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A developmentally regulated spliced variant of PTBP1 is upregulated in Type 1 diabetic hearts

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Abstract

Alternative splicing (AS) is dysregulated in Type 1 diabetic (T1D) hearts but mechanisms responsible are unclear. Here, we provide evidence that the RNA binding protein (RBP) PTBP1 is modulated in adult T1D hearts contributing to AS changes. We show that a spliced variant of PTBP1 that is highly expressed in normal newborn mouse hearts is aberrantly expressed in adult T1D mouse hearts. Comparing known PTBP1-target datasets to our T1D mouse transcriptome datasets, we discovered a group of genes with PTBP1 binding sites in their pre-mRNAs that are differentially spliced in T1D mouse hearts. We demonstrated that inducible expression of diabetes-induced PTBP1 spliced variant has less repressive splicing function. Notably, PTBP1 regulates AS of some of its targets antagonistically to RBFOX2. In sum, our results indicate that diabetic conditions disrupt developmental regulation of PTBP1 leading to differential AS of PTBP1 target genes. Identification of PTBP1 and PTBP1-regulated RNA networks can provide RNA-based therapies for the treatment of diabetes cardiac complications.

Keywords

Diabetic heart; PTBP1; alternative splicing; RNA binding proteins

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by hyperglycemia due to very little or no insulin production by the pancreas [1]. T1D patients can develop cardiovascular complications that can lead to heart failure [2–4]. Aberrant alternative splicing (AS) regulation has been implicated in cardiovascular complications of diabetes, however the molecular mechanisms responsible for AS changes in T1D hearts are not well defined. RNA binding proteins (RBPs) regulate AS via binding to specific motifs in the intronic and exonic regions of pre-mRNAs and interacting with the splicing machinery. Recent studies indicate that RBPs have emerging roles in systemic complications of diabetes [5].

PTBP1 belongs to a family of RBPs including PTBP1, PTBP2 (also known as neuronal PTB) and PTBP3 (ROD1) that bind to polypyrimidine tract sequences in the RNA. They have well-established roles as repressors of exon inclusion [6–13], though there is evidence that PTBP can activate exon inclusion of certain pre-mRNAs [14, 15]. PTBP1 is regulated during heart development and implicated in coordinated AS transitions during heart development [16–18]. It is widely unknown if it is modulated in T1D hearts.

In this study, we found that PTBP1 is differentially spliced in adult T1D hearts increasing the expression of a PTBP1 isoform predominantly expressed in newborn mouse hearts. We identified a group of PTBP1 targets aberrantly spliced in T1D mouse hearts. We found that inducible expression of diabetes-induced PTBP1 spliced variant with less repressive function changes AS patterns similar to that in diabetic hearts. PTBP1 and RBFOX2 antagonistically regulate AS of some of their targets.

Methods

Animal Experiments

Streptozotocin (STZ) induced T1D and non-obese diabetic (NOD) mice were used for our experiments as described previously [19–21]. Animal experiments approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee (Protocol # 1101001) were conducted in compliance with the NIH Guidelines. Mice were sacrificed after hyperglycemia induction (blood glucose levels >400mg/dL for at least 3 weeks) and the left ventricles were isolated. For mouse heart developmental, hearts were isolated from adult (6 months old) mice, embryonic day 18 embryos, and newborn pups.

Differential alternative splicing analysis

Paired end (2×15 cycles) RNA sequencing was done at the UTMB Next-Generation Sequencing Core facility with an Illumina HiSeq 1000 system and yielded about 200 million reads per sample [20, 21]. Changes in AS were determined by DAS analysis as we previously described [19, 22]. To determine PTBP1-binding sites on transcripts mis-spliced in diabetic hearts, we overlapped the differential cassette exon events identified in our diabetic heart RNA-seq data with the binding sites of PTBP1 that were defined by merging two sets of PTBP1 CLIP-Seq peaks from GSM2259090 and GSM2259091[23]. PTBP1

binding sites were examined within the 250bp intronic regions flanking the alternative exon and 50bp of the 5' and 3' ends of the alternative exons.

