The Transcription Factor Myt3 Acts as a Pro-Survival Factor in β-cells

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Abstract

Aims/Hypothesis: We previously identified the transcription factor Myt3 as specifically expressed in pancreatic islets. Here, we sought to determine the expression and regulation of Myt3 in islets and to determine its significance in regulating islet function and survival.

Methods: Myt3 expression was determined in embryonic pancreas and adult islets by qPCR and immunohistochemistry. ChIP-seq, ChIP-qPCR and luciferase assays were used to evaluate regulation of Myt3 expression. Suppression of Myt3 was used to evaluate gene expression, insulin secretion and apoptosis in islets.

Results: We show that Myt3 is the most abundant MYT family member in adult islets and that it is expressed in all the major endocrine cell types in the pancreas after E18.5. We demonstrate that Myt3 expression is directly regulated by Foxa2, Pdx1, and Neurod1, which are critical to normal β-cell development and function, and that Ngn3 induces Myt3 expression through alterations in the Myt3 promoter chromatin state. Further, we show that Myt3 expression is sensitive to both glucose and cytokine exposure. Of specific interest, suppressing Myt3 expression reduces insulin content and increases β-cell apoptosis, at least in part, due to reduced Pdx1, Mafa, Il-6, Bcl-xl, c-ιap2 and Igfr1 levels, while over-expression of Myt3 protects islets from cytokine induced apoptosis.

Conclusion/Interpretation: We have identified Myt3 as a novel transcriptional regulator with a critical role in β-cell survival. These data are an important step in clarifying the regulatory networks responsible for β-cell survival, and point to Myt3 as a potential therapeutic target for improving functional β-cell mass.

Introduction

Our understanding of the transcriptional networks regulating gene expression during β-cell genesis and function is rapidly expanding [1,2,3,4]. The importance of these networks is exemplified by the fact that several monogenic forms of diabetes are linked to defects in transcription factors, namely Hnf1, Hnf4, Igfr1, Myt1, Myt1l, Myt3 and Neurod1, which are critical to normal β-cell genesis and function [5,6,7,8,9]. In addition, transcription factors play critical roles in glucose-stimulated insulin secretion, via the regulation of vesicle docking (Foxa2), glucose sensing (Pdx1), glucose–, KCl– and arginine-induced insulin secretion (Maf6), oxidative metabolism of glucose, and insulin secretion complex formation (Neurod) [10,11,12,13]. Despite this, our understanding of these processes is far from complete and we anticipated that the identification of novel transcriptional regulators expressed specifically in β-cells, and the determination of their functional roles would help further elucidate these complex networks.

In previous work [2], we identified 2,536 genes with pancreas-enriched expression, including Myelin transcription factor 3 (Myt3), also known as Suppression of tumourogenicity 18 (St18). Myt3 is part of the C2HC-type zinc-finger, or MYT, family of transcription factors that in vertebrates is composed of three genes: Myt1, Myt1l and Myt3 [14,15,16]. These factors function as both positive and negative regulators of gene expression [14,15,16,17]; and both Myt1 and Myt1l have been implicated in the regulation of neuronal cell fate determination, proliferation and differentiation [14,15,18,19]. Myt3 was initially identified as a transcriptional repressor in rat brain that strongly bound to bipartite AASTT motif [16]. Myt3 suppression and promoter hypermethylation were subsequently determined to be prevalent in primary breast tumours [20], while Myt3 degradation by miR-125b-2 was implicated in the development of megakaryoblastic leukaemia [21]. In addition, in dermal fibroblasts MYT3 regulates TNFα induced pro-inflammatory and pro-apoptotic gene expression, including Il-1α and Il-6 [22].
In pancreas, to date, studies on the MYT family of transcription factors have been limited to Myt1. These studies determined that a Myt1/Ngn3 feed forward loop is required for pancreatic endocrine cell specification, and as a result Myt1 disruption results in impaired endocrine cell function, including glucose tolerance and insulin secretion [23,24,25]. Interestingly, Myt3 expression is up-regulated in endocrine cells lacking Myt1 [25], suggesting Myt3 plays a compensatory role. Despite these findings no previous studies have assessed Myt3’s significance in pancreatic islet function. To address this deficit we assess Myt3’s expression in pancreas development, it’s regulation by key transcription factors, and its role in islet function and survival.

Methods

In situ Hybridisation and Immunofluorescence
Probes for in situ hybridization were generated using the primers: Myt3 forward: 5’-gggtccagacagcagaa-3’; reverse: 5’-agttcctagcctggac-3’ and cloned into pCRII-TOPO (Invitrogen). RNA probes were subsequently labeled with DIG-UTP using T7/SP6 polymerase reactions with 1 µg of linearized plasmid (Roche). In situ hybridization of E9.5, E14.5 embryo and isolated islet sections was performed as described in Prando et al. [26]. In short, cryostat sections (10 µm) were treated with 1 µg/ml proteinase K and fixed in 4% paraformaldehyde. Sections were hybridized with 1 µg/ml of probe overnight at 70°C. High stringency washes were used to remove unbound probe. Sections were subsequently blocked with 10% FBS, 1% Blocking Reagent (Roche) and incubated with anti-digoxigenin-alkaline phosphatase antibody diluted 1:1000. Slides were washed and color developed using BM purple as a substrate (Roche).

Immunohistochemistry was performed on islet cryo-sections following in situ hybridisation. Sections were stained with guinea pig anti-Insulin (1/100; Stem Cell Technologies Inc.) or guinea pig anti-Glucagon (1/500; Linco). Immunohistochemistry was also performed on paraffin sections of E14.5 mouse embryos, as well as E16.5, E18.5 and adult ICR pancreata. Sections were co-stained with rabbit anti-Myt3 (1/250) and guinea pig anti-Insulin (1/1000; Linco), guinea pig anti-Glucagon (1/1000; Linco), guinea pig anti-PP (1/100; Linco), goat anti-Somatostatin (1/1000; Santa Cruz) or mouse anti-Pdx1 (1/500; DSHB). Primary antibodies were detected using donkey anti-rabbit Alexa 488, goat anti-glucagon pig Alexa 546, goat anti-mouse Alexa 546 or donkey anti-goat Alexa 546 (1/2000; Invitrogen). The Myt3 antibody was generated by OpenBiosystems and was raised against the synthetic peptide RGKKGKMTPTKKEEDSLR. The serum from the terminal bleed of two rabbits was affinity purified.

