MicroRNA expression profiles and type 1 diabetes mellitus: systematic review and bioinformatic analysis

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Abstract

Growing evidence indicates that microRNAs (miRNAs) have a key role in processes involved in type 1 diabetes mellitus (T1DM) pathogenesis, including immune system functions and beta-cell metabolism and death. Although dysregulated miRNA profiles have been identified in T1DM patients, results are inconclusive; with only few miRNAs being consistently dysregulated among studies. Thus, we performed a systematic review of the literature on the subject, followed by bioinformatic analysis, to point out which miRNAs are dysregulated in T1DM-related tissues and in which pathways they act. PubMed and EMBASE were searched to identify all studies that compared miRNA expressions between T1DM patients and non-diabetic controls. Search was completed in August, 2017. Those miRNAs consistently dysregulated in T1DM-related tissues were submitted to bioinformatic analysis, using six databases of miRNA–target gene interactions to retrieve their putative targets and identify potentially affected pathways under their regulation. Thirty-three studies were included in the systematic review: 19 of them reported miRNA expressions in human samples, 13 in murine models and one in both human and murine samples. Among 278 dysregulated miRNAs reported in these studies, 25.9% were reported in at least 2 studies; however, only 48 of them were analyzed in tissues directly related to T1DM pathogenesis (serum/plasma, pancreas and peripheral blood mononuclear cells (PBMCs)). Regarding circulating miRNAs, 11 were consistently dysregulated in T1DM patients compared to controls: miR-21-5p, miR-24-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-375 and miR-1275. The bioinformatic analysis retrieved a total of 5867 validated and 2979 predicted miRNA–target interactions for human miRNAs. In functional enrichment analysis of miRNA target genes, 77 KEGG terms were enriched for more than one miRNA. These miRNAs are involved in pathways related to immune system function, cell survival, cell proliferation and insulin biosynthesis and secretion. In conclusion, eleven circulating miRNAs seem to be dysregulated in T1DM patients in different studies, being potential circulating biomarkers of this disease.

Key Words
- systematic review
- microRNA
- type 1 diabetes mellitus
- bioinformatic analysis

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Introduction

Type 1 diabetes mellitus (T1DM) is characterized by autoimmune destruction of pancreatic beta-cells by T lymphocytes and macrophages (1). The disease is usually diagnosed when over 80–90% of beta-cells have been destructed by the infiltrating immune system. T1DM development is slow, providing a potentially long window of time in which it is possible to identify and theoretically treat individuals at risk (2, 3).

The first sign of autoimmunity against beta-cells, frequently detectable a few months/years before the appearance of clinical symptoms, is the occurrence of antibodies against beta-cell antigens (4). These autoantibodies are used as biomarkers of T1DM risk and are directed against insulin, glutamic acid decarboxylase, zinc cation efflux transporter and tyrosine phosphatases-2 and -2β (4). The presence of more than two of these autoantibodies indicates high risk for T1DM development (5, 6). However, the use of islet autoantibodies as biomarkers of T1DM progression has some limitations, especially because a subset of children with new-onset T1DM is negative for islet autoantibodies (6), and many autoantibody-positive subjects will never develop T1DM (2, 7). Moreover, autoantibodies cannot be used as markers to initiate a potential treatment at earlier stages of the disease when many beta-cells are still present (2, 7). Thus, new biomarkers of T1DM are necessary to complement the information obtained from the presence of autoantibodies together with genetic and environmental risk factors (8).

In this context, several microRNAs (miRNAs) are released in the circulation and might be used as biomarkers to evaluate health status and disease progression (2). miRNAs are a class of small noncoding RNAs that negatively regulate gene expression by partially pairing to the 3′, 5′ untranslated regions of their target mRNAs, leading to translation repression and/or transcript degradation (9, 10, 11). They have recognized roles in the regulation of various processes, such as cellular differentiation, proliferation, metabolism, aging and apoptosis (10, 12). miRNAs are estimated to regulate the expression of more than 60% of protein-coding genes (9); consequently, changes in their expressions have been linked to many diseases, including cancer, endocrine disorders and autoimmune diseases (13, 14, 15).

