1 and HDAC2 completely blocked early β-cell development eluding to HDAC1's function in β-cell expansion [26]. Therefore, suggesting HDAC1 overexpression may upregulate the β-cell proliferation pathway. Our preliminary data and the literature suggests HDAC1 plays

an essential role in the β-cell proliferation pathway and overexpression could activate this highly regulated pathway to increase functional β-cell mass.

HDAC enzymes have been classified into four groups based on function and DNA sequence similarities [38]. HDAC1 is a member of the class I HDAC family and plays a specific role in cell proliferation and regulation. Class I HDACs are primarily expressed in the nucleus and are responsible for removing acetyl groups from lysine residues predominantly on histones [27]. Histones are proteins found in the nucleus of each cell and are responsible for packaging the DNA into chromatin complexes [37]. Posttranslational modification of these chromatin complexes regulates gene transcription and other cellular functions. Two enzymes involved in post-translational modifications are histone acetyl transferase [34] and HDAC [38]. HAT is an enzyme responsible for transferring acetyl groups to the histones while HDACs balance this act by removing these acetyl groups. HATs generally increase gene expression while HDACs generally result in a more compact chromatin structure resulting in decreased gene expression. HDACs critical role in chromatin remodeling makes this class of enzymes important regulators of gene transcription, cell proliferation, and apoptosis.

Studies report HDAC1 as an essential modulator for cell survival and proliferation [44]. To determine the need for HDAC1 in cell proliferation, studies disrupted HDAC1 and observed deleterious effects on animals. Knockdown of HDAC1 led to embryo lethality and decreased embryonic stem cell proliferation [27, 28]. In addition to these findings, deletion of HDAC1 in mouse embryonic fibroblast cells showed slowed proliferation and increased apoptosis [28]. Other studies showed that during times of increased proliferation, HDAC1 expression was increased and was necessary for normal cell cycle progression [45]. Each of these studies verifies

HDAC1's essential role in cell survival and proliferation and suggests HDAC1 could be a viable target to induce functional β-cell proliferation.

HDAC1 also play an essential role in the inflammatory pathway; however, it's beneficial effects are controversial. Diabetes is categorized as an inflammation related disease and therefore reducing levels of inflammation could lead to greater β-cell proliferation and survival. Studies report HDACs as regulators of the inflammatory pathway by controlling macrophage and dendritic cell function. HDAC1 has direct connections to the inflammation signaling pathway and acts as a controller for interferon and hypoxia-inducible factor-1 alpha inflammatory responses [26]. A direct link between HDAC1 and inflammatory cytokine production has been observed and again emphasized the important regulatory role of HDAC1 in the inflammatory pathway [46]. Few studies explore the effects of HDAC overexpression and in fact, many studies see positive effects of HDAC inhibitors on inflammatory diseases. Contrary to what we desire, in many inflammatory related diseases HDAC inhibition can rescue cells from inflammation and reduce apoptosis [30-33]. However, these discrepancies many be related to diverse cell lines, varying HDAC inhibition and differing apoptotic stimuli. Nonetheless, subsequent studies have reported in β-cells other HDACs as pro-apoptotic but concluded HDAC1 concomitantly does not add to the apoptotic phenotype and may in fact be counterproductive [34]. Taken together, these studies suggest a direct link between HDAC1 and the inflammatory pathway which could lead to increased β-cell survival rates and provide apoptotic protection.

HDAC1 has the ability to directly modulate the cell cycle by regulating specific cell cycle genes making it an ideal target for β-cell proliferation. It has been observed that deletion of HDAC1 and HDAC2 leads to upregulation of p21 and p57 which are regulators of the G1 phase in the cell cycle [47]. Upregulation of these cell cycle regulators decreases cell cycle progression

by blocking cyclin-CDK complexes and inhibits cell growth. Other studies show a direct correspondence between HDAC1 and suppression of cell cycle inhibitors [42, 43], therefore suggests a possible mechanism by which HDAC may potentiate a way for mature β-cells to proliferate. This regulator quality of HDAC1 makes it an ideal target for enhancing β-cell proliferation.

In conclusions, many studies have revealed the correlation between HDAC1 and cell proliferation. We have seen HDAC1 plays a key role in the development of β-cells and could act as potential target for mature β-cell propagation. It is also inevitable that HDAC1 plays an essential role in the inflammatory pathway and that overexpression of HDAC1 may have a protective effect on β-cell apoptosis. HDAC1 also has the ability to directly regulate the cell cycle and can therefore modulate this pathway to allow for greater cell cycle progression. Together, these properties suggest HDAC1 could be a potential target for β-cell proliferation and could provide protection against apoptotic stimuli, therefore increasing functional β-cell mass.

