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ORIGINAL ARTICLE

Persistent coxsackievirus B4 infection induces microRNA dysregulation in human pancreatic cells

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Abstract Enterovirus infections are implicated in the development of type 1 diabetes (T1D). MicroRNAs as regulators of gene expression are involved in many physiological and pathological processes. Given that viral infections dysregulate cellular microRNAs, we investigated the impact of persistent coxsackievirus B4 infection on microRNA expression of human pancreatic cells. Next-generation sequencing was used to determine microRNA expression in PANC-1 cells persistently infected (for several weeks) with coxsackievirus B4 and uninfected control cells. Target prediction restricted to T1D risk genes was performed with miRWalk2.0. Functional annotation analysis was performed with DAVID6.7. Expression of selected microRNAs and T1D risk genes was measured by quantitative reverse-transcription polymerase chain reaction. Eighty-one microRNAs were dysregulated in persistently infected PANC-1 cells. Forty-nine of the known fifty-five T1D risk genes were predicted as putative targets of at least one of the dysregulated microRNAs. Most functional annotation terms that were enriched in these 49 putative target genes were related to the immune response or autoimmunity. mRNA levels of AFF3, BACH2, and IL7R differed significantly between persistently infected cells and uninfected cells. This is the first characterization of the microRNA expression profile changes induced by persistent coxsackievirus B4 infection in pancreatic cells. The predicted targeting of genes involved in the immune response and autoimmunity by the dysregulated microRNAs as well as the dysregulated expression of diabetes risk genes shows that persistent coxsackievirus B4 infection profoundly impacts the host cell. These data support the hypothesis of a possible link between persistent coxsackievirus B4 infection and the development of T1D.

Keywords miRNA · Persistence · Latency · Gene expression · Latent infection

Abbreviations

CVB	Type B coxsackieviruses
CVB4	Coxsackievirus B4
Ct	Cycle threshold
FCS	Fetal calf serum
miRNA	MicroRNA
T1D	Type 1 diabetes

Introduction

Type 1 diabetes (T1D) is thought to be caused by an interplay between genetic factors, the immune system, and environmental factors [1]. Among the later, enterovirus infections, especially with type B coxsackieviruses (CVB), have been linked to the development of T1D [2, 3]. Signs of enteroviral infection have frequently been detected in pancreatic cells of T1D patients [4]. Therefore, a persistent enteroviral infection of the pancreas could be one of the causes responsible for the development of T1D [1, 3, 5, 6].

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CVB are small non-enveloped RNA viruses that belong to the *Enterovirus* genus of the *Picornaviridae* family. CVB can establish persistent infections in vitro as well as in vivo [7]. Persistence of CVB4 in human pancreatic ductal cells resulted in an impaired formation and viability of islet-like cell aggregates [8]. CVB might initiate or accelerate the development of T1D by different mechanisms, including direct destruction of beta-cells, inflammatory cytokine induction, molecular mimicry, or dysregulation of host cell microRNA (miRNA) expression [1, 9].

miRNAs are small 19–23 nucleotide RNA molecules that regulate gene expression by inducing translational arrest and/or degradation of messenger RNAs [10]. miRNAs are involved in many key regulatory processes in cells and have also been shown to play an important role in beta-cell function [11]. Dysregulation of miRNA expression has been found in many diseases, including viral infections and T1D [9, 12].

The impact of enterovirus infection on host cell miRNA expression has been described in several recent studies [13–16]. However, these studies focused on acute enterovirus infection. To our knowledge, the impact of persistent enterovirus infection on pancreatic cell miRNA expression has not been investigated to date. Given that persistent enterovirus infection of the pancreas may be one underlying mechanism of T1D development, the aim of the current study was to investigate the impact of persistent CVB4 infection on the miRNA expression profile of pancreatic cells. To this end, we took advantage of pancreatic ductal cells persistently infected with CVB4, in which a stable persistent infection was obtained beyond 5 weeks post-inoculation of the virus [8, 17].

We further investigated whether the dysregulated miRNAs potentially target T1D risk genes.

Methods

Cells and virus

The human pancreatic ductal cell line PANC-1 (ATCC, LGC Standards, Molsheim, France) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% of heat inactivated fetal calf serum (FCS), 1% of L-glutamine, penicillin, and streptomycin (Thermofisher Scientific, Courtaboeuf, France). The diabetogenic CVB4E2 strain (kindly provided by Ji-Won Yoon, Julia McFarlane Diabetes Research Center, Calgary, Alberta, Canada) was propagated in Hep-2 cells (BioWhittaker, Vervier, Belgium). The CVB4E2 strain was isolated by Yoon et al. from the pancreas of a child with recently diagnosed type 1 diabetes and is diabetogenic in mice [18]. Briefly, after three freeze–thaw cycles, the suspension was collected and clarified at 2000 g for 10 min at 4 °C. Aliquots of virus preparations were stored at −80 °C.

Persistent CVB4 infection in PANC-1 cells

A persistent CVB4 infection was established in PANC-1 cells [17]. Briefly, a 25 cm^2 Nunc® cell culture flask (Thermofisher Scientific, Villebon, France) containing an average of 10^6 cells was inoculated with CVB4E2 at a multiplicity of infection (MOI) of 0.01. During the acute lytic infection, cells were regularly washed to remove excess virus. A stable equilibrium developed between viral replication and cell proliferation. The CVB4 infected and uninfected PANC-1 cells were scraped and subcultured once a week.

Cells were collected from four independent biological replicates of PANC-1 cells persistently infected with CVB4 (after 13 passages, i.e., 13 weeks ($n = 3$), and after 20 passages, i.e., 20 weeks ($n = 1$), of persistent CVB4 infection) and four replicates of uninfected PANC-1 cells. Cells were washed with PBS. RNA was extracted with the miRNeasy Mini kit (Qiagen, Courtaboeuf, France) with on-column DNA digestion using DNase I (Qiagen, Courtaboeuf, France). Extracted RNA was quantified with a Nanodrop® spectrophotometer (Thermofisher Scientific, Courtaboeuf, France) and RNA quality was assessed using the 2100 Bioanalyzer (Agilent technologies, Courtaboeuf, France).