Growing evidence suggests that miRNAs also play a key role in immune system functions as well as in beta-cell metabolism, proliferation and death, which are processes involved in T1DM pathogenesis (2, 10, 16, 17). Indeed, IL-1β and TNF inflammatory cytokines were reported to induce miR-21-5p, miR-30b-3p, miR-34, miR-101a and miR-146a-5p expressions in MIN6 cells and human pancreatic islets (18, 19), suggesting that these miRNAs may have a role in cytokine-mediated beta-cell destruction. miRNA-specific profiles were observed in PBMCs or serum from T1DM patients (20, 21, 22, 23, 24), and some miRNAs seem to modulate mRNA expressions of the major T1DM autoantigens (24, 25).

Several studies identified a large number of miRNAs as being differentially expressed in T1DM samples (2, 10). These studies were performed in cultured cells, body fluids or solid tissue samples from T1DM patients or murine models of the disease, using different techniques to quantify gene expression. Consequently, findings are inconsistent among studies; with only few miRNAs actually being important signatures of T1DM. Therefore, to further investigate which miRNAs may be used as new potential biomarkers of T1DM, we performed a systematic review of the literature on the subject. Additionally, bioinformatic analyses were performed to investigate the regulatory and functional roles of miRNAs in T1DM. For this, six databases of miRNA–target gene interactions were queried, including experimentally validated and computationally predicted miRNA–target gene interactions. The functional enrichment analysis of miRNAs target genes was performed using pathways annotation from the KEGG Pathway Database.

Methods

Search strategies and eligibility of relevant studies

This systematic literature search was designed and described in accordance with current guidelines (26, 27). PubMed and EMBASE repositories were searched to identify all studies that evaluated miRNA expressions in T1DM samples. The following medical subject headings (MeSH) were used: (‘diabetes mellitus’ OR ‘type 1, diabetes mellitus’) AND (‘microRNA’ OR ‘RNA, small untranslated’). The search was restricted to English, Portuguese or Spanish language papers and was completed on August, 2017. We also manually checked the reference lists of all articles retrieved to identify other important citations. To ensure that relevant studies were not overlooked, searches in Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) and Array Express (www.ebi.ac.uk/arrayexpress) databases were also performed.

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We included original reports that analyzed miRNA expressions in T1DM patients (cases) and non-diabetic subjects (controls) or in murine model of this disease. Studies that did not have a control group or studies performed in cell lines were excluded. Two investigators (T S A and B M S) independently reviewed titles and abstracts of articles retrieved in order to evaluate whether the studies were eligible for inclusion in this review.

Data extraction and quality assessment of each individual study

Data were independently extracted by two investigators (B M S and T S A) using a standardized abstraction form (26), and consensus was sought in all extracted items. Information extracted from each study in humans were as follows: (1) characteristics of studies and samples; (2) information regarding miRNA expression (method used for quantification, tissue analyzed, number of miRNAs analyzed) and (3) miRNA expression in groups. For those studies performed in mice/rats, we also collected information about the murine model analyzed. All miRNA names were standardized based on miRBase v21 prior to analysis.

Two investigators (T S A and B M S) assessed the quality of each eligible study using The Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) (28). This tool comprises 4 key domains (patient selection, index test, reference standard and flow/timing) supported by 7 questions to aid judgment on risk of bias, rating risk of bias and concerns about applicability of studies. Each question can be answered with 'yes', 'no' or 'unclear'. Then, a score of 1 is given for each 'yes' (low risk/high concern), a score of 0.5 for each 'unclear' and a score of 0 for each 'no' (high risk/low concern). Quality scores range from 0 to 7, with studies being classified as having 'good quality' (scores 6–7), 'fair' (scores 4–5) and 'poor quality' (scores <3).

Additionally, we checked if the articles were performed in accordance to Minimum Information about a Microarray Experiment (MIAME) guideline, version 2.0 (29) or Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) guideline (30). Only articles in accordance with these guidelines were included in the systematic review.

Bioinformatic prediction and analysis of miRNA's target genes

To investigate in greater depth the functional involvement of miRNAs in T1DM, we selected those miRNAs that were consistently dysregulated in T1DM-related tissues (PBMCs, serum/plasma and pancreas) and performed bioinformatic analysis to retrieve their putative targets and identify potentially affected biological pathways under their regulation (Supplementary Fig. 1, see section on supplementary data given at the end of this article). For this, we queried the databases miRTarbase release 6.1 (31) and starBase, v2.0 (32) of experimentally validated data concerning miRNA–target interactions, restricting the search for interactions classified as functional in miRTarBase (33), and interactions predicted by two or more software with at least one supporting experiment in starBase. Moreover, we obtained the complete collection of validated targets provided by miRecords. The union of all interactions retrieved from the 3 queried sources was considered as the set of validated miRNA–target gene interactions in our study.