Mouse Maintenance, Islet Isolations and Cell Culture
Mice were maintained according to the guidelines of the Canadian Council on Animal Care. All protocols were approved by the UBC Animal Care Committee. Hand-picked pancreatic islets were isolated as previously described [27] and cultured in RPMI 1640 (2g/L Glucose) supplemented with 10% FBS, 50U/ml Penicillin/Streptomycin and 2mM L-Glutamine at 37° in a 5% CO2 humidified incubator. mPAC cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM, 4.5 g/L Glucose) supplemented with 10% FBS, 50 U/ml Penicillin/Streptomycin and 2mM L-Glutamine (DMEM Complete) at 37° in 5% CO2 humidified incubator. Islets were cultured in 3 mM, 7 mM, 11 mM, 16.7 mM and 33 mM glucose, or with various cytokine combinations [INFγ (1000U/ml), IL-1β (17.5 ng/ml) and TNFα (10 ng/ml)] as appropriate. For cycloheximide (CHX) experiments, islets were preincubated in 3 mM glucose for 6 hrs and CHX (10 µg/ml) or DMSO was added 1 hr prior to transferring islets to fresh 3 mM or 16.7 mM glucose supplemented with CHX or DMSO.

Database Analysis (SAGE and ChIP-seq)
Serial Analysis of Gene Expression (SAGE) data were obtained from the Mouse Atlas of Gene Expression Database (www.mouseatlas.org) [2]. Foxa2 and Pdx1 Chromatin Immunoprecipitation (ChIP) sequencing data were obtained from the Short Read Archive (SRX003306 and SRX003296) [26]. Mafa and Neurod1 ChIP sequencing data were obtained from the Gene Expression Omnibus (GSE30296). Data were analyzed as previously described [2,28].

Adenoviral Mediated Knockdown and Over-expression
pLKO.1 vectors containing short hairpin constructs targeting Myt3 under control of the hU6 promoter and a scramble shRNA construct were purchased from OpenBiosystems. U6-shRNA expression cassettes for three of these were cloned into pAdTrack using InFusion cloning (Clontech) and sequence verified (TRCN0000042478: CCGGCGAACAATCACGAGTGAATGCAGTGGTTTGGTCAGTGTCTGTCTTTTG, TRCN0000042479: CCGGGCCAGCAATGATCATTAACTGCAAGTAAAGCTGGATACGTGCTGTCTTTTGG, TRCN0000042481: CGGGCGAATCCGACAGTCTATGCTACGATTTTGTGACTTGGTCGATGATTTTTGG). pAd-Track-hU6-shRNAs were linearized with PmeI and inserted into the pAdEasy viral genome by homologous recombination to generate pAdV-shMyt3 and pAdV-shScramble [29]. Full length Myt3 was cloned into pDNA3.1-V5/His6 (Invitrogen) and pAdV-Myt3 was generated as above. Islets were transduced with these adenoviruses at the indicated MOI’s for 3 hours and analyzed 48 hrs later. For pMPC studies, cells were plated at 40000 cells/well and transduced with pAdV-Ngn3 or pAdV-βgal for 3 hrs at an MOI of 50 and analysed 48 hrs later [30].

ChIP-qPCR
Islets were used in ChIP reactions as previously described [28], with 3 µg of anti-Foxa2 (Santa Cruz, sc-6554), anti-Pdx1 (Upstate, 07-6969), or anti-Neurod1 (Cell Signalling, D352G). mPAC cells transduced with pAdV-Ngn3 or pAdV-βgal, as described above, were used in ChIP reactions with 3 µg anti-H3K1me1 (Abcam, Ab8895), anti-H3K4me3 (Abcam, Ab5800), anti-H3K27ac (Abcam, Ab4729), anti-H3K27me3 (Abcam, Ab6002) or rabbit IgG (Santa Cruz, sc-2027). Fold enrichment was calculated relative to the IgG ChIP and percent recovery was calculated relative to sample input.

Reporter Constructs
A 1200 bp region upstream of the Myt3 transcriptional start site (TSS) was amplified from mouse genomic DNA and cloned into pGL3-Basic (Promega) to generate the Myt3 reporter construct. The Foxa2, Pdx1 and Neurod1 binding site mutagenesis primers were designed using the Agilent QuikChange Primer Design tool. Site directed mutagenesis PCR was performed using Phusion Taq (Finnzymes).

Luciferase Assays
mPAC cells were transfected with 400 ng of pGL3-Myt3-promoter dual luciferase reporter construct, pGL3-Myc3-promoter mutant constructs or a control pGL3-Basic vector, with or without 200 ng of Foxa2, Pdx1 or Neurod1. An EGFP vector was used to
ensure equal amounts of DNA were transfected into each well. After 48 hrs reporter activity was analyzed using the Promega Dual Luciferase kit as per manufacturer’s instructions using a Spectramax L luminometer (Molecular Devices).

qPCR Analysis

Islets were transduced with pAdv-shMyt3 clone 2 and pAdv-shScrable as above. After 48 hrs islets were dispersed and sorted to obtain EGFP positive cells (i.e. transduced cells) using a FACSVantage BD Biociences. RNA was isolated from pancreata of E11.5, E13.5, E15.5 and E18.5 embryos as well as ducts and adult islets using Trizol (Invitrogen) and the Qiagen RNA purification kit. cDNA was generated using Superscript III (Invitrogen). Taqman probes were used to quantify Myt3, Myle1, Ins1, Ins2, Pdx1, Neurog3, Pax4, NeuroD1, B-1β, B-1α, B-1μ, B-6, Tnf-a, iNOS, β-actin, and Gapdh (Applied Biosystems), all other primers were designed using Primer3plus. A Via7 real-time PCR system (Applied Biosystems) and SYBR Green supermix or Universal PCR Master Mix (Applied Biosystems) was used for all reactions. 10 ng of cDNA was used in each reaction with all reactions done in triplicate. 10 ng of cDNA and SYBR Green supermix or Universal PCR Master Mix (Applied Biosystems) was used for all reactions. 10 ng of cDNA was used in each reaction with all reactions done in triplicate. Data are presented as relative quantification values as fold-enrichment over a negative region and data are represented as mean ± SEM. P-values for TUNEL positive cells were calculated using paired, two-tailed Student’s t-tests on percent TUNEL positive values. Data are represented as mean ± SEM. In all cases * indicates a statistically significant difference at p<0.05, ** at p<0.01, *** at p<0.001.