Sequencing of microRNAs

Small RNA libraries were prepared from 1 µg of total RNA using the Ion Total RNA-Seq Kit v2.0 (Life Technologies, Carlsbad, CA, USA). Each sample was ligated to two barcodes to detect and bypass PCR biases. Barcoded libraries were quantified and assessed for quality using the Agilent 2100 BioAnalyzer (Agilent technologies France, Courtaboeuf, France). Libraries were pooled in equimolar amounts and sequenced on an Ion PROTON™ Platform using an Ion P1™ Chip Kit v2 and the Ion P1™ Sequencing 200 kit v3 (Life Technologies, Thermofisher Scientific, Courtaboeuf, France).

Primary analysis transforming signal to DNA sequences was done with the default parameters on a Torrent Server 4.0.2 (Life Technologies, Thermofisher Scientific, Courtaboeuf, France). Demultiplexing was done with 0 errors allowed in barcodes. Raw reads were analyzed with ncPRO-Seq 1.5.1 [19]. For each sample, reproducibility of counts within the two barcodes was investigated by Spearman correlation and visual inspection of counts showing no biases between the barcodes. Each pair of barcodes was then pooled and a new ncPRO-Seq analysis

was performed on all samples ($n = 8$). The differential expression of the raw counts obtained from ncPRO-Seq was performed with DESeq2 [20].

Quantification of miRNAs by RT-PCR

The expression of hsa-miR-663b, hsa-miR-1913, hsa-miR-10a-5p, hsa-miR-23a-3p, hsa-miR-23b-3p, hsa-miR-125b-5p, hsa-miR-146a-5p, and hsa-miR-138-5p was quantified using the TaqMan® MicroRNA kits (Life Technologies, Thermofisher Scientific, Courtaboeuf, France). The Taqman MicroRNA Reverse Transcription Kit was used for cDNA synthesis, and real-time PCRs were performed with the Taqman Small RNA assays (primers and probe) and the Taqman Universal PCR Master Mix II according to the manufacturer's recommendations, on a Mx3000p® thermocycler (Agilent technologies France, Courtaboeuf, France).The expression of hsa-miR-6087 and hsa-miR-4516 was quantified using the miScript PCR Starter Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's recommendations, on a Mx3000p® thermocycler (Agilent technologies France, Courtaboeuf, France). RNU6B was used for normalization and the relative expression was determined using the $2^{-\Delta\Delta Ct}$ formula [21].

MiRNA target prediction

miRWalk 2.0 was used to predict potential target genes of the 81 dysregulated miRNAs [22]. The following settings were applied: "species: human"; "restricted to 3′UTR"; "minimum seed length = 7"; "p value = 0.05"; and using the databases: miRWalk, RNA22, miRanda, and Targetscan. The list of genes predicted as targets by at least three of the four databases was compared with the list of type 1 diabetes risk genes obtained from T1Base [23] to identify T1D risk genes that are potentially targeted by the dysregulated miRNAs.

Functional annotation analysis

The 49 T1D risk genes that were potential targets of the 81 dysregulated miRNAs were functionally annotated using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.7 [24]. This analysis yields functional annotations that are enriched in these genes and indicates an enrichment p value.

Gene expression analysis

2 μg of total RNA from PANC-1 cells persistently infected with CVB4 and uninfected control cells were DNAse treated (RQ1 RNase-Free DNase, Promega France, Charbonnières-les-Bains, France). cDNA synthesis was performed using the Superscript III first strand synthesis system for RT-PCR kit (Thermofisher Scientific, Courtaboeuf, France) and random hexamer primers. cDNA was diluted 1:10 and quantitative PCR was performed with the Power SYBR green master mix (Applied Biosystems, Thermofisher Scientific, Courtaboeuf, France). The following primers were used (Eurofins Genomics, Ebersberg, Germany): *CLEC16A*: CATCAAGACGAGTGGGGAGAGT and TCCTCGTCCGTGGTGTTCTG; *GLIS3*: CAACCAGATCAGTCCTAGCTTACA and GCGAAATAAGGGACCTGGTATC; *IKZF1*: CACAGTGAAATGGCAGAAGACC and GGCCCTTGTCCCCAAGAAAT; *SH2B3*: AACCACCAGGTTCCTGCAAC and GGACAGCCAGAAGAACTAAGGTG [16]; *BACH2*: GACTTTGATCGTGGAGAGGAA and GCAAGCTGACCACCAAATC; *beta-Actin*: ACCGAGCGCGGCTACAG and CTTAATGTCACGCACGATTTCC; *AFF3*: ACTCAACAGGATGATGGCACG and TGCCTAAAGTGTTCTGGATCCG; *IKZF4*: ACGAAATACGTGACCTGGAGATG and CTTGCGTTTGGTGAGGCTATTG; *IL7R*: CTGGTTTGTAGGCAGCAGAAGA and GGAGCCTCCTATTTGAGACTTGAC.

Cycling was performed on an ABI7500 (Applied Biosystems, Thermofisher Scientific, Courtaboeuf, France) with the following thermal profile: 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Cycle thresholds (Cts) were normalized to *beta-Actin* levels. Relative mRNA expression was expressed as fold change in PANC-1 cells persistently infected with CVB4 compared to uninfected control cells. Relative mRNA expression in persistently infected versus non-infected cells was compared using the Mann–Whitney test. A p value <0.05 was considered statistically significant. Statistical analyses and graphs were performed with the Graph Pad Prism 6.03 software (GraphPad Software, La Jolla, CA).