To complement the information derived from experimental validation and search for additional miRNA targets, we also applied *in silico* target prediction algorithms for selected miRNA sequences using web-based tools TargetScan, v7.1 (34), Diana MicroT-CDS (35) and miRanda-mirSVR (August 2010 Release) (36, 37). To control for false-positive rates, we adopted the following filtering criteria: (1) for TargetScan, v7.1, we considered interactions involving conserved miRNA sites and with context++ scores <−0.1; (2) for Diana MicroT-CDS, we kept interactions with prediction scores ≥0.7; (3) for miRanda-mirSVR, we selected interactions involving conserved miRNAs and with scores <−0.1; (4) the compilation of miRNA–target interactions gathered from *in silico* analysis was built based on target genes predicted by at least 2 adopted computational tools. The combination of validated and predicted miRNA–target interactions was used for further analyses. miRNAs and gene identifiers were mapped to miRBase, v21 and Human Gene Nomenclature Committee (38, 39) or Mouse Genome Information nomenclature (40, 41).

Next, we implemented functional enrichment analysis of miRNAs target genes using pathways annotation from the KEGG Pathway Database (42, 43) and the clusterProfiler package in R/Bioconductor environment (44). This investigation was performed for targets of each individual miRNA as well as for targets of miRNAs grouped by tissue (PBMCs, serum/plasma or pancreas). Significance for KEGG pathways enrichment was estimated with a hypergeometric test and adjusted to account for multiple hypotheses using the false discovery rate (FDR) procedure implemented in the q-value R package (45). Pathways with a q-value <0.05 were considered strongly enriched for the genes targeted by selected miRNAs.

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Results

Literature search, characteristics of the eligible studies and quality assessment

The flow diagram showing the strategy used to identify and select studies for inclusion in this systematic review is depicted in Fig. 1. According to the search criteria, a total of 1738 publications were retrieved from databases; however, after full text analysis, only 33 articles (20, 22, 23, 24, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74) fulfilled the eligibility criteria and were included in the review. The main characteristics of these 33 articles are shown in Table 1. Among these studies, 19 reported miRNA expression profiles in human, 13 focused on miRNA profiles in murine models, and only one analyzed both human and murine samples (46). Sample sizes ranged from 10 to 162 in studies that analyzed human samples and from 6 to 60 in studies with murine models. The number of miRNAs analyzed ranged from 1 to 847, with the number of miRNAs differentially expressed between groups varying from 1 to 136 (Table 1).

Regarding tissues analyzed, 24.1% of the studies evaluated miRNA expression in serum/plasma samples, 20.7% in PBMCs/T cells, and 6.9% in pancreas tissue. The remaining studies evaluated other tissues related to T1DM chronic complications, such as urine, kidney, heart and retina (Table 1). Two articles analyzed different tissues (46, 52) and were considered separately, totalizing 36 studies.

Quality of each study included in this review was assessed using QUADAS-2, as reported in the Methods section. Overall, most studies were considered as having a good quality since 62.5% of studies received QUADAS-2 scores between 6 and 7 (Table 1). No study scored less than 5.0.

Dysregulated miRNAs in T1DM-related tissues

Out of 278 dysregulated miRNAs reported in 36 studies that compared T1DM patients and controls, 72 miRNAs (25.9%) were reported in at least two studies (Supplementary Table 1). However, only 48 of them were analyzed in tissues directly related to T1DM pathogenesis (PBMCs, serum/plasma and pancreas). Hence, these 48 miRNAs were chosen for further evaluation (Table 2).

Eight miRNAs were consistently downregulated in T1DM-related tissues from patients compared to controls (miR-100-5p, miR-1275, miR-150-5p, miR-151-3p, miR-146a-5p, miR-151-3p, miR-574-3p and miR-720), while 10 miRNAs were upregulated in cases (miR-21-5p, miR-24-3p, miR-25-3p, miR-27b-3p, miR-148a-3p, miR-181a-5p, miR-210-5p, miR-375, miR-450a-2-3p and miR-454-3p) (Fig. 2A and Table 2). Thirty miRNAs were reported as being downregulated in cases from one study and upregulated in cases from another study, possibly due to the different tissues or species that were analyzed (Table 2).

miRNA expression profiles according to species

In subgroup analysis of species, 19 studies reported expressions of 139 miRNAs in different tissues from T1DM patients and controls, with 36 of these miRNAs being