Results

Myt3 is the Dominant MYT Family Member in Mature Islets

In previous studies Myt3 was reported to be absent from the developing pancreas [25], although our data suggested its enriched expression in mature pancreatic islets [2]. To confirm our previous data, and clarify the expression of Myt3 in the pancreas, we assessed its expression in 205 serial analysis of gene expression (SAGE) libraries. We found Myt3 SAGE tags (representing Myt3 expression) in neural tissue, as well as at low levels in pancreatic and endocrine precursor cells. However, in confirmation of our previous results, maximal Myt3 levels were found in pancreatic islets (Figure 1A). To further validate these data, we performed in situ hybridisation on mouse embryo’s at embryonic day 9.5 (E9.5) and 14.5 (E14.5), as well as on adult islets (Figure 1B-D). Whole mount in situ hybridisation with E9.5 embryo’s showed strong Myt3 staining in the telencephalon, the second and fourth rhombomeres, as well as in the ventral neural tube (Figure 1B). At E14.5 we found relatively strong Myt3 staining in the anterior of the neocortex, with weaker staining in the thalamus and tectum (Figure 1C). In agreement with previous studies [25], no staining was found in the pancreas at this time point. Despite this, we found strong Myt3 staining in mature pancreatic islets, which co-localized with both insulin and glucagon (Figure 1D). These data demonstrate that although Myt3 expression is minimal in the developing pancreas it is relatively abundant in mature islets.

Given this degree of similarity between the MYT family members [16], and their possible functional redundancy [16,31], we wanted to determine which family member was most abundant in developing pancreatic tissues and in adult islets. Using our SAGE data we determined that while Myt1 is more highly expressed in Neurog3 expressing endocrine precursor cells, Myt3 is more abundant in mature islets (Figure 2A). Myt1 could not be assessed as it does not produce any SAGE tags that uniquely map to it. In agreement, qPCR analysis of Myt1, Myt3 and Myt3 in developing pancreas tissues and adult islets showed that Myt1 was clearly more abundant than Myt1 or Myt3 in the developing pancreas, particularly at E15.5 and E18.5 (Figure 2B-D). However, all three family members showed maximal expression in adult islets (Figure 2B-D), likely due to the higher proportion of cells expressing these factors in islets as compared to within the whole developing pancreas. To clarify which of the MYT family members is dominant in adult islet cells, we determined the total copy number of Myt1, Myt1, and Myt3 transcripts in islets, as well as in MIN6 (β-cell) and 2TC1 (α-cell) cells using absolute quantification qPCR. Myt3 was expressed at a 15-fold higher level in islets, a 4-fold higher level in MIN6 cells, and a 2.5-fold higher level in 2TC1 cells than Myt1, and a 23-fold higher level in islets, a 3-fold higher level in MIN6 cells, and a 2.5-fold higher level in 2TC1 cells than Myt1 (Figure 2E). Last, we sought to determine the expression level of Myt3 in human islets relative to mouse islets, and found that although Myt3 is expressed in human islets, Myt3 expression is 4-fold higher in mouse islets (Figure 2F).

Together, these data demonstrate that Myt3 is more abundantly expressed in mature pancreas endocrine cell types than either Myt1 or Myt1, and confirm its expression in α- and β-cells.
Myt3 is Expressed in Maturing and Adult Endocrine Cells

The above data indicate that Myt3 expression occurs predominantly in adult islet cell types. To determine whether Myt3 protein levels match this pattern, and to identify the cell types that Myt3 is expressed in, we developed an antibody against it. Using this antibody we found no evidence of Myt3 protein in the developing pancreas at either E14.5 or E16.5 (Figure 3). At E18.5 however, Myt3 protein was found in both insulin (β-cells) and glucagon (α-cells) expressing cells (Figure 3). Similarly, Myt3 staining was evident throughout the islet in adult pancreas sections, while no Myt3 staining was evident in the surrounding exocrine tissue (Figure 4). Similar to what we observed in our in situ experiments with whole islets, co-staining of adult sections with endocrine cell markers showed that Myt3 co-localizes in cells expressing insulin (β-cells), glucagon (α-cells), somatostatin (δ-cells) and pancreatic polypeptide (PP-cells) (Figure 4). High magnification confocal microscopy confirmed the co-localization of Myt3 with endocrine markers, and indicated that in mature endocrine cell types Myt3 is primarily cytoplasmic, with only a fraction of total protein localizing to the nucleus (inset), similar to other β-cell transcription factors such as Pdx1 and Neurod1 [32,33]. These data indicate that...
Myt3 is first evident at E18.5, and that it is expressed in mature α-, β-, δ-, and PP-cell types. Myt3 expression is Regulated by Foxa2, Pdx1 and Neurod1