Results

The miRNA expression profile differed between PANC-1 cells with persistent CVB4 infection and uninfected PANC-1 cells. A total of 81 miRNAs showed a significant differential expression (defined as a fold change ≥ 3, $p < 0.05$), and among them, 65 miRNAs were up-regulated and 16 were down-regulated in persistently infected cells (Table 1). The highest induction was seen for hsa-miR-6087, hsa-miR-663b, hsa-miR-4516, hsa-miR-4532, hsa-miR-1913, and hsa-miR-3621 that showed a more than hundredfold higher expression in persistently infected compared to uninfected cells. In contrast, the down-regulated miRNAs displayed less than tenfold down-regulation in persistently infected cells compared to uninfected cells. The expression of selected miRNAs that showed a high

Table 1 MiRNAs differentially expressed in PANC-1 cells persistently infected with CVB4 compared to uninfected cells

miRNA (Mirbase-v20)	Dysregulation[a]	Fold change[b]	Adjusted *p* value[c]
hsa-miR-6087	Up	340.51	1.37E−125
hsa-miR-663b	Up	208.55	1.53E−27
hsa-miR-4516	Up	156.42	2.16E−22
hsa-miR-4532	Up	133.15	9.88E−22
hsa-miR-1913	Up	120.21	6.09E−18
hsa-miR-3621	Up	115.92	9.22E−16
hsa-miR-6730-3p	Up	95.54	3.41E−15
hsa-miR-4492	Up	85.40	1.08E−14
hsa-miR-6800-3p	Up	78.41	4.11E−12
hsa-miR-6087	Up	73.40	1.19E−11
hsa-miR-1292-3p	Up	65.85	9.49E−21
hsa-miR-3687	Up	58.50	3.22E−16
hsa-miR-6858-3p	Up	46.37	1.17E−08
hsa-miR-4449	Up	41.62	1.92E−14
hsa-miR-663a	Up	37.51	1.79E−07
hsa-miR-885-3p	Up	32.61	6.28E−36
hsa-miR-7641	Up	32.51	4.84E−13
hsa-miR-4488	Up	31.76	1.51E−06
hsa-miR-3656	Up	30.96	8.46E−10
hsa-miR-1470	Up	25.14	4.34E−18
hsa-miR-4497	Up	23.67	2.36E−06
hsa-miR-6858-5p	Up	23.14	1.64E−14
hsa-miR-4508	Up	21.92	9.26E−25
hsa-miR-3182	Up	17.61	9.72E−05
hsa-miR-3654	Up	16.42	3.86E−04
hsa-miR-4477a	Up	16.14	1.83E−06
hsa-miR-664b-5p	Up	13.57	1.17E−08
hsa-miR-6821-5p	Up	11.83	2.70E−03
hsa-miR-6808-3p	Up	11.18	2.65E−03
hsa-miR-4758-5p	Up	11.05	2.74E−04
hsa-miR-664a-5p	Up	10.88	1.77E−04
hsa-miR-922	Up	10.60	4.21E−03
hsa-miR-6834-3p	Up	10.51	4.65E−03
hsa-miR-4710	Up	9.17	8.41E−03
hsa-miR-6892-5p	Up	8.79	9.80E−03
hsa-miR-204-5p	Up	8.54	2.24E−04
hsa-miR-582-5p	Up	7.94	1.17E−02
hsa-miR-1268b	Up	7.59	4.39E−03
hsa-miR-551a	Up	7.17	1.05E−02
hsa-miR-4417	Up	6.76	2.73E−02
hsa-miR-668-5p	Up	6.72	2.77E−02
hsa-miR-483-3p	Up	6.65	4.19E−03
hsa-miR-8072	Up	6.59	1.92E−02
hsa-miR-137	Up	6.07	3.80E−02
hsa-miR-30d-3p	Up	5.61	4.11E−02
hsa-miR-15a-3p	Up	5.59	1.38E−03
hsa-miR-483-5p	Up	5.58	2.96E−02
hsa-miR-5701	Up	5.57	3.43E−02
hsa-miR-146a-5p	Up	5.41	2.06E−09
hsa-miR-1908-3p	Up	5.31	2.02E−02
hsa-miR-412-5p	Up	5.30	4.14E−02
hsa-miR-1227-3p	Up	5.23	2.20E−02
hsa-miR-380-5p	Up	5.03	1.27E−02
hsa-miR-147b	Up	4.91	6.80E−08
hsa-miR-138-5p	Up	4.61	4.34E−02
hsa-miR-7704	Up	4.13	7.64E−08
hsa-miR-668-3p	Up	3.73	4.21E−03
hsa-miR-376b-3p	Up	3.66	2.06E−15
hsa-miR-222-3p	Up	3.60	3.85E−18
hsa-miR-3117-3p	Up	3.55	7.65E−03
hsa-miR-665	Up	3.50	3.88E−08
hsa-miR-1248	Up	3.42	5.00E−04
hsa-miR-376a-3p	Up	3.30	4.66E−08
hsa-miR-675-3p	Up	3.23	2.19E−02
hsa-miR-1307-5p	Up	3.04	1.61E−10
hsa-let-7f-1-3p	Down	−8.20	7.85E−03
hsa-miR-23b-5p	Down	−7.90	9.65E−03
hsa-let-7b-3p	Down	−6.78	4.86E−07
hsa-miR-335-3p	Down	−6.17	2.96E−02
hsa-miR-194-5p	Down	−5.53	4.41E−02
hsa-miR-3178	Down	−5.39	2.22E−02
hsa-miR-34c-5p	Down	−5.31	2.45E−05
hsa-miR-187-3p	Down	−5.25	7.60E−06
hsa-let-7d-3p	Down	−5.21	9.63E−04
hsa-miR-132-5p	Down	−4.64	4.17E−02
hsa-miR-10a-5p	Down	−4.28	1.66E−33
hsa-miR-10b-5p	Down	−3.55	1.35E−20
hsa-miR-98-3p	Down	−3.44	2.91E−02
hsa-miR-23b-3p	Down	−3.22	4.62E−22
hsa-miR-335-5p	Down	−3.11	1.53E−03
hsa-miR-1260b	Down	−3.07	2.17E−02