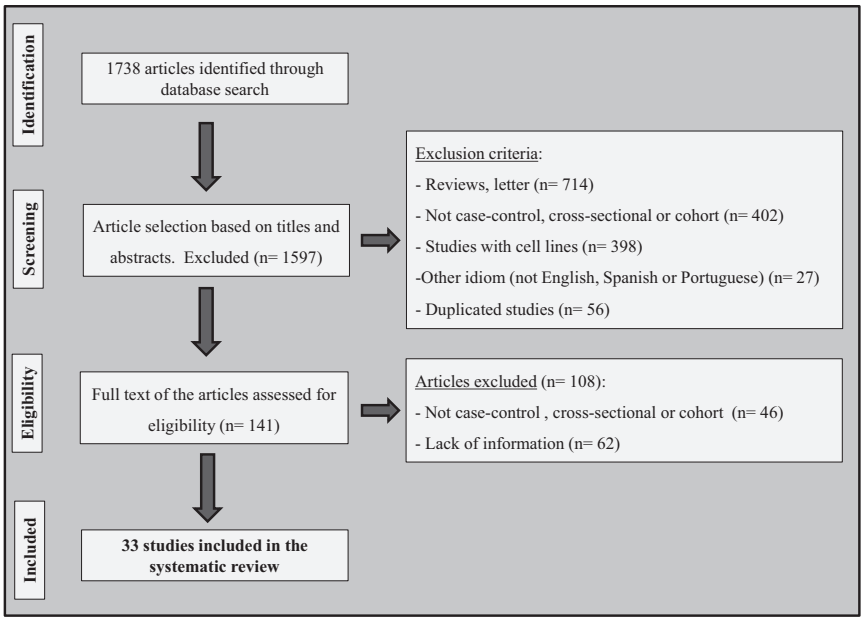

Figure 1
Flowchart illustrating the search strategy used to identify association studies of miRNAs expression with type 1 diabetes for inclusion in the systematic review.

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Table 1 Characteristics of studies included in the systematic review.

		T1DM				Differentially expressed microRNAs				
First author, year (Ref)	**Country**	Diabetic sample	Tissue	Sample size Case/Control	Method	Cut-off criteria	Total	Increased	Decreased	**Quality/ QUADAS-2**
Alipour *et al.* 2013 (58)	Iran	Male Sprague-Dawley rats induced with STZ	Kidney	6/6	RT-PCR	0.05	1	1	0	5.5
Bacon *et al.* 2015 (59)	Ireland	T1DM patients	Urine	44/26	RT-PCR	N/A	2	2	0	6.0
Barutta *et al.* 2013 (46)	Italy	Normoalbuminuric T1DM patients	Urinary Exossome	12/12	RT-PCR	2-fold	2	2	0	6.0
Barutta *et al.* 2013 (46)	Italy	Male C57BL6/J mice induced with STZ	Glomeruli	30/30	RT-PCR	2-fold	1	1	0	5.5
Barutta *et al.* 2013 (46)	Italy	Male C57BL6/J mice induced with STZ	Exossome	30/30	RT-PCR	2-fold	1	1	0	5.5
Diao *et al.* 2011 (47)	China	Male C57BL/6 mice induced with STZ	Heart	15/10	Microarray analysis	0.05	16	10	6	6.0
Emadi *et al.* 2014 (48)	Iran	Male Wistar rats	Aorta	6/6	RT-PCR	0.05	1	0	1	6.0
Erener *et al.* 2013 (63)	Canada	C57BL/6 mice induced with STZ and NOD mice	Plasma	6/6	RT-PCR	0.05	1	1	0	5.5
Erener *et al.* 2017 (71)	Canada	Recent-onset T1DM patients	Serum	10/7	RT-PCR	N/A	35	27	8	6.0
Estrella *et al.* 2016 (65)	Chile	T1DM patients	PBMCs	20/20	RT-PCR	N/A	2	1	1	6.0
Garcia de la Torre *et al.* 2015 (64)	Spain	T1DM patients without DR	EPC	76/38	RT-PCR	2-fold	1	1	0	6.0
Garcia-Contreras *et al.* 2017 (72)	USA	T1DM patients	Plasma-derived exosome	36/36	Microarray and RT-PCR	N/A	7	1	6	5.5
Hezova *et al.* 2010 (49)	Czech Republic	T1DM patients	T cells	5/5	TLDA	0.05	3	1	2	5.5
Kato *et al.* 2010 (50)	USA	C57BL/6 mice induced with STZ	Kidney	3/3	RT-PCR	0.05	1	1	0	6.0
Kovacs *et al.* 2011 (51)	USA	Male Sprague-Dawley rats induced with STZ	Retina	3/3	Microarray	0.05	17	14	3	5.5
Li *et al.* 2009 (67)	China	C57BL/6 mice induced wit STZ	Liver	8/8	Microarray	0.05	2	1	1	5.5
Ma *et al.* 2016 (61)	China	NOD mice	Pancreas	6/6	RT-PCR	N/A	1	0	1	6.0
Marchand *et al.* 2016 (66)	France	T1DM patients recently diagnosed	Serum	22/10	RT-PCR	0.05	1	1	0	6.0
Nabih *et al.* 2016 (62)	Egypt	Children and adolescents with T1DM	Serum	40/40	RT-PCR	N/A	1	1	0	6.0
Nielsen *et al.* 2012 (22)	Denmark	Children with newly diagnosed T1DM	Serum	108/54	Solexa sequencing/ RT-PCR	2-fold	24	24	0	6.5
Osipova *et al.* 2014 (52)	Germany	T1DM pediatric patients	Serum	68/79	RT-PCR	0.05	2	2	0	6.0
Osipova *et al.* 2014 (52)	Germany	T1DM pediatric patients	Urine	68/79	RT-PCR	0.05	3	2	1	6.0
*Perez-Bravo *et al.* 2014 (69)	Chile	T1DM patients	PBMCs	5/5	RT-PCR	N/A	1	0	1	–
Qing *et al.* 2014 (56)	China	T1DM patients	Retina	90/20	TLDA	N/A	3	3	0	5.0
Salas-Perez *et al.* 2013 (20)	Chile	T1DM pediatric patients	PBMCs	20/20	RT-PCR	0.05	2	0	2	5.0
*Sebastiani *et al.* 2012 (68)	Italy	T1DM patients	Serum	20/20	TLDA	0.05	64	21	43	–