To characterise the factors responsible for the regulation of Myt3 expression we first assessed Foxa2, Pdx1, Neurod1 and Mafa ChIP-seq data generated from islets [28]. We identified Foxa2, Pdx1 and Neurod1 enrichment, or peaks, in the Myt3 promoter region (Figure 5A) suggesting its expression is directly regulated by these factors. No enrichment of Mafa was noted. To validate these data we used ChIP-qPCR. Using an antibody against Foxa2 we obtained a 250-fold (p < 0.01, n = 3) enrichment of an Nkx2.2 positive control region [2], and a 500-fold (p < 0.01, n = 3) enrichment of the Myt3 promoter (Figure 5B). Meanwhile, using an antibody against Pdx1 we obtained a 180-fold (p < 0.01, n = 3) enrichment in a Pdx1 positive control region [2], and a 90-fold (p < 0.01, n = 3) enrichment of the Myt3 promoter (Figure 5C); and using an antibody against Neurod1 we obtained a 21-fold (p < 0.001, n = 3) enrichment of an Abcc8 control region, and a 70-fold (p < 0.001, n = 3) enrichment of the Myt3 promoter (Figure 5D). In all cases less than a 5-fold enrichment was obtained using primers for regions upstream of the Myt3 promoter. To further confirm the direct regulation of Myt3 expression by these factors we generated a Myt3-promoter luciferase reporter. In co-transfections with this reporter, Foxa2 reduced Myt3 promoter activity by 1.3-fold (p < 0.001, n = 3), while Pdx1 and Neurod1 increased promoter activity by 1.3-fold (p < 0.001, n = 3) and 9-fold (p < 0.001, n = 3), respectively (Figure 5E–G). Mutation of the Foxa2 binding site reversed the suppressive effect of Foxa2 by 2-fold (p < 0.001, n = 3), while mutation of the Pdx1 and Neurod1 binding sites reduced the relative luciferase activity by 3-fold (p < 0.001, n = 3) and 3.4-fold (p < 0.001, n = 3), respectively, over the non-mutated promoter (Figure 5E–G). Together, these data show that Foxa2, Pdx1 and Neurod1 directly regulate Myt3 expression, and that Neurod1 is likely a primary determinant of Myt3 promoter activity.

Genes regulated by Neurod1 in mature tissues are often initially induced during development by the related bHLH transcription factor Ngn3, which is critical to pancreas endocrine cell specification [34], as both bind to E-box elements [35,36]. Thus, to test whether Ngn3 induces Myt3, we treated mPAC cells with an Ngn3 over-expressing adenovirus, or control βgal expressing virus. Ngn3 over-expression resulted in a 963-fold (p < 0.0001, n = 4) increase in Myt3 expression relative to cells treated with the βgal virus (Figure 5H). We next evaluated the ability of Ngn3 over-expression to induce Myt3 expression in adult islets. A) A heatmap showing the relative gene expression of Myt1, Myt1L and Myt3 in pancreatic and endocrine precursor cells, whole pancreas, duct cells and mature islets as determined by analysis of 10 SAGE libraries (www.mouseatlas.org). Expression levels for B) Myt1, C) Myt1L and D) Myt3 in the pancreas as determined by qPCR at various stages of embryonic development as well as in ductal cells and whole islets from adult mice (8–10 weeks of age). Expression is relative to β-actin and is normalised to expression levels in whole islets. E) Absolute level of Myt1, Myt1L, and Myt3 transcripts in islets, MIN6 cells (a β-cell line) and αTC1 cells (an α-cell line) as determined by qPCR. Absolute quantification expressed as number of copies per μg cDNA. F) Expression of Myt3 in human and mouse islets as a percentage of Gapdh expression. doi:10.1371/journal.pone.0051501.g002

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expression to alter the histone modification status of the Myt3 promoter to establish the mechanism of Myt3 induction. We performed ChIP-qPCR for mono-methylated Histone 3 Lysine 4 (H3K4me1) (Figure 5I), tri-methylated Histone 3 Lysine 4 (H3K4me3) (Figure 5J) and acetylated Histone 3 Lysine 27 (H3K27ac) (Figure 5K), which demarcate active cis-regulatory loci [37,38,39,40]; as well as, for tri-methylated Histone 3 Lysine 27 (H3K27me3) (Figure 5L), which is associated with repressed chromatin [41,42,43]. Our data demonstrate Ngn3 over-expression in mPAC cells increased the levels of H3K4me1 and H3K27ac by 2-fold (p<0.0001, n = 3) and 3-fold (p<0.05, n = 3) respectively. Meanwhile levels of tri-methylated Histone 3 Lysine 27 (H3K27me3) were reduced 5.0-fold (p<0.01, n = 3) relative to βgal expressing cells. Levels of tri-methylated Histone 3 Lysine 4 (H3K4me3) were unchanged. These data suggest that Ngn3 expression alters the epigenetic landscape around the Myt3 promoter from an inactive, to an active chromatin state, thereby initiating its expression.

**Myt3 Expression is Regulated by Glucose and Cytokines**

Under normal physiological conditions islets are exposed to fluctuating concentrations of glucose and many genes with critical roles in controlling islet function, such as Insulin, Iapp and Mafa, are regulated by glucose [32,44,45,46]. To determine whether Myt3 is

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**Figure 3. Myt3 protein is detected in endocrine cells from E18.5.** Sagittal sections of E14.5, E16.5 and E18.5 pancreata were analysed for expression of Insulin, Glucagon or Pdx1, as indicated (red), and Myt3 (green). Nuclei were stained with Hoechst (blue). Arrows indicate co-localisation of Myt3 with indicated endocrine markers.

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similarly regulated we assessed its expression in islets at various glucose concentrations 24 hrs after transfer from 3 mM glucose. Exposure of islets to 7 mM, 11 mM, 16.7 mM and 33 mM glucose increased *Myt3* expression by 1.78- (p < 0.001, n = 4), 2.74- (p < 0.001, n = 4), 2.71- (p < 0.001, n = 4) and 2.86-fold (p < 0.001, n = 4), respectively, over 3 mM glucose (Figure 6A). We next sought to determine the timing of the increase in *Myt3* expression in response to glucose. 3 hr after transfer to 16.7 mM glucose there was no change in *Myt3* expression, and only a slight but significant (1.2-fold, p < 0.05, n = 4) change by 6 hrs; however, by 12 hrs *Myt3* had reached maximal induction (1.84-fold, p < 0.001, n = 4) and this was maintained at 24 hrs (1.70-fold, p < 0.001, n = 4) (Figure 6B). The delay in glucose induced *Myt3* expression suggests that it may be dependent on the synthesis of additional regulatory proteins in addition to the translocation of transcription factors to the nucleus. To test this we treated islets with cycloheximide (CHX, 10 μg/ml) to inhibit protein synthesis. Interestingly, treatment with CHX increased basal *Myt3* expres-