APPENDIX C

Presentations

EXPERIMENTAL BIOLOGY 2016

Overexpression of HDAC1 induces functional β-cell mass.
Carrie Draney, Amanda Hobson, Jeffery Tessem

β-cell mass is lost in both major forms of diabetes. Mature β-cells have restricted proliferative capacity. Studies aimed at increasing β-cell mass frequently have the unwanted side effects of decreased glucose stimulated insulin secretion (GSIS) or increased apoptotic rates. Understanding the molecular pathways that enhance β-cell mass while maintaining or improving GSIS and the response to apoptotic stimuli could be used as a potential treatment for diabetes. Overexpression of the β-cell transcription factor Nkx6.1 induces β-cell proliferation, enhances GSIS and protects against apoptosis. Here we present data demonstrating that HDAC1 is induced by Nkx6.1, in sufficient to induce β-cell proliferation, and is necessary for Nkx6.1 mediated proliferation. HDAC1 overexpression maintains GSIS and increases β-cell survival when exposed to apoptotic stimuli. Overexpression of HDAC1 results in increased expression of cell cycle controlling genes and down-regulation of the cell cycle inhibitor p15/INK4b. We present a model by which HDAC1 is necessary for Nkx6.1 mediated β-cell proliferation by down-regulating p15/INK4b expression.

Overexpression of HDAC1 induces functional β-cell mass

Carrie Draney, Amanda Hobson and Jeffery Tessem

Nutrition, Dietetics and Food Science Department, College of Life Sciences, Brigham Young University, Provo, UT

Abstract

β-cell mass is lost in both major forms of diabetes. Mature β-cells have restricted proliferative capacity. Studies aimed at increasing β-cell mass frequently have the unwanted side effects of decreased glucose stimulated insulin secretion (GSIS) or increased apoptotic rates. Understanding the molecular pathways that enhance β-cell mass while maintaining or improving GSIS and the response to apoptotic stimuli could be used as a potential treatment for diabetes. Overexpression of the β-cell transcription factor Nkx6.1 induces β-cell proliferation, enhances GSIS and protects against apoptosis. Here we present data demonstrating that HDAC1 is induced by Nkx6.1, in sufficient to induce β-cell proliferation, and is necessary for Nkx6.1 mediated proliferation. HDAC1 overexpression maintains GSIS and increases β-cell survival when exposed to apoptotic stimuli. Overexpression of HDAC1 results in increased expression of cell cycle controlling genes and down-regulation of the cell cycle inhibitor $p15^{INK4b}$. We present a model by which HDAC1 is necessary for Nkx6/1 mediated β-cell proliferation by down-regulating $p15^{INK4b}$ expression.

Overexpression of HDAC1 induces *β*-cell proliferation

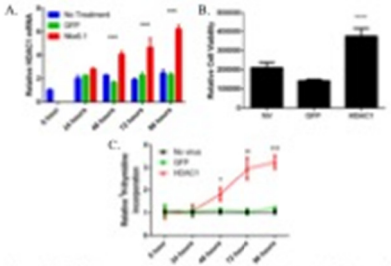

Figure 1. **HDAC1 overexpression induces β-cell proliferation.** (A) Primary rat islets were transduced with AdCMV-GFP or AdCMV-Nkx6.1. Cells were harvest every 24 hours from isolation to 96 hours after transduction and mRNA was measured using RT-PCR. (B) 832/13 INS-1 cells were transduced with AdCMV-GFP or AdCMV-HDAC1. Cell viability was determined at 96 hours by comparing the untreated population to the treated population. (C) Primary rat islets were transduced with AdCMV-GFP or AdCMV-Nkx6.1 and were cultured in the presence of ^{3}H-thymidine for 24 hour periods. Cells were harvest at 24, 48, 72, or 96 hours. Overexpression of HDAC1 in primary islets and the cell line induced β-cell proliferation.