(Continued)

Table 1 Continued.

		T1DM				Differentially expressed microRNAs				
First author, year (Ref)	Country	Diabetic sample	Tissue	Sample size Case/Control	Method	Cut-off criteria	Total	Increased	Decreased	Quality/ QUADAS-2
Sebastiani *et al.* 2017 (74)	Italy	T1DM patients	T-cells from pancreatic lymphnodes		Megaplex RT-stem-loop microRNA Pool A v2.1	N/A	1	1	0	6.0
Seyhan *et al.* 2016 (70)	USA	T1DM patients	Plasma	16/27	RT-PCR	0.05	4	4	0	6.5
Silva *et al.* 2011 (53)	Brazil	Male Wistar rats, induced with STZ	Retina	3/3	RT-PCR	0.05	1	1	0	6.5
Takahashi *et al.* 2014 (23)	Brazil	T1DM patients	PBMCs	6/6	Microarray	N/A	44	35	9	6.0
Tian *et al.* 2015 (60)	China	Male NIH mice induced with STZ	Pancreas	3/3	Microarray	2-fold	136	64	72	6.0
Wang *et al.* 2014 (54)	USA	Male Long Evans rats induced with STZ	Retina	3/3	RT-PCR	N/A	1	1	0	5.5
Wang *et al.* 2017 (73)	China	T1DM patients	PBMCs	78/56	RT-PCR	N/A	3	0	3	6.0
Xiong *et al.* 2014 (57)	China	Rat induced with STZ	Retina	6/6	RT-PCR	0.05	17	15	2	6.0
Yang *et al.* 2015 (24)	China	Newly diagnosed T1DM patients	PBMCs	12/10	Microarray/ RT-PCR	0.05	24	5	19	6.5
Yousefzadeh *et al.* 2015 (55)	Iran	Male Sprague-Dawley rats induced with STZ	Sciatic nerve	6/6	RT-PCR	0.05	1	1	0	6.0

DR, diabetic retinopathy; EPC, Endothelial Progenitor cells; N/A, not available; QUADAS-2, Quality Assessment of Diagnostic Accuracy Studies 2; RT-PCR, Reverse transcription polymerase chain reaction; STZ, streptozotocin; T1DM, Type 1 diabetes mellitus; TLDA, TaqMan Low Density Array. *Abstract from Congress.

reported by at least two studies (Supplementary Table 1). One study analyzed both human and murine samples. Additionally, 13 miRNA profile studies were performed in murine models of T1DM, identifying 173 dysregulated miRNAs in different tissues, with only 45 of them being reported by at least two studies (Supplementary Table 1).