[a] miRNAs up-regulated or down-regulated in PANC-1 cells persistently infected with CVB4 compared to uninfected cells are listed

[b] Fold change of miRNA expression in PANC-1 cells persistently infected with CVB4 compared to uninfected cells

[c] Adjusted *p* value calculated between PANC-1 cells persistently infected with CVB4 and uninfected cells obtained with DESeq2 (based on negative binomial generalized linear models)

fold change in the sequencing data or that are known to be implicated in enteroviral infection (hsa-miR-6087, hsa-miR-663b, hsa-miR-4516, hsa-miR-1913, hsa-miR-10a-5p, hsa-miR-23a-3p, hsa-miR-23b-3p, hsa-miR-125b-5p, and hsa-miR-146a-5p, hsa-miR-138-5p) [13–15, 25] was additionally analyzed by RT qPCR. Five of the six miRNAs

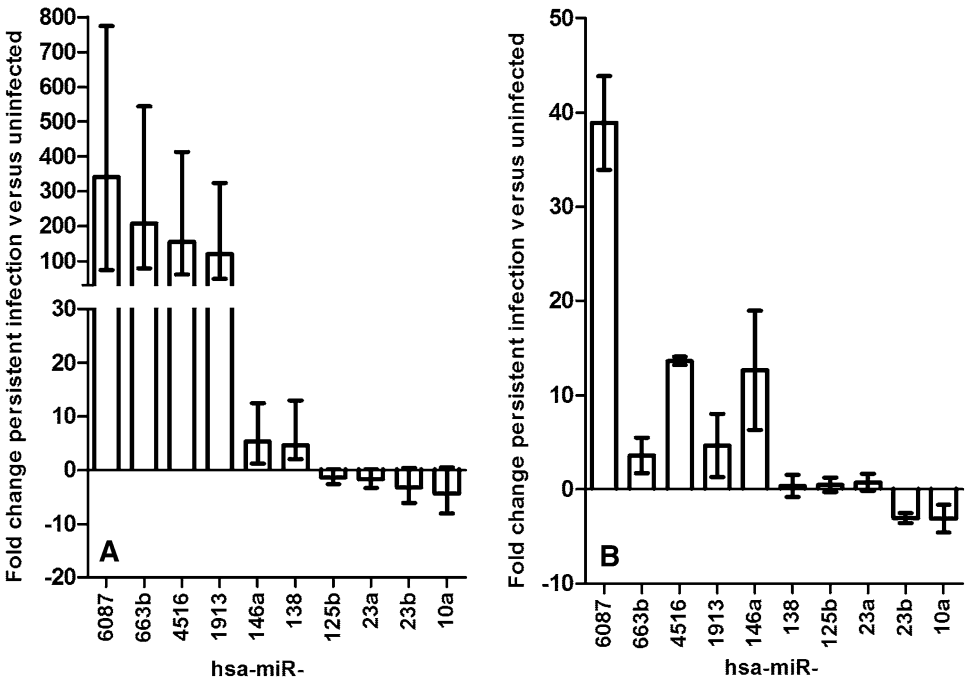

Fig. 1 Changes of miRNA expression in PANC-1 cells persistently infected with CVB4. **a** Expression of selected miRNAs expressed as fold changes of persistently infected compared to uninfected control cells. Data of four independent biological replicates are shown as mean ± standard error obtained from sequencing data analyzed by DESeq2. **b** Expression of selected miRNAs determined by RT qPCR expressed as fold changes of persistently infected compared to uninfected control cells. Data of six independent biological replicates are shown except for hsa-miR-6087 and hsa-miR-4516, where data of two independent biological replicates are shown as mean ± standard error of the mean

analyzed that showed an up-regulation in the sequencing data (Fig. 1a) and also showed an up-regulation by RT qPCR, whereas one showed no change (Fig. 1b). Two of the four miRNAs analyzed that showed a down-regulation in the sequencing data (Fig. 1a) and also showed a down-regulation by RT qPCR (Fig. 1b). The extent of the dysregulation was different between the sequencing and the RT qPCR data, for example hsa-miR-6087 showed a 341-fold up-regulation by sequencing and a 39-fold up-regulation by RT qPCR (Fig. 1).

We next investigated the potential impact of the miRNA expression changes induced by persistent CVB4 infection on genes that are known to be implicated in T1D. To this end, miRNA target prediction was performed using miRWalk and target genes were restricted to the 55 T1D risk genes contained in the T1D database [23]. Forty-nine of the fifty-five T1D risk genes were predicted as putative targets of at least one of the dysregulated miRNAs (Table 2). Most of the genes were potentially targeted by several of the dysregulated miRNAs, and the genes targeted by the highest number of dysregulated miRNAs ($n = 24$) were AFF3 and BACH2. Most of the dysregulated miRNAs were also predicted to target several of the T1D risk genes (Table 2).

mRNA levels of the house-keeping gene *beta-Actin* and selected T1D risk genes were measured by RT qPCR in PANC-1 cells with persistent CVB4 infection and uninfected PANC-1 cells. *Beta-Actin* mRNA levels were similar in persistently infected and uninfected cells (mean difference of 0.5 Cts, data not shown). mRNA levels of *AFF3* and *IL7R* were significantly higher in persistently infected cells, whereas *BACH2* levels were significantly lower (Fig. 2). The mRNA levels of the other analyzed genes were similar in infected and uninfected cells.

Functional annotation analysis of the 49 putative target genes was studied with DAVID [26]. "Autoimmune thyroid disease" showed that the highest enrichment score and genes associated with viral myocarditis and T1D were also enriched. Most functional annotation terms that were enriched in these 49 putative target genes were related to the immune response or autoimmunity (Table 3).