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**Figure 4. Myt3 co-localises with endocrine cell markers in adult pancreas.** Saggital sections of adult pancreata were analysed for expression of Insulin, Glucagon, Somatostatin and Pancreatic Polypeptide, as indicated (red), and Myt3 (green). Nuclei were stained with Hoechst (blue). Arrows indicate co-localisation of Myt3 with indicated endocrine markers. High magnification confocal images of individual cells showing co-localization of Myt3 with Insulin, Glucagon, Somatostatin and Pancreatic Polypeptide, representative cells are shown (Inset), note that Myt3 staining is predominately cytoplasmic but can also be found within the nucleus. doi:10.1371/journal.pone.0051501.g004
Figure 5. Myt3 expression in islets is under the control of known regulators of β-cell function. A) A screenshot of the Myt3 promoter region in the UCSC genome browser showing Foxa2, Pdx1, Mafa and Neurod1 ChIP-seq data from islets. Peaks indicate binding sites. ChIP-qPCR was used to validate B) Foxa2, C) Pdx1 and D) Neurod1 binding within the Myt3 promoter region. Nkx2.2, Pdx1 and Abcc8 are positive controls for Foxa2, Pdx1 and Neurod1 binding respectively. E–G) Relative luciferase activity of the indicated luciferase reporter vectors co-transfected with empty vector or with Foxa2, Pdx1 or Neurod1 expressing vectors. Mutant vectors had the indicated transcription factor binding sites altered by site-directed mutagenesis. Wild type and mutant binding site sequences are as indicated. H) Myt3 expression relative to β-actin following treatment of mPAC cells with pAdV-Ngn3. ChIP-qPCR was used to determine I) H3K4me1, J) H3K4me3, K) H3K27ac and L) H3K27me3 histone modifications at the Myt3 promoter. * indicates a statistically significant difference at p<0.05, ** at p<0.001, and *** at p<0.001 based on student’s t-test for luciferase data and a Kruskal-Wallis test with a Dunn’s multiple comparison for ChIP-qPCR data. doi:10.1371/journal.pone.0051501.g005
sion by 4.2-fold (p≤0.001, n = 3) relative to 3 mM glucose with DMSO. Induction with 16.7 mM glucose increased Myt3 levels a further 3.6-fold (p<0.01, n = 3), similar to the level of Myt3 induction by 16.7 mM glucose in DMSO (3.2 fold, p≤0.001, n = 3) (Figure 6C). These data indicate that Myt3 expression is positively regulated by the glucose signals responsible for insulin secretion, and suggest that Myt3 is repressed by some factor that requires continued protein synthesis.

In both type 1 and type 2 diabetes β-cell exposure to cytokines can induce dysfunction by altering the expression of genes responsible for regulating normal β-cell function [47,48]. In fibroblasts Myt3 was found to be up-regulated by exposure to TNFα [22], but to be down-regulated in a microarray study of genes affected by exposure to IL-1β and IFNγ in rat islets [48]. To clarify this discrepancy, we examined the expression level of Myt3 following exposure of islets to different combinations of IL-1β, IFNγ, and TNFα. Myt3 expression was reduced by exposure of islets to IL-1β (1.7 fold, p≤0.05, n = 3) but not by IFNγ or TNFα, while a combination of IL-1β and IFNγ reduced Myt3 expression 3-fold (p≤0.01, n = 3). Treatment of islets with IL-1β, IFNγ and TNFα together had the most significant effect, reducing Myt3 expression 5-fold (p≤0.001, n = 3) (Figure 6D). Similar to what was seen following exposure of islets to glucose, the reduction in Myt3 expression was also time dependent. At 3 hrs post transfer into a full dose of cytokine mix Myt3 expression was unchanged. By 6 hrs post transfer Myt3 expression was significantly reduced (1.35-fold, p≤0.001, n = 3) with maximal suppression being reached by 24 hrs (2.9-fold, p≤0.001, n = 3) (Figure 6E).

To determine how Myt3 expression varied with cytokine dose dependent we treated islets with varying concentrations of the triple cytokine mix. Our data demonstrate that maximal reduction in Myt3 levels was evident at 1/8 the concentration of IL-1β, IFNγ and TNFα used above (i.e 125 U/ml INFγ, 2.15 ng/ml IL-1β and 1.25 ng/ml TNFα) (Figure 6F).

As IL-1β, IFNγ and TNFα are important cytokine effectors of β-cell death in type 1 diabetes [47,48], we next sought to determine whether Myt3 is reduced by immune-cell attack in non-obese diabetic (NOD) mice. We isolated RNA from whole pancreata from 4-week old pre-diabetic and 12-week old diabetic female NOD mice and analysed Myt3 expression. Our data demonstrate

![Figure 6. Myt3 expression is sensitive to both glucose and cytokine exposure. A) Whole cultured islets were treated with the indicated glucose concentrations for 24 hrs, after being pre-incubated for 24 hr in 3 mM glucose. Subsequently qPCR was used to determine the relative expression of Myt3 as compared to β-actin. B) Myt3 expression was determined at the indicated time points following transfer into 16.7 mM glucose. C) Myt3 expression was determined for islets incubated in low or high glucose following treatment with DMSO or cycloheximide (10 μg/ml). Expression is expressed relative to β-actin. D) Whole cultured islets were treated with the indicated cytokine combinations, or E) with triple cytokine mix at the indicated time points. Myt3 expression is expressed relative to Gapdh. F) Whole cultured islets were treated with the indicated cytokine doses (a dose of “1” equals 1000U/ml INFγ, 17.5 ng/ml IL-1β and 10 ng/ml of TNFα) for 24 hours. Subsequently qPCR was used to determine the relative expression of Myt3. Expression is expressed relative to Gapdh. * indicates a statistically significant difference at p≤0.05, ** at p≤0.01, and *** at p≤0.001, based on student’s t-test. doi:10.1371/journal.pone.0051501.g006](https://www.plosone.org/article/doi/10.1371/journal.pone.0051501.g006)
that in pancreata from diabetic mice undergoing immune infiltration, Myt3 expression is reduced by 2.5-fold (p ≤ 0.05, n = 4) (Figure 7A). We also assessed Myt3 expression relative to the level of immune infiltration by immunofluorescence. For this, we independently scored insulin levels and changes in Myt3 signal in pancreas sections from 12-week old female NOD mice (Figure 7B, C). From this analysis it was evident that as insulin progresses there is a concomitant decrease in Myt3 expression. Together, these data indicate that cytokines that cause β-cell dysfunction and apoptosis negatively regulate Myt3 expression and that this may be relevant to the progression of diabetes in NOD mice.