Considering only the 48 miRNAs expressed in serum/plasma, PBMCs or pancreas, 12 miRNAs were dysregulated exclusively in human samples, with 4 miRNAs (miR-100-5p, miR-146a-5p miR-150-5p and miR-1275) being downregulated and 8 upregulated (miR-10a-5p, miR-21-5p, miR-24-3p miR-26b-5p, miR-27b-3p, miR-148a-3p, miR-181a-5p and miR-210-5p) in T1DM patients compared to controls. Only one miRNA (mmu-miR-26a-5p) was consistently downregulated in pancreas from murine models of T1DM. Four miRNAs (miR-151-3p, miR-324-5p, let-7a-5p and let-7c-5p) were shown to be downregulated in tissues from both human and mice with diabetes, and only miR-375 was upregulated in tissues from human and mice (Fig. 2B and Table 2).

Dysregulated miRNAs as circulating and tissue biomarkers of T1DM

Several miRNAs are released into the bloodstream or expressed in blood cells and might be used as circulating biomarkers of T1DM (2). Among miRNAs that were analyzed in more than one study, 21 (miR-15b, miR-20b-5p, miR-21-5p, miR-22-3p, miR-24-3p, miR-25-3p, miR-26b-5p, miR-27b-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-200c-3p, miR-210-5p, miR-335-5p, miR-342-3p, miR-375, miR-1275, let-7f-5p and let-7g-5p) were expressed in serum/plasma or PBMCs/T cells and, therefore, have the potential to be circulating biomarkers of T1DM (Table 2). Nevertheless, only 11 of them were consistently dysregulated, being analyzed in the same tissue by at least two studies: miR-146a-5p, miR-150-5p, miR-342-3p and miR-1275 were downregulated in PBMCs from T1DM cases compared to controls, while miR-21-5p, miR-24-3p, miR-100-5p, miR-148a-3p, miR-181a-5p, miR-210-5p and miR-375 were upregulated in serum/plasma from cases (Fig. 2C and Table 2).

Two studies evaluated miRNA expression profiles in pancreas from murine models of T1DM (60, 61) and showed that miR-26a-5p expression was downregulated in pancreas from NOD or streptozotocin (STZ)-induced diabetes mice compared to control mice (Fig. 2C and Table 2). No study has evaluated miRNA expressions in human pancreas from T1DM patients and non-diabetic controls.

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Table 2 miRNAs differently expressed in tissues related to T1DM analyzed in at least two studies.

miRNA ID	**First author** (ref.)	Species	Sample type	Change of expression
let-7a-5p	Tian *et al*. (60)	Mice	Pancreas	Down
	Yang *et al*. (24)	Human	Pancreas	Down
let-7c-5p	Tian *et al*. (60)	Mice	Pancreas	Down
	Yang *et al*. (24)	Human	Pancreas	Down
let-7f-5p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Yang *et al*. (24)	Human	PBMCs	Down
	Tian *et al*. (60)	Mice	Pancreas	Down
let-7g-5p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Erener *et al*. (71)	Human	Serum	Up
	Yang *et al*. (24)	Human	PBMCs	Down
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-10a-5p	Nielsen *et al*. (22)	Human	Serum	Up
	Takahashi *et al*. (23)	Human	PBMCs	Up
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-100-5p	Hezova *et al*. (49)	Human	T cells	Down
	Erener *et al*. (71)	Human	Serum	Down
miR-126-3p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-1275	Takahashi *et al*. (23)	Human	PBMCs	Down
	Yang *et al*. (24)	Human	PBMCs	Down
miR-146a-5p	Yang *et al*. (24)	Human	PBMCs	Down
	Hezova *et al*. (49)	Human	T cells	Up
	Sebastiani *et al*. (68)	Human	Serum	Down
	Perez-Bravo *et al*. (69)	Human	PBMCs	Down
	Wang *et al*. 2017 (73)	Human	PBMCs	Down
miR-148a-3p	Nielsen *et al*. (22)	Human	Serum	Up
	Takahashi *et al*. (23)	Human	PBMCs	Up
	Seyhan *et al*. (70)	Human	Plasma	Up
miR-148b-3p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-150-5p	Estrella *et al*. (65)	Human	PBMCs	Down
	Wang *et al*. 2017 (73)	Human	PBMCs	Down
miR-151-3p	Hezova *et al*. (49)	Human	T cells	Down
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-154-3p	Tian *et al*. (60)	Mice	Pancreas	Up
	Erener *et al*. (71)	Human	Serum	Down
miR-15b	Takahashi *et al*. (23)	Human	PBMCs	Up
	Yang *et al*. (24)	Human	PBMCs	Down
miR-16-5p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Garcia-Contreras *et al*. (72)	Human	Plasma-derived exosome	Down
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-181a-5p	Nielsen *et al*. (22)	Human	Serum	Up
	Nabih *et al*. (62)	Human	Serum	Up
miR-199a-3p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Sebastiani *et al*. (68)	Human	Serum	Down
miR-19a-3p	Takahashi *et al*. (23)	Human	PBMCs	Up
	Tian *et al*. (60)	Mice	Pancreas	Down
miR-200c-3p	Nielsen *et al*. (22)	Human	Serum	Up
	Yang *et al*. (24)	Human	PBMCs	Down
miR-20b-5p	Hezova *et al*. (49)	Human	T cells	Down
	Takahashi *et al*. (23)	Human	PBMCs	Up
miR-210-5p	Nielsen *et al*. (22)	Human	Serum	Up
	Osipova a *et al*. (52)	Human	Serum	Up
miR-21-5p	Seyhan *et al*. (70)	Human	Plasma	Up
	Nielsen *et al*. (22)	Human	Serum	Up
	Osipova *et al*. (52)	Human	Serum	Up
	Takahashi *et al*. (23)	Human	PBMCs	Up
miR-221-3p	Erener *et al*. 2017 (71)	Human	Serum	Up
	Yang *et al*. (24)	Human	PBMCs	Down