Generation of stable cell lines

Triple flag-tagged human PTBP1 including exon 9 (PTBP1+ex9) and excluding exon 9 (PTBP1 -ex9) in pcDNA5/FRT/TO expression vectors. Flp-in T-REx 293 cells plated on 6-well plates were co-transfected with 4µg pOG44 recombinase and 0.4µg PTBP1+ex9, PTBP1 -ex9, or empty vector using Lipofectamine 2000 (Invitrogen). Cells stably expressing the constructs were selected in 100µg/mL hygromycin as described in [13].

Transfections

Flp-in T-REx 293 cells stably expressing empty vector, Flag-tagged PTBP1+ex9, or Flag-tagged PTBP1 -ex9 were transfected with a pool of 10µM PTBP1/PTBP2 targeting siRNAs (Qiagen siRNA Catalog# SI02649206 and SI04255146) or scrambled siRNA (Invitrogen AM4611) using Lipofectamine RNAiMAX (Invitrogen). 24 hours post-transfection, media was replaced and expression of PTBP1 was induced with 1µg/mL doxycycline. Cells were harvested 48 hours after induction.

H9C2 cells were cultured and transfected as described previously [20, 21]. H9C2 cells plated on 100mm dish were transfected with a pool of 10µM PTBP1/PTBP2 targeting siRNAs, RBFOX2 specific siRNA (Invitrogen siRNA ID# s96620) or scrambled siRNA (Invitrogen AM4611) using Lipofectamine RNAiMAX (Invitrogen). Cells were harvested 72 hours after siRNA transfections. H9c2 cells plated on 150mm dish were transfected with 10µg GFP-tagged PTBP1, RBFOX2, or 5µg GFP control plasmid using Neon nucleofection system (Invitrogen) and harvested 48 hours post-transfection.

Quantitative RT-PCR and statistical analysis

qRT-PCR was performed as described previously [20, 21]. TRIzol (Invitrogen 15596-018) was used to extract RNA from cells and mouse hearts according to the manufacturer's protocol with the modification that RNA was precipitated overnight at -80°C. Primer information used for qRT-PCR is provided in Supplemental Table 1. Student t-test was used to determine statistical significance between two groups and one-way Anova for comparisons of more than two groups with Bonferroni correction using the Prism software.

Results

PTBP1 is differentially spliced in Type 1 diabetic mouse hearts

We have previously identified genome wide AS changes in STZ induced T1D (STZ^{T1D}) mouse hearts [20, 21]. To determine the mechanism responsible for T1D induced AS dysregulation, we examined T1D heart AS data and identified a splicing change in PTBP1 where exon 8 (exon 9 in human) is more skipped in T1D mouse hearts indicated by lower number of reads mapped to this exon (Fig. 1A). This PTBP1 isoform lacking exon 8 is reported to have low splicing repressor function [13, 24, 25]. qRT-PCR analysis showed increased exon 8 skipping of PTBP1 in two different T1D mouse models: STZ and non-obese diabetic (NOD) when compared to non-diabetic controls (Fig. 1B and 1C).

Regulation of diabetes-induced and developmentally regulated PTBP1 spliced variant in the heart

The expression of this PTBP1 spliced variant has been shown to regulate AS of brain specific genes during neuronal development [13]. For that reason, we tested whether PTBP1 exon 8 splicing is regulated during mouse heart development. We found that PTBP1 exon 8 is more skipped in WT newborn hearts in comparison to WT adult and embryonic hearts (Fig. 1D). To determine how PTBP1 splicing is modulated in T1D mouse hearts and during mouse heart development, we tested whether CELF1 and RBFOX2 are involved. We have previously shown that both CELF1 and RBFOX2 protein levels are modulated in the diabetic heart [19–21]. These proteins are involved in developmental regulation of AS in the heart [16, 26, 27]. Therefore, we reasoned that changes in PTBP1 AS might be due to alterations in CELF1 and RBFOX2. We depleted CELF1 or RBFOX2 then tested PTBP1 exon 8 AS. We found that depletion of these proteins leads to more exon 8 skipping (Fig. 1E) similar to the AS pattern in diabetic mouse hearts (Fig. 1B and 1C). This result is consistent with low CELF1 and RBFOX2 AS function in the diabetic heart [20, 21].