**Myt3 Suppression Reduces Insulin Content in β-cells**

To determine whether Myt3 plays a role in regulating glucose-stimulated insulin secretion, we generated three independent adenoviruses expressing shRNA sequences targeting Myt3 (shMyt3) (see methods and materials) or a scramble sequence (shScramble). RT-qPCR analysis of FACS-sorted islets indicated that clone TRCN0000042479 resulted in the highest level of Myt3 suppression (data not shown) and this clone was used in all subsequent experiments. Our analysis also showed the shMyt3 virus had no effect on Gapdh expression, but reduced Myt3 levels by approximately 5-fold (p ≤ 0.001, n = 10) as compared with islets treated with the shScramble virus (Figure 8A). Treatment of whole islets with the shMyt3 virus also significantly reduced Myt3 protein level by 2-fold (p ≤ 0.01, n = 3) (Figure 8B, C). Myt3 suppression in islets modestly, but significantly [1.4-fold, p ≤ 0.05, n = 3] reduced cellular insulin levels (Figure 8D), but had no effect on their ability to secrete insulin following stimulation with glucose, KCl or arginine (Figure 8E-G).

To determine how suppression of Myt3 reduces cellular insulin levels we assessed the effect of Myt3 suppression on the expression of selected transcriptional regulators important in pancreas development or function, or genes with well-established roles in β-cell function. Myt3 suppression in ex vivo islets had a significant effect on several transcription factors and cofactors known to regulate β-cell function, including Hnf4α, Hnf1β, Hnf1α, Ins1m, Sox9, Pdx1, and Mafa, which were all reduced by at least 1.6-fold (Figure 9H). Of the genes involved in β-cell function, Myt3 suppression reduced Atoh1 and Skl209a the most, by 1.54-fold and 1.67-fold respectively (Figure 8I). Myt3 suppression also impaired Ins1 and Ins2 expression, while the expression levels of the other islet hormones were unaltered (Figure 8I). Treating MIN6 cells with siRNA targeting Myt3 produced similar results for selected genes, in particular for Pdx1 and Mafa (data not shown). Given this, and as Pdx1 and Mafa have well-established roles in β-cell function [10,12], we attempted to validate their repression at the protein level. Western blot analysis of islets transduced with adenovirus expressing shMyt3 reduced Mafa levels by 1.67-fold (p < 0.001, n = 3) and Pdx1 levels by 1.48-fold (p < 0.001, n = 3) (Figure 8J, K), consistent with our qPCR data. These results suggest that Myt3 affects cellular insulin content via the regulation of several genes including Ins1, Ins2, Pdx1 and Mafa.

**Myt3 Regulates β-cell Survival**

Exposure of islets to cytokines both in vitro and in vivo suppresses Myt3 expression suggesting a potential role for Myt3 in β-cell survival. To test this hypothesis we transduced MIN6 cells with our adenoviruses expressing shRNA’s targeting Myt3 or a scramble sequence and incubated the cells with propidium iodide (PI). Increasing shMyt3 virus concentration significantly increased β-cell death over time (p ≤ 0.0001, n = 4) (Figure 9A). Similarly, Myt3 suppression increased Annexin-V positive cells by 2-fold (p ≤ 0.001, n = 3) (Figure 9B), and the level of cleaved caspase 3 (Figure 9C). To validate these results we performed TUNEL analysis on dispersed islets treated with either the shScramble or shMyt3 virus. Our data show that apoptosis was increased by approximately 2-fold (p ≤ 0.01, n = 4) (Figure 9D), similar to our results in MIN6 cells. This was also confirmed in whole islets (Figure 9E). As cytokine exposure results in reduced Myt3 expression, and adenoviral mediated suppression of Myt3 increases apoptosis, we examined the ability of Myt3 over-expression to protect islets from cytokine mediated cell death. Dispersed islets treated with an adenovirus over-expressing Myt3 had a greater than 2-fold (p ≤ 0.01, n = 4) decrease in cytokine induced apoptosis, as compared to islets treated with a control adenovirus expressing eGFP, as revealed by TUNEL staining (Figure 9F).

To determine how Myt3 regulates apoptosis in β-cells we examined the expression of a number of different anti-apoptotic and pro-apoptotic genes in shMyt3 and shScramble treated islets. Our data demonstrate that Myt3 suppression leads to a 1.25-fold (p ≤ 0.01, n = 3) reduction in Bcl-x1, a 1.54-fold (p ≤ 0.01, n = 3) reduction in Ikg1, and a 1.4-fold (p ≤ 0.05, n = 3) reduction in c-Ikap2 (Figure 9G). To determine whether endoplasmic reticulum (ER) stress played a role in these changes we assessed the expression of genes characteristic of ER stress [49,50]. We found that Bip, CHOP, Gadd34 and IRE1α were unchanged, however, Xbp1 was reduced 2-fold (p ≤ 0.05, n = 3) (Figure 9H). Finally, as Myt3 plays a role in pro-inflammatory gene expression in fibroblasts, we further assessed the expression of selected β-cell expressed cytokines. Myt3 suppression caused a 2-fold (p ≤ 0.05, n = 3) reduction in Il-6 expression but had no effect on the expression levels of Il-1α, Il-1β, Il-1m or Tgfα (Figure 9I). Together, these results indicate that Myt3 plays a significant role in regulating β-cell survival and pro-inflammatory gene expression.