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Table 2 Continued.

miRNA ID	**First author** (ref.)	Species	Sample type	Change of expression
miR-22-3p	Yang *et al.* (24)	Human	PBMCs	Down
	Estrella *et al.* (65)	Human	PBMCs	Up
miR-24-3p	Seyhan *et al.* (70)	Human	Plasma	Up
	Erener *et al.* 2017 (71)	Human	Serum	Up
	Nielsen *et al.* (22)	Human	Serum	Up
miR-25-3p	Nielsen *et al.* (22)	Human	Serum	Up
	Garcia-Contreras *et al.* (72)	Human	Plasma-derived exosome	Up
	Erener *et al.* 2017 (71)	Human	Serum	Up
	Yang *et al.* (24)	Human	PBMCs	Down
miR-26a-5p	Nielsen *et al.* (22)	Human	Serum	Up
	Ma *et al.* (61)	Mice	Pancreas	Down
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-26b-5p	Nielsen *et al.* (22)	Human	Serum	Up
	Takahashi *et al.* (23)	Human	PBMCs	Up
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-27a-3p	Nielsen *et al.* (22)	Human	Serum	Up
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-27b-3p	Nielsen *et al.* (22)	Human	Serum	Up
	Takahashi *et al.* (23)	Human	PBMCs	Up
miR-30b-3p	Tian *et al.* (60)	Mice	Pancreas	Up
	Yang *et al.* (24)	Human	PBMCs	Down
miR-324-3p	Tian *et al.* (60)	Mice	Pancreas	Down
	Erener *et al.* 2017 (71)	Human	Serum	Up
miR-324-5p	Takahashi *et al.* (23)	Human	PBMCs	Down
	Erener *et al.* 2017 (71)	Human	Serum	Up
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-32-5p	Takahashi *et al.* (23)	Human	PBMCs	Up
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-335-5p	Hezova *et al.* (49)	Human	T cells	Down
	Takahashi *et al.* (23)	Human	PBMCs	Up
miR-342-3p	Takahashi *et al.* (23)	Human	PBMCs	Down
	Sebastiani *et al.* (68)	Human	Serum	Up
	Yang *et al.* (24)	Human	PBMCs	Down
miR-375	Erener *et al.* (63)	Mice	Plasma	Up
	Marchand *et al.* (66)	Human	Serum	Up
	Sebastiani *et al.* (68)	Human	Serum	Down
	Seyhan *et al.* (70)	Human	Plasma	Up
miR-377-3p	Sebastiani *et al.* (68)	Human	Serum	Down
	Erener *et al.* 2017 (71)	Human	Serum	Up
miR-378	Erener *et al.* (63)	Mice	Plasma	Up
	Garcia-Contreras *et al.* (72)	Human	Plasma-derived exosome	Down
miR-424-5p	Wang *et al.* 2017 (73)	Human	PBMCs	Down
	Takahashi *et al.* (23)	Human	PBMCs	Up
miR-450a-2-3p	Takahashi *et al.* (23)	Human	PBMCs	Up
	Tian *et al.* (60)	Mice	Pancreas	Up
miR-454-3p	Takahashi *et al.* (23)	Human	PBMCs	Up
	Erener *et al.* 2017 (71)	Human	Serum	Up
miR-490-5p	Tian *et al.* (60)	Mice	Pancreas	Up
	Erener *et al.* 2017 (71)	Human	Serum	Down
miR-574-3p	Garcia-Contreras *et al.* (72)	Human	Plasma-derived exosome	Down
	Tian *et al.* (60)	Mice	Pancreas	Down
miR-720	Takahashi *et al.* (23)	Human	PBMCs	Down
	Erener *et al.* 2017 (71)	Human	Serum	Down
miR-9-3p	Tian *et al.* (60)	Mice	Pancreas	Up
	Sebastiani *et al.* (68)	Human	Serum	Down
miR-98-5p	Takahashi *et al.* (23)	Human	PBMCs	Up
	Tian *et al.* (60)	Mice	Pancreas	Down