PTBP1 targets undergo AS changes in diabetic hearts

Since PTBP1 is modulated in diabetic hearts, we wanted to determine the contribution of PTBP1 to genome wide AS changes in the diabetic heart. For that reason, we overlaid our Type 1 diabetic heart RNA-Seq data [19, 21] with the available PTBP1 CLIP-Seq datasets obtained from mouse embryonic stem cells [23]. We examined PTBP1 binding sites within 250nt of the upstream and downstream intronic sequences and 50nt of the 5' and 3' ends of the alternative exons, which are typically the regions for AS regulation. Out of 1318 differential cassette exon events [19], 58 exhibited PTBP1 binding sites (Fig. 2A). Our result is consistent with results from Blencowe lab, which has also identified around 58 cassette exons regulated by this PTBP1 spliced variant in chicken DT40 B cells [13]. When we examined transcripts with PTBP1 binding sites, PTBP1 regulated alternative exons were equally excluded and included in diabetic hearts (Fig. 2B).

To better comprehend how this spliced variant of PTBP1 impacts AS outcomes, we examined AS of the well-known and extensively studied target of PTBP1 (*Tpm2* exon 6b) in diabetic hearts. PTBP1 is a repressor of *Tpm2* exon 6b inclusion [28], but we find that this exon was more included in diabetic hearts based on RNA-seq reads (Fig. 2C). This is consistent with expression of a PTBP1 spliced variant with low repressor activity. To determine the consequences of PTBP1-mediated AS changes in diabetic mouse hearts, we performed the KEGG pathway analysis of PTBP1 targets using Enrichr (<http://amp.pharm.mssm.edu/Enrichr>). Top categories for PTBP1-regulated genes (total: 58) are RNA transport, MAPK signaling and endocytosis (Fig. 2D).

Diabetes-induced PTBP1 spliced variant regulates AS of transcripts differentially spliced in diabetic hearts

We identified *CLIP1* gene as a target of PTBP1 differentially spliced in the diabetic heart (Supplemental Table 2). The genome browser image shows that *CLIP1* exon 9 is more included in STZ:T1D mouse hearts (Fig. 3A). To determine how diabetes-induced PTBP1 spliced variant regulates AS of *CLIP1*, we generated inducible human 293 cells stably

expressing WT PTBP1 that includes exon 9 (exon 8 in mouse): PTBP1ex9 and that excludes exon 9: PTBP1⁻ex9. We treated these cells with PTBP1/2 siRNA to remove the endogenous PTBP activity, and then induced expression of empty vector or PTBP1 (+ex9, ⁻ex9) using doxycycline. We examined AS patterns of *CLIP1* and *Med23* (a known *PTBP1* target used as a positive control). Depletion of PTBP1/2 led to increased inclusion of alternative exons of *CLIP1* and *MED23*, indicating that PTBP1 is a repressor of these exons (Fig. 3B). Cells stably expressing WT PTBP1+ex9 displayed lower inclusion of *MED23* and *CLIP1* alternative exons consistent with its role as a repressor (Fig. 3B, dark gray bars), whereas expression of PTBP1⁻ex9 spliced variant caused more inclusion (Fig. 3B, black bars vs dark gray bars). Efficiency of knockdown of endogenous PTBP1 and expression of PTBP1+ex9 or PTBP1⁻ex9 were verified by qRT-PCR (Fig. 3C) and by WB (Fig. 3D). These results suggest that a spliced variant of PTBP1 with less repressive function is generated in diabetic hearts and contributes to AS of its targets.