Discussion

miRNAs are major regulators of gene expression and are involved in many physiological and pathological processes. Inhibition of miRNA production by disruption of the gene Dicer leads to development of diabetes in mice [27]. Disruption of Dicer leads to altered islet morphology, marked decreased beta-cell mass, and reduced insulin production [27]. Several miRNAs have been described to be involved

Table 2 Type 1 diabetes risk genes predicted as targets of the 81 dysregulated miRNAs

Type 1 diabetes risk gene	Up-regulation	Down-regulation	Number of miRNAs[a]
AFF3	hsa-miR-4492, hsa-miR-6087, hsa-miR-6800-3p, hsa-miR-4417, hsa-miR-4710, hsa-miR-582-5p, hsa-miR-1227-3p, hsa-miR-138-5p, hsa-miR-146a-5p, hsa-miR-222-3p, hsa-miR-30d-3p, hsa-miR-380-5p, hsa-miR-1227-3p, hsa-miR-222-3p, hsa-miR-30d-3p, hsa-miR-1248, hsa-miR-665, hsa-miR-675-3p	hsa-let-7b-3p, hsa-let-7f-1-3p, hsa-miR-194-5p, hsa-miR-23b-3p, hsa-miR-335-3p, hsa-miR-98-3p	24
BACH2	hsa-miR-4492, hsa-miR-4516, hsa-miR-663b, hsa-miR-885-3p, hsa-miR-204-5p, hsa-miR-582-5p, hsa-miR-922, hsa-miR-137, hsa-miR-147b, hsa-miR-3117-3p, hsa-miR-380-5p, hsa-miR-483-3p, hsa-miR-5701, hsa-miR-668-3p, hsa-miR-137, hsa-miR-147b, hsa-miR-380-5p, hsa-miR-483-3p, hsa-miR-1248	hsa-miR-23b-3p, hsa-miR-23b-5p, hsa-miR-335-3p, hsa-miR-335-5p, hsa-miR-34c-5p	24
BAD	hsa-miR-4449, hsa-miR-663a	hsa-miR-132-5p, hsa-miR-34c-5p	4
C1QTNF6	hsa-miR-4492, hsa-miR-4516, hsa-miR-1913, hsa-miR-3656, hsa-miR-4532, hsa-miR-6087, hsa-miR-663a, hsa-miR-663b, hsa-miR-6858-5p	hsa-miR-132-5p, hsa-miR-23b-5p	11
CCR5	hsa-miR-15a-3p, hsa-miR-204-5p, hsa-miR-3182, hsa-miR-6800-3p, hsa-miR-6834-3p	hsa-miR-335-3p, hsa-miR-335-3p	7
CD226	hsa-miR-204-5p	hsa-miR-335-3p	2
CD69		hsa-miR-23b-3p	1
CLEC16A	hsa-miR-4492, hsa-miR-1248, hsa-miR-138-5p, hsa-miR-1913, hsa-miR-1914-3p, hsa-miR-222-3p, hsa-miR-3182, hsa-miR-380-5p, hsa-miR-4488, hsa-miR-4497, hsa-miR-4508, hsa-miR-4710, hsa-miR-4758-5p, hsa-miR-483-3p, hsa-miR-663b, hsa-miR-664a-5p, hsa-miR-6800-3p, hsa-miR-922	hsa-miR-3178, hsa-miR-335-3p	20
COBL	hsa-miR-3117-3p, hsa-miR-664a-5p, hsa-miR-668-3p, hsa-miR-6808-3p	hsa-miR-23b-3p	5
CTLA4	hsa-miR-1248, hsa-miR-664a-5p	hsa-miR-23b-5p, hsa-miR-335-3p	4
CTSH	hsa-miR-4710		1
CYP27B1	hsa-miR-146a-5p, hsa-miR-15a-3p	hsa-miR-335-3p	3
DEXI	hsa-miR-137, hsa-miR-15a-3p, hsa-miR-3182, hsa-miR-4497, hsa-miR-6800-3p	hsa-miR-1260b, hsa-miR-23b-5p	7
EFR3B	hsa-miR-1268b, hsa-miR-15a-3p, hsa-miR-1913, hsa-miR-4492, sa-miR-4516, hsa-miR-6087, hsa-miR-663a, hsa-miR-664a-5p, hsa-miR-664b-5p, hsa-miR-665, hsa-miR-6730-3p, hsa-miR-6858-5p, hsa-miR-922	hsa-miR-10a-5p, hsa-miR-10b-5p, hsa-miR-1260b, hsa-miR-34c-5p	17
ERBB3	hsa-miR-137, hsa-miR-204-5p, hsa-miR-3182, hsa-miR-4492, hsa-miR-4516, hsa-miR-664a-5p, hsa-miR-665	hsa-miR-335-5p	8
FUT2	hsa-miR-1268b, hsa-miR-664a-5p, hsa-miR-675-3p	hsa-miR-1260b, hsa-miR-23b-5p	5
GLIS3	hsa-miR-137, hsa-miR-138-5p, hsa-miR-146a-5p, hsa-miR-204-5p, hsa-miR-3117-3p, hsa-miR-3182, hsa-miR-380-5p, hsa-miR-4710, hsa-miR-582-5p, hsa-miR-665	hsa-let-7b-3p, hsa-let-7f-1-3p, hsa-miR-335-3p, hsa-miR-98-3p	14
GPR183	hsa-miR-582-5p		1
GSDMB		hsa-miR-34c-5p	1
HLA-A	hsa-miR-6800-3p	hsa-miR-23b-3p	2
HLA-B	hsa-miR-30d-3p, hsa-miR-4417, hsa-miR-6800-3p	hsa-miR-23b-3p	4
HLA-DQB1	hsa-miR-137, hsa-miR-1913, hsa-miR-30d-3p, hsa-miR-6858-3p, hsa-miR-7641	hsa-miR-335-3p	6
HLA-DRB1	hsa-miR-15a-3p		1
IKZF1	hsa-miR-137, hsa-miR-138-5p, hsa-miR-146a-5p, hsa-miR-1470, hsa-miR-15a-3p, hsa-miR-3117-3p, hsa-miR-3182, hsa-miR-3654, hsa-miR-376a-3p, hsa-miR-376b-3p, hsa-miR-380-5p, hsa-miR-4488, hsa-miR-4516, hsa-miR-4710, hsa-miR-483-3p, hsa-miR-582-5p, hsa-miR-663a, hsa-miR-6858-3p, hsa-miR-665, hsa-miR-664a-5p	hsa-miR-3178, hsa-miR-335-3p, hsa-miR-34c-5p	23