**Discussion**

We anticipated that the identification of transcription factors specifically expressed in developing endocrine cells, or in adult pancreatic islets, would provide insight into the transcriptional networks that regulate β-cell genesis and function [2]. In trying to find such factors we identified Myt3. Myt3 has a high degree of similarity to other MYT family members, particularly Myt1, with both genes encoding proteins with conserved zinc-finger, and MYT family domains [16]. Furthermore, both transcription factors recognise similar synthetic oligonucleotides, with Myt1 recognizing the consensus sequence RRRAGTT, and Myt3 recognizing the related AAATTT consensus sequence, suggesting some degree of functional redundancy [16,31]. Previous reports indicated that the MYT family of transcription factors is highly expressed in neural tissue [51,52], but that only Myt1 is expressed in developing pancreas cells [23]. Our data agree with these reports and indicate that Myt3 and Myt3 have little or no expression early in pancreas development [25]; however our SAGE, qPCR, and IHC data indicate that Myt3 is relatively abundant in mature pancreatic islets. In fact, Myt3 is greater than 10-fold more highly expressed in islets than either Myt1 or Myt1. Furthermore, Myt3 is expressed in human islets, albeit at a lower level than in mouse islets, suggesting that Myt3 is important not only for islet function in rodents, but also in humans.

In the pancreas, endocrine progenitors are specified by the expression of Ngn3 during the secondary transition (~E13.5) [34,53]. During this time frame Ngn3 expressing cells differentiate and expand. Subsequently, from ~E16.5 until several days after birth, these cells coalesce into islet structures and increase their expression of key maturation factors such as Neurod1 and Mafa that
Figure 7. Exposure of islets to cytokines in a mouse model of T1D decreases Myt3 expression. A) RNA was isolated from whole pancreas of female 4-week and 12-week old NOD mice and Myt3 expression was determined relative to Gapdh. B) Insulitis levels and Myt3 expression in islets were scored by analysing sections from 12-week old NOD mice. C) Representative images of NOD sections showing Myt3 (green) and Insulin (red) expression in islets that are unaltered, intermediately altered and altered. Nuclei were stained with Hoechst (blue). * indicates a statistically significant difference at p≤0.05 based on student’s t-test.

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Figure 8. *Myt3* regulates insulin content and gene expression in β-cells. Islets were transduced with adenoviruses expressing shRNA’s targeting *Myt3* or a scrambled sequence. A) *Myt3* expression was determined relative to β-actin. *Gapdh* was used as a control for off target effects of the virus. B) Western blot analysis of *Myt3* and β-actin protein levels in islets. C) Results of the densitometry of triplicate western blot analyses from B.

E) 2.8mM Glucose 16.7mM Glucose
F) 2.8mM Glucose 30mM KCl
G) 2.8mM Glucose 10mM Arginine

H) Relative Expression (β-actin)

I) Relative Expression (β-actin)

J) Western blot analysis of Mafa, Pdx1, and β-actin protein levels in islets.

K) Relative Amount (β-actin)
relative to β-actin. Ex vivo islets were transduced as above and D) cellular insulin content and insulin secretion induced with E) 16.7 mM Glucose, F) 30 mM KCl or G) 10 mM Arginine were determined 48 hrs later by Insulin ELISA. qPCR was used to determine the relative expression of H) transcription factors and cofactors and I) Genes involved in β-cell function/physiology as compared to β-actin. J) Western blot analysis of Mafa, Pdx1, and β-actin protein levels. K) Results of the densitometry of triplicate western blot analyses from J relative to β-actin. * indicates a statistically significant difference at p≤0.05, ** at p≤0.01, *** at p≤0.001 based on students t-test.

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Figure 9. Myt3 is critical for β-cell survival. A) The percent of MIN6 cells that were PI positive at the indicated time points after being transduced with varying amounts of adenoviruses expressing shRNA’s targeting Myt3 or a scrambled sequence. B) Quantification of Annexin-V APC positive cells in virus treated MIN6 cells. Untransfected cells were used as a negative control while cytokine treated cells acted as a positive control. C) Western blot analysis of cleaved Caspase 3 and β-actin levels in transduced MIN6 cells. Numbers indicate separate biological replicates. D) Quantification of TUNEL positive cells in dispersed islets treated with shScramble or shMyt3 viruses. E) Representative images of TUNEL staining (red) of transduced islets. Transduced cells are stained in green and nuclei are labelled with Hoechst (blue). F) Quantification of TUNEL positive cells in dispersed islets treated with eGFP or Myt3 over-expression viruses. G) Expression of pro- and anti-apoptotic genes relative to β-Actin. * indicates a statistically significant difference at p≤0.05, ** at p≤0.01, *** at p≤0.001 based on students t-test.

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drive their maturation into fully functional endocrine cells [33,54]. 
Myt3 protein first appears in endocrine cells at ~E18.5 during the period of islet maturation and is maintained in mature α-, β-, δ-, and PP-cell types. The expression of Myt3 from E18.5 onwards suggests that it may play an important role in the regulation of this maturation step and in the maintenance of mature β-cell function.

The tightly controlled spatiotemporal expression of Myt3 suggests precise, tissue specific transcriptional regulation. We show that the Myt3 promoter is bound and directly regulated by Foxa2, Pdx1 and Neurod1. Foxa2 is a critical initiator of Pdx1 expression [55] and loss of either transcription factor leads to impaired pancreas development and perinatal lethality [55,56]. Foxa2 and Pdx1 are both expressed in mature β-cells where they function to regulate insulin vesicle docking to the plasma membrane and insulin biosynthesis respectively [11,57]. Meanwhile, Neurod1 is essential for specification and differentiation of endocrine cell types and also functions in mature β-cells to regulate insulin biosynthesis and secretion [13,57,58]. The regulation of the Myt3 promoter by Foxa2, Pdx1 and Neurod1 suggests that it may play an important role in mediating the downstream effects of these transcription factors.

Genes that are maintained in the adult islet by Neurod1 are often induced by Ngn3 during development, as both bind similar E-box elements [35]. In concordance, Neurod1 and Ngn3 induce similar sets of genes when over-expressed in mPAC cells [36]. The importance of the identified E-box element in the Myt3 promoter in initiating and maintaining its expression is exemplified by the fact that not only does Ngn3 induce Myt3 expression in mPAC cells but Neurod1 over-expression also has the most significant affect on Myt3 promoter activity relative to Foxa2 and Pdx1. In addition, ectopic expression of Ngn3 induces a more open and active chromatin state around the Myt3 promoter, through an increase in the enrichment of the activating H3K4me1 and H3K27ac marks, with a concomitant decrease in repressive H3K27me3 enrichment levels. These data suggest that Ngn3 induced changes to the histone modification state around the Myt3 promoter may allow it to become activated by other factors, and that once activated its expression is maintained in mature islets, at least in part, by Neurod1 and Pdx1.