PTBP1 regulates AS in diabetic hearts antagonistically to RBFOX2

So far, our published results and results shown here indicate that 3 RBPs CELF1 [21] RBFOX2, and PTBP1 are altered in the diabetic heart [20] (Fig. 1). Thus, to determine how changes in these 3 RBPs affect AS decisions in the diabetic heart, we focused on AS of *Tmem184b* gene as *Tmem184b* pre-mRNA exhibits binding sites for all three RNA binding proteins: PTBP1, CELF1 and RBFOX2 ([20] and Fig. 4A). We have shown that CELF1 regulates AS of this exon, which is excluded in T1D hearts [19]. Thus, here we tested the roles of PTBP1 and RBFOX2 in AS of *Tmem184b*. Knockdowns of PTBP1/2 resulted in inclusion of *Tmem184b* exon 9 suggesting that PTBP is a repressor of this alternative exon (Fig. 4B). On the other hand, RBFOX2 overexpression increased exon inclusion, but its knockdown led to exon exclusion, suggesting that RBFOX2 is an activator of this alternative exon (Fig. 4B). In sum, PTBP1 and RBFOX2 antagonistically regulate AS of *Tmem184b*.

Discussion

We have previously shown that RBFOX2 and CELF1 are contributors to AS dysregulation in the diabetic heart [20]. In this study, our goal was to determine whether other RBPs play a role in AS changes in the diabetic heart. Indeed, both computational and molecular analyses identified a splicing change in PTBP1 in diabetic hearts. PTBP proteins are splicing repressors with well-defined roles in cancer, neuronal and heart development, and pluripotency of embryonic stem cells [23, 29–33]. Developmentally regulated AS events in the heart are enriched for adjacent PTBP1 binding sites [34]. However, PTBP1's role in AS regulation in diseased hearts is not clear. Here, we provide evidence that PTBP1 contributes to AS changes in the diabetic heart (Fig. 2). Here, we show for the first time that a group of PTBP1 targets are affected in diabetic hearts.

Importantly, we found that PTBP1 undergoes a splicing change in diabetic hearts that generates a spliced variant with less repressive splicing function. Consistent with this finding, a well-known *Tpm2* alternative exon repressed by PTBP1 was more included in diabetic hearts (Fig. 2). Diabetes-induced PTBP1 spliced isoform lacking exon 8 in mouse (exon 9 in human) has been shown to display less repressive splicing function [13, 24, 25]

and activation of a group of brain-specific AS networks during neurogenesis [13]. We showed that diabetes-induced spliced variant of PTBP1 displays less repressive function (Fig. 3). In addition, this PTBP1 spliced variant was more favored in the newborn mouse heart (Fig. 1D). Newborn heart undergoes hypertrophic growth during post-natal heart development that increases as an adaptation to stress in adult hearts, but overtime hypertrophy can lead to heart dysfunction [40]. Cardiac hypertrophy is associated with adverse outcomes in diabetic patients [41]. Since we found the PTBP1 spliced variant is aberrantly expressed in adult diabetic hearts, it is possible that expression of this PTBP1 variant might contribute to cardiac hypertrophy by activating newborn-like AS patterns. In support of this, PTBP1 targets that are mis-spliced in diabetic hearts are within the MAPK signaling pathway, which has a major role in cardiac hypertrophy.

Our results indicate that PTBP1, CELF1, and RBFOX2 are all altered in diabetic hearts [20, 21]. PTBP1 is a well-known repressor of exon inclusion [6–13], but it has been shown to also activate exon inclusion [14, 15]. PTBP1 has been shown to repress differentiated muscle-specific alternative exons [10, 29, 35–39]. PTBP1 and CELF1 similarly regulate AS of some targets [39], but they also antagonistically regulate AS [35, 42]. PTBP1 and RBFOX2 antagonistically control AS [39, 43]. CELF1 and RBFOX2 oppositely regulate AS [39, 44, 45]. Using the *Tmem184b* exon 9 AS as a model, we found that both CELF1 [19] and PTBP1 are repressors and RBFOX2 is an activator of *Tmem184b* exon 9 inclusion. Thus, we propose the model that modulation of RBFOX2, CELF1, and PTBP1 jointly contribute to genome wide AS changes in the diabetic heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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4. Diabetes-induced PTBP1 spliced variant expression is controlled by CELF1 and RBFOX2.
5. PTBP1 regulates alternative splicing of some targets antagonistically to RBFOX2.