Table 2 continued

Type 1 diabetes risk gene	Up-regulation	Down-regulation	Number of miRNAs[a]
IKZF4	hsa-miR-137, hsa-miR-146a-5p, hsa-miR-1913, hsa-miR-1914-3p, hsa-miR-30d-3p, hsa-miR-3117-3p, hsa-miR-3182, hsa-miR-4516, hsa-miR-4710, hsa-miR-483-3p, hsa-miR-582-5p, hsa-miR-663b, hsa-miR-665, hsa-miR-6858-3p, hsa-miR-6858-5p, hsa-miR-885-3p, hsa-miR-922	hsa-miR-10a-5p, hsa-miR-10b-5p, hsa-miR-34c-5p, hsa-miR-98-3p	21
IL10	hsa-miR-3182, hsa-miR-4492	hsa-miR-194-5p, hsa-miR-23b-5p	4
IL21		hsa-miR-194-5p	1
IL27	hsa-miR-30d-3p, hsa-miR-4492		2
IL2RA	hsa-miR-1248, hsa-miR-138-5p, hsa-miR-1913, hsa-miR-1914-3p, hsa-miR-204-5p, hsa-miR-3182, hsa-miR-6808-3p, hsa-miR-922		8
IL7R	hsa-miR-1227-3p, hsa-miR-1248, hsa-miR-137, hsa-miR-138-5p, hsa-miR-146a-5p, hsa-miR-1913, hsa-miR-1914-3p, hsa-miR-204-5p, hsa-miR-3182, hsa-miR-483-3p, hsa-miR-5701, hsa-miR-665	hsa-miR-194-5p, hsa-miR-23b-3p, hsa-miR-335-3p	15
INS	hsa-miR-3656, hsa-miR-8072		2
ITGB7	hsa-miR-668-3p		1
NAA25	hsa-miR-15a-3p, hsa-miR-3656, hsa-miR-6800-3p, hsa-miR-922	hsa-let-7b-3p, hsa-let-7f-1-3p, hsa-miR-194-5p, hsa-miR-23b-5p, hsa-miR-335-5p	9
NRP1	hsa-miR-1248, hsa-miR-137, sa-miR-1914-3p, hsa-miR-204-5p, hsa-miR-3654, hsa-miR-376a-3p, hsa-miR-376b-3p, hsa-miR-4492, hsa-miR-483-3p, hsa-miR-5701, hsa-miR-582-5p, hsa-miR-668-3p	hsa-miR-194-5p, hsa-miR-335-3p, hsa-miR-98-3p	15
ORMDL3	hsa-miR-15a-3p, hsa-miR-1913, hsa-miR-204-5p, hsa-miR-3182, hsa-miR-4516, hsa-miR-4710, hsa-miR-663b, hsa-miR-665, hsa-miR-6800-3p	hsa-miR-23b-3p, hsa-miR-34c-5p	11
PHTF1	hsa-miR-7641		1
PRKCQ	hsa-miR-137, hsa-miR-30d-3p, hsa-miR-4488, hsa-miR-4492, hsa-miR-4516, hsa-miR-665, hsa-miR-668-5p, hsa-miR-7641	hsa-miR-34c-5p	9
PTPN2	hsa-miR-137, hsa-miR-138-5p, hsa-miR-1913, hsa-miR-4417, hsa-miR-582-5p, hsa-miR-675-3p, hsa-miR-7641	hsa-miR-194-5p, hsa-miR-23b-5p, hsa-miR-335-3p	10
PTPN22	hsa-miR-30d-3p	hsa-miR-335-5p	2
RAC2	hsa-miR-146a-5p, hsa-miR-1914-3p, hsa-miR-4492, hsa-miR-922		4
RASGRP1	hsa-miR-146a-5p, hsa-miR-222-3p, hsa-miR-30d-3p, hsa-miR-376a-3p, hsa-miR-582-5p	hsa-let-7f-1-3p	6
RBM17	hsa-miR-1248, hsa-miR-15a-3p	hsa-miR-23b-3p	3
RNLS	hsa-miR-137, hsa-miR-204-5p, hsa-miR-222-3p, hsa-miR-4516	hsa-miR-194-5p, hsa-miR-335-3p	6
SH2B3	hsa-miR-1248, hsa-miR-138-5p, sa-miR-147b, hsa-miR-1913, hsa-miR-204-5p, hsa-miR-30d-3p, hsa-miR-3656, hsa-miR-380-5p, hsa-miR-4417, hsa-miR-668-3p, hsa-miR-6858-3p, hsa-miR-6892-5p	hsa-miR-10a-5p, hsa-miR-10b-5p, hsa-miR-3178	15
STAT4	hsa-miR-1227-3p, hsa-miR-30d-3p, hsa-miR-3182, hsa-miR-6858-5p	hsa-miR-335-3p	5
TAGAP	hsa-miR-6858-5p	hsa-miR-335-3p	2
TNFAIP3	hsa-miR-1248, hsa-miR-15a-3p, hsa-miR-1914-3p, hsa-miR-204-5p, hsa-miR-30d-3p, hsa-miR-380-5p, hsa-miR-4477a, hsa-miR-4516, hsa-miR-664a-5p, hsa-miR-6808-3p, hsa-miR-6858-3p, hsa-miR-885-3p, hsa-miR-922	hsa-miR-23b-3p, hsa-miR-335-3p	15
TYK2	hsa-miR-4710, hsa-miR-922		1
UBASH3A	hsa-miR-138-5p, hsa-miR-4492	hsa-miR-132-5p, hsa-miR-23b-3p	4