Pancreatic islets respond to elevated glucose levels following feeding, not only by secreting insulin, but also by increasing insulin, and other, gene expression [59]. These functional responses are mediated, in part, through the glucose-induced translocation of Pdx1 and Neurod1 to the nucleus where they can affect gene expression changes [33,60]. As we determined that both of these factors are direct regulators of Myt3 expression we evaluated the role of glucose in the regulation of Myt3 expression. In fact, increasing glucose concentrations resulted in increased Myt3 expression. Exposure of islets to elevated glucose levels increases Insulin expression within one hour of exposure [33,60,61]; however, Myt3 expression was only up-regulated after 6 hrs post transfer to 16.7 mM glucose. To determine whether this delay was due to a need for synthesis of regulatory proteins we inhibited protein synthesis with cycloheximide. Surprisingly, the delay was due to a need for synthesis of regulatory proteins we evaluated the role of glucose in the regulation of Myt3 expression. In addition to being glucose responsive, both Pdx1 and Neurod1 are also repressed by exposure to the pro-inflammatory cytokines IL-1β, TNFα and IFNγ [62,63]; we therefore further examined the effect of these cytokines on Myt3 expression. Exposure of islets to IL-1β, TNFα and IFNγ in vitro resulted in a significant reduction in Myt3 expression. Furthermore, in a mouse model of T1D, immune infiltration into the islet results in a concomitant reduction in Myt3 expression likely due to exposure of the islets to pro-inflammatory cytokine secretion from the infiltrating immune cells confirming the in vivo relevance of our cytokine results.

Prolonged exposure to cytokines, which occurs in type 1 (T1D) and type 2 (T2D) diabetes, results in β-cell dysfunction and apoptosis [64,65]. We initially wondered whether cytokine-induced β-cell dysfunction may be mediated through Myt3 suppression. To determine this we assessed whether shRNA mediated Myt3 suppression could impair islet function. Although, Myt3 suppression did not cause any change in glucose-, KCl-, or arginine-stimulated insulin secretion, Myt3 suppression did reduce intra-cellular insulin content. To begin to assess the mechanism underlying the reduced insulin content we interrogated the gene expression of several factors with known roles in regulating β-cell function and insulin gene expression. We show that Myt3 regulates many of these factors, including Pdx1 and Mafa, which function synergistically to regulate insulin expression levels [57]. In agreement, Myt3 suppression also reduced Ins1 and Ins2 expression levels. Also, consistent with the lack of impaired insulin secretion, genes involved in insulin secretion were mostly unaltered. Thus, we suggest that while the level of Myt3 suppression we are able to achieve in whole islets is unable to induce defects in glucose-, KCl-, and arginine-stimulated insulin secretion, it is sufficient to alter cellular insulin levels due, at least in part, to reduced Pdx1 and Mafa levels.

We next assessed whether cytokine induced β-cell apoptosis might be mediated through Myt3 suppression. In fact, our data clearly show that Myt3 suppression leads to increased apoptosis in islets and MIN6 cells, suggesting that IL-1β, TNFα and IFNγ induced Myt3 repression may be a significant factor in cytokine induced β-cell apoptosis. We further demonstrate that adenoviral mediated Myt3 over-expression largely prevents cytokine-induced apoptosis in islets. In agreement with Myt3 having a pro-survival role in β-cells, suppression of Myt3 resulted in a significant reduction in the expression of Bel-αl, which alters the localisation of the pro-apoptotic Bax from the mitochondrial membrane to the cytoplasm thus preventing cytochrome c release and subsequently apoptosis [66], and c-Iap2 that regulates cell survival via inhibition of effector caspase activity [67]. Also, IL-6 and Igf1 expression were significantly reduced by Myt3 suppression. IL-6 induces α-cells to secrete the incretin hormone GLP-1 [68]. GLP-1 stimulates β-cell Igf1 expression, which regulates β-cell survival via Akt signalling [69,70]. This suggests that Myt3 may indirectly affect β-cell survival by reducing levels of IL-6 induced GLP-1 secretion from α-cells, thereby reducing Igf1 and thus increasing β-cell apoptosis; although, further work is required to validate this model. Further, IL-6 has been shown to protect islets from pro-inflammatory cytokine exposure both in vivo and in vitro [71]. In addition, Pdx1 and Mafa also play pro-survival roles in β-cells [72,73]. In fact, similar to our findings, increased β-cell apoptosis in Pdx1 heterozygous mice is due to reduced expression of the pro-survival factors Bel2 and Bel-αl [72]. In further agreement, insulin secretion is similarly unimpaired in these mice [72]. Thus, the Myt3 suppression induced reductions in Pdx1 levels that we note, and the phenotype we see, are consistent with the phenotype of Pdx1 heterozygous mice that have similar levels of Pdx1 in their islets. Together, these data clearly demonstrate that changes in
Myt3 expression levels are sufficient to alter apoptosis in islets, likely through the regulation of pro-survival genes such as Pdk1, Bcl-xL, e-Ifp2, and Igf1.

In summary, we have identified Myt3 as the predominant MYT family member in mature islets, and show that it is present in all major endocrine cell types. We show that Myt3 expression is regulated by the transcription factors Foxa2, Pdx1, and NeuroD1, and that its expression is responsive to both glucose and cytokines. We demonstrate that Myt3 suppression reduces cellular insulin levels, and significantly increases the rate of β-cell apoptosis. Importantly, over-expression of Myt3 is able to protect cells from cytokine-induced apoptosis. These data are an important step in clarifying the regulatory networks responsible for β-cell function and survival, and suggest that Myt3 may be an interesting therapeutic target for improving β-cell survival in diabetic patients and islet graft recipients.

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