Knockdown of HDAC1 inhibits *β*-cell proliferation

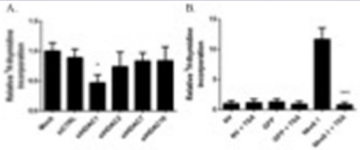

Figure 2. **Genetic and chemical knockdown of HDAC1 decreases β-cell proliferation rate.** (A) 832/13 INS-1 cells were transfected with siRNA for HDAC1, HDAC2, HDAC7, HDAC10, or siCTRL sequences and were cultured in the presence of ^{3}H-thymidine. Genetic knockdown of HDAC1 resulted in decreased ^{3}H-thymidine incorporation. (B) Primary rat islets were transduced with AdCMV-GFP or AdCMV-Nkx6.1. Islets were cultured for 72 hours at which ^{3}H-thymidine was added. Islets were harvest at 96 hours. Islets were treated with vehicle or 200nmol/L of HDAC1 inhibitor Trichostatin A (TSA) for the final 24 hours of the labeling period. Chemical knockdown of HDAC1 resulted in decreased Nkx6.1 mediated β-cell proliferation.

Overexpression of HDAC1 induces mRNA expression of many cell cycle genes

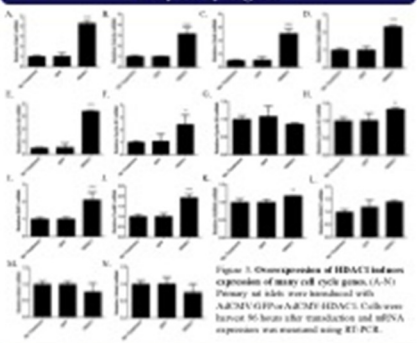

Figure 3. **Overexpression of HDAC1 induces expression of many cell cycle genes.** (A-N) Primary rat islets were transduced with AdCMV-GFP or AdCMV-HDAC1. Cells were harvest 96 hours after transduction and mRNA expression was measured using RT-PCR.

HDAC1 protects against *β*-cell drug induced apoptosis

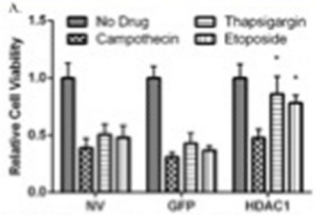

Figure 4. **HDAC1 overexpression protects INS-1 β-cells from apoptosis.** (A) 832/13 INS-1 cells were transduced with AdCMV-GFP or AdCMV-HDAC1. Twenty-four hours after adenoviral transduction, cells were cultured for 18 hours in the presence of camptothecin, thapsigargin, etoposide, or the vehicle control. Cell counts were completed 18 hours after drug treatment. Cell viability was determined by comparing the untreated population to the treated population. Cells overexpressing HDAC1 presented greater viability when treated with thapsigargin or etoposide than control cells.

Overexpression of HDAC1 maintains Glucose Stimulated Insulin Secretion

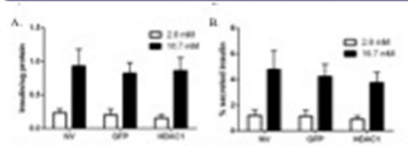

Figure 5. **Overexpression of HDAC1 maintains glucose stimulated insulin secretion.** (A) Total insulin content was measured in 832/13 INS-1 cells at 96 hours after transduction with AdCMV-GFP or AdCMV-HDAC1. No significant difference was observed in total insulin content. (B) Glucose stimulated insulin secretion was measured 96 hours after transduction with AdCMV-GFP or AdCMV-HDAC1 in media containing 2.5 mM glucose or 16.7 mM glucose for 1 hour. No significant decrease in glucose stimulated insulin rate was observed with HDAC1 overexpression.

Overexpression of HDAC1 decreases p15 expression

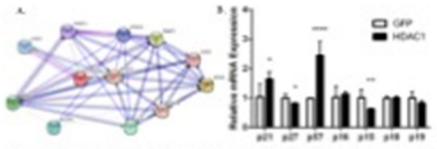

Figure 6. **Overexpression of HDAC1 results in decreased p15 mRNA levels.** (A) Previous published microarrays measured cycle cycle genes turned on in primary rat islets overexpressing HDAC1. Cdkn2b (p15) expression is connected to HDAC1 overexpression. (B) Primary rat islets were transduced with AdCMV-GFP or AdCMV-HDAC1. Cells were harvest 96 hours after transduction and mRNA was measured using RT-PCR. Cells overexpressing HDAC1 had significantly lower levels of p15.

Conclusion

Acknowledgements

A special thanks to J. Tessem and my fellow lab members for being mentors throughout this course of this project and the BYU Office of Research and Creative Activities for funding this research project.