Figure 1: Regulation of PTBP1 spliced variant expression in the heart under diabetic conditions and during development.

A) Representative genome browser images of *PTBP1* exon 8 exclusion in diabetic mouse hearts in comparison to the control mouse hearts. qRT-PCR analysis of *PTBP1* exon 8 inclusion in **(B)** control or streptozotocin induced diabetic (STZ:T1D), **(C)** in control or non-obese diabetic (NOD:T1D) mouse hearts. qRT-PCR analysis of *PTBP1* exon 8 inclusion **(D)** during mouse heart development at different stages: embryonic day 18 (E18), newborn (NB), or adult (n = 3) and **(E)** in H9c2 cells depleted of RBFOX2 or CELF1. (n = 3). Data

represent means $\pm$ SD. Statistical significance was calculated using Student's t-test for two groups and one-way ANOVA to compare three different groups. P-values are represented as **** < 0.0001 , *** < 0.001 and ** < 0.01 .

Figure 2: PTBP1 binding sites are present within transcripts that are mis-spliced in Type 1 diabetic mouse hearts.

A) Number of transcripts differentially spliced in Type 1 diabetic mouse hearts that display PTBP1 binding sites. PTB binding sites were identified by extracting data from previously published CLIP-Seq data and overlapping these binding sites within transcripts (1318) mis-spliced in diabetic hearts. 58 of these transcripts exhibit PTBP1 binding sites. **B)** Distribution of cassette exon inclusion or exclusion of PTBP1 targets in diabetic hearts. **C)** Representative genome browser images of a known PTBP1 target *Tpm2* exon 6b (E6B) in control vs STZ:T1D mice hearts. **D)** The KEGG pathway categories for PTBP1-regulated AS events in diabetic hearts using *Enrichr*.

Figure 3: PTBP1 spliced variant regulates AS of transcripts differentially spliced in diabetic hearts.

A) Representative genome browser images of PTBP1 target *Clip1* exon 9 in control vs STZ:T1D mice hearts. AS analysis of **B)** *Clip1*, *Med23* and **C)** *PTBP1* in 293 cells depleted of PTBP1/2 and stably expressing inducible WT (PTBP1+ex9) and diabetes-induced spliced variant (PTBP1 Δ ex9) using qRT-PCR. **D)** Validation of endogenous PTBP1 depletion and ectopic expression of PTBP1+ex9 and PTBP1 Δ ex9 by Western blot (WB) using an antibody against PTBP1. Total protein staining was used to confirm even protein loading. Data

represent means $\pm$ SD. Statistical significance was calculated using one-way ANOVA to compare four different groups. P-values are represented as *** < 0.001 , ** < 0.01 , * < 0.05 .

Figure 4: PTBP1 regulates alternative splicing of *Tmem184b* mis-spliced in diabetes antagonistically to RBFOX2.

A) Diagram of PTBP1 (light gray boxes), and RBFOX2 binding sites (black box) within the *Tmem184b* pre-mRNA derived from CLIP-seq data and PTBP1 motif analysis. **B)** AS analysis of endogenous *Tmem184b* exon 9 in H9c2 cells transfected with scrambled (Control) or RBP specific siRNA pools (PTBP1/PTBP2siPool or RBFOX2siPool) (n=3) and transfected with GFP (Control), PTBP1GFP or RBFOX2GFP (n=3). Data represent means $\pm$ SD. Statistical significance was calculated using one-way ANOVA to compare three different groups. P-values are represented as *** < 0.001, ** < 0.01