Type 1 diabetes risk genes are shown with the miRNAs that are predicted to target them and that have been found up-regulated or down-regulated by persistent CVB4 infection in the current study

[a] The total number of miRNAs found dysregulated in the current study that are predicted to target the type 1 diabetes risk genes

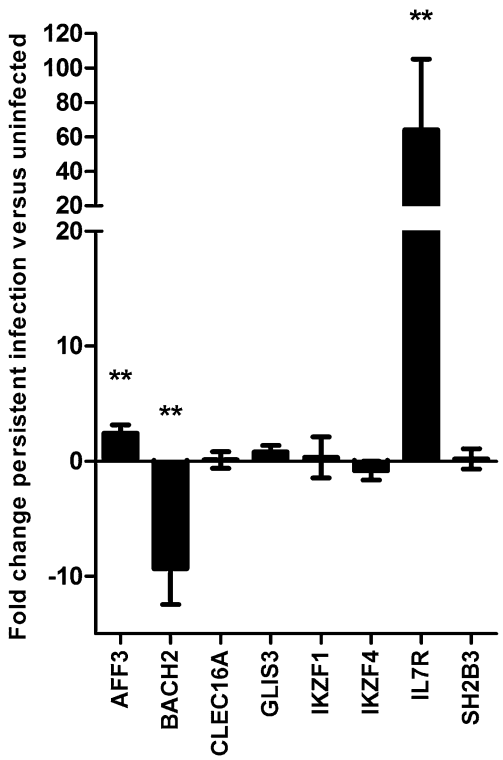

Fig. 2 mRNA expression of T1D risk genes in PANC-1 cells persistently infected with CVB4. mRNA expression of selected T1D risk genes was measured by RT qPCR and expressed as fold changes in cells persistently infected with CVB4 compared to uninfected control cells. Data of five independent biological replicates are shown as mean ± standard error of the mean. $^{**}p < 0.01$

in pancreas development, glucose sensing, insulin production, and diabetes [9, 28, 29]. miRNA expression changes induced by persistent enterovirus infection could thus impact on the function or regeneration of pancreatic beta-cells.

We, therefore, investigated the miRNA expression profile changes associated with persistent CVB4 infection in a human pancreatic cell line (PANC-1). To our knowledge, our study is the first to describe the impact of persistent enterovirus infection on miRNA expression in pancreatic cells. As far as acute enterovirus infection is concerned, two studies have analyzed the impact of CVB infection on pancreatic miRNA expression [16, 30]. Kim et al. reported a dysregulation of 33 miRNAs out of a total 754 analyzed in human pancreatic islets during acute CVB5 infection [16], and Lam et al. investigated the impact of acute CVB4 infection on miRNA expression in rat beta-cells [30]. When we compared the miRNAs that were reported to be dysregulated in these two studies with the list of 81 dysregulated miRNAs identified in the study presented here, there was no overlap with the study by Lam et al. and only one miRNA was dysregulated in our study and the study by Kim et al.: hsa-miR-663b. The function and targets of hsa-miR-663b are unknown, and to date, no reports on its involvement in viral infection or T1D have been published. The small overlap of dysregulated miRNAs between our study and the two previous studies might partly be due to methodological differences of the miRNA expression analysis [31] and/or the use of different cells. Furthermore, the effect on cellular miRNA expression may depend on the virus strain. The small overlap between the miRNA expression changes identified in our study and the two previous studies also demonstrate that acute CVB infection has a different impact on the cellular miRNA expression profile and thus on the resulting gene expression changes than persistent CVB infection. Concerning persistent CVB infection, a recent study compared miRNA expression profiles in endomyocardial biopsies of patients with CVB3 cardiomyopathy who spontaneously eliminated CVB3 with those who had virus persistence on follow-up. 16 miRNAs were differentially expressed and were proposed as markers for the risk of CVB3 persistence in CVB3 cardiomyopathy [32]. Interestingly, none of these 16 miRNAs was found dysregulated in our study, underlining again that the changes in miRNA expression profiles are cell-specific and virus-specific.

MiRNAs play an important role in the regulation of insulin expression and glucose homeostasis. Hsa-miR-204 is highly expressed in beta-cells and overexpressed in diabetes. Its overexpression reduces insulin expression in

Table 3 Functional annotation analysis of 49 T1D risk genes that are potential targets of 81 dysregulated miRNAs

Functional annotation term	*p* value
Autoimmune thyroid disease	4.66E−06
Allograft rejection	2.83E−05
Cell adhesion molecules	4.10E−05
Type I diabetes mellitus	5.27E−05
Viral myocarditis	4.14E−04
Graft-versus-host disease	9.60E−04
Jak-STAT signaling pathway	9.66E−04
MHC class II receptor activity	1.24E−03
Intestinal immune network for IgA production	1.87E−03
Growth factor binding	3.02E−03
Antigen processing and presentation	8.32E−03
Asthma	9.38E−03
Endocytosis	1.33E−02
T cell receptor signaling pathway	1.70E−02
Cytokine binding	3.66E−02
Cytokine-cytokine receptor interaction	4.21E−02
MHC class I receptor activity	4.61E−02

The 49 type 1 diabetes risk genes that were potential targets of the 81 dysregulated miRNAs were functionally annotated using DAVID 6.7. Functional annotations that are enriched with an enrichment *p* value <0.05 are shown

human islets by targeting a key insulin transcription factor, MafA [33]. We found that hsa-miR-204 is induced by persistent CVB4 infection (Table 1). Induction of this miRNA by persistent CVB4 infection may, therefore, reduce insulin expression and contribute to the insulin deficiency observed in T1D. The let-7 family is also involved in the control of glucose homeostasis and insulin sensitivity [34]. Several members of the let-7 family were found down-regulated in our study (Table 1), suggesting that persistent CVB4 infection may have an impact on glucose homeostasis. Hsa-miR-10a-5p was also found down-regulated during persistent CVB4 infection in our study (Table 1; Fig. 1a). The mouse homolog of this miRNA is expressed to a lower level in regulatory T cells of non-obese diabetic mice (NOD) as compared to autoimmunity-resistant mice [35] suggesting a potential link of this miRNA to autoimmunity. The link of the miRNAs dysregulated during persistent CVB4 infection to autoimmunity is further supported by the results of the functional annotation analysis, because most of the enriched functional annotation terms were related to the immune response or autoimmunity (Table 3). Hsa-miR-10a-5p was also shown to be up-regulated during differentiation of human induced pluripotent stem cells to insulin-producing cells [36]. The down-regulation of hsa-miR-10a-5p that we observed during persistent CVB4 infection (Table 1) might thus be implicated in the impaired formation of islet-like cell aggregates of PANC-1 cells reported previously [8]. A recent study found that EV-71 infection induced expression of miR-146a in human cells and in a mouse model. MiR-146a was further shown to target TRAF6 and IRAK1 [37]. Inhibition of mir-146a restored IRAK1 and TRAF6 expression, increased IFN-α production, inhibited viral propagation, and improved mouse survival. The authors concluded that miR-146a facilitates enteroviral pathogenesis by suppressing IFN production [37]. Interestingly, proinflammatory cytokines induce miR-146a in MIN6 cells and human islets via the NfκB pathway [38, 39]. Moreover, induction of miR-146 promotes beta-cell apoptosis, while miR-146 inhibition reduces beta-cell death [38, 40]. Expression of this miRNA is increased in islets of prediabetic NOD mice [38]. Taken together, these findings suggest that overexpression of miR-146a during persistent CVB4 infection as shown in the current study (Table 1; Fig. 1) might link enteroviral infection to the development of T1D. In agreement with this hypothesis, hsa-miR-146a has been reported to be dysregulated in the plasma of prediabetic patients and in PBMCs of patients with T1D [41].

Persistent CVB4 infection induced dysregulation of 81 miRNAs in pancreatic cells (Table 1). A single miRNA can potentially regulate hundreds of genes [42]; therefore, the changes in miRNA expression are expected to have a strong impact on the transcriptome and biology of these cells. Importantly, the miRNAs dysregulated by persistent CVB4 infection were found to potentially target several T1D risk genes (Table 2). Indeed, gene expression of selected genes investigated by RT qPCR analysis confirmed dysregulation of *AFF3*, *BACH2,* and *IL7R* in PANC-1 cells persistently infected with CVB4 (Fig. 2), whereas the other investigated genes showed similar mRNA expression. However, the expression of these genes might be impacted at the protein level.

Basic leucine zipper transcription factor 2 (BACH2) is a key regulation factor in B and T cell differentiation and function [43]. BACH2 is also involved in the regulation of apoptosis. Inhibition of BACH2 sensitized beta-cells to cytokine-induced apoptosis [44]. BACH2 is a predicted target of 24 of the dysregulated miRNAs in our study and dysregulation of its expression has been found by RT qPCR (Table 2; Fig. 2). Cells persistently infected with CVB4 might, therefore, be more susceptible to apoptosis.

AF4/FMR2 family member 3 (AFF3) is a nuclear transcriptional activator that is preferentially expressed in lymphoid tissue [45]. *AFF3* polymorphisms have been found associated with autoimmune diseases [46]. AFF3 is a predicted target of 24 of the dysregulated miRNAs and dysregulation of its expression has been found by RT qPCR (Table 2; Fig. 2).

Interleukin-7 receptor (IL7R) is indispensable for normal lymphocyte development and *IL7R* polymorphisms have been found associated with autoimmune diseases [47, 48]. Binding of IL7 to the IL7R activates multiple pathways that regulate lymphocyte survival, glucose uptake, proliferation, and differentiation [49]. Blocking of IL7R in non-obese diabetic (NOD) mice prevented autoimmune diabetes and reversed disease in new-onset diabetic mice by modulation of effector/memory T cell function [50]. The soluble form of IL7R antagonizes IL7 signaling and is increased in patients at the onset of T1D [51]. IL7R is a predicted target of 15 of the dysregulated miRNAs and dysregulation of its expression has been found by RT qPCR in our study (Table 2; Fig. 2). Given that dysregulation of IL7/IL7R signaling is involved in the development of T1D, our results suggest that persistent CVB4 infection has an impact on IL7/IL7R signaling in pancreatic cells that might be linked to the development of T1D.

Our previous studies showed that during persistent CVB4 infection, approximately 50% of cells harbored CVB4 RNA and 1–5% cells were VP1 positive [8]. Therefore, it is noteworthy, that although only approximately 50% of the cells are infected during persistent infection, expression of some miRNAs and mRNAs changed strongly. In fact, miRNAs can be secreted and taken up by surrounding cells and these miRNAs can be

functional [52, 53]. Therefore, it is possible that the persistently infected cells influence the miRNA expression profile of surrounding non-infected cells and consequently also their gene expression profile.

In conclusion, our study presents the first characterization of the miRNA expression profile changes induced by persistent CVB4 infection in pancreatic cells. The predicted targeting of genes involved in the immune response and autoimmunity by the dysregulated miRNAs as well as the dysregulated expression of the diabetes risk genes *AFF3*, *IL7R,* and *BACH2* show that persistent CVB4 infection profoundly impacts the host cell and link persistent CVB4 infection to the development of T1D. To fully understand the impact of miRNA dysregulation induced by persistent CVB4 infection on the cell physiology, it will be interesting to study the global gene and protein expression profiles of these cells using transcriptomic and proteomic approaches.

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Compliance with ethical standards

Conflict of interest The authors declare that no conflict of interest exists.

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