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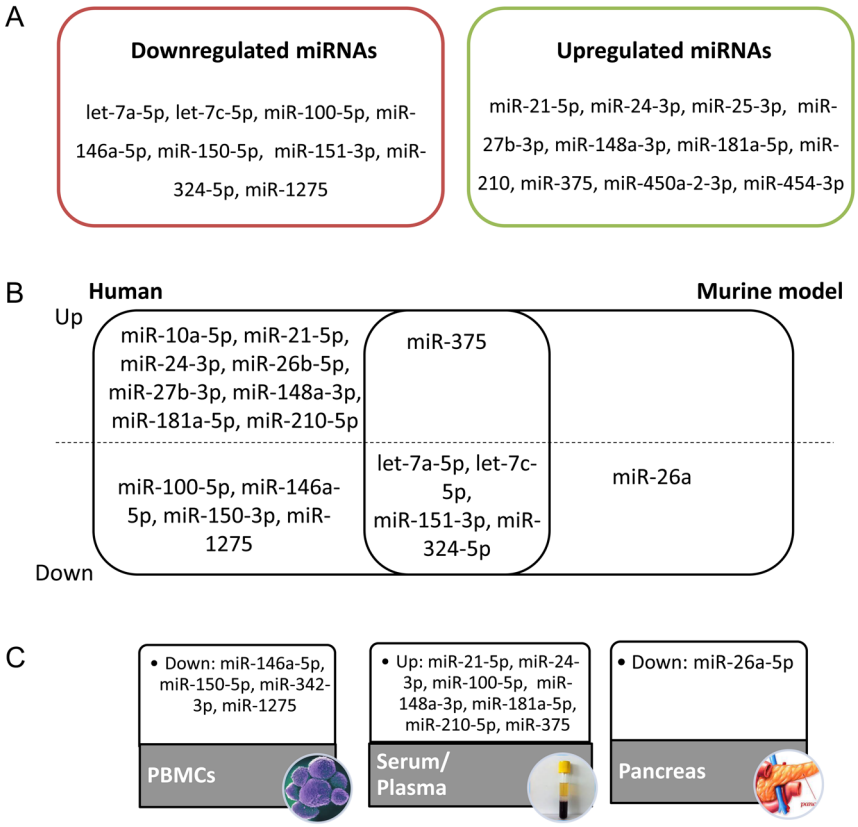

Figure 2
miRNA expression analyzed in at least two studies considering their expression profile in all tissues analyzed independently of the species (A). miRNA expression profile between the species (B). miRNA expression profile in tissues related to T1DM pathogenesis (C).

Moreover, 3 miRNAs (miR-151-3p, let-7a-5p and let-7c-5p) were downregulated in pancreas from diabetic mice as well as PBMCs/T cells from T1DM patients compared to the respective control groups. Inversely, several miRNAs were downregulated in pancreas from diabetic mice but upregulated in PBMCs or serum/plasma from T1DM patients (Table 2), which might reflect differential expression in tissues and/or species.

Perturbed pathways in type 1 diabetes mellitus

Bioinformatic analyses were performed to retrieve putative targets and pathways potentially modulated by 12 miRNAs (hsa-miR-21-5p, hsa-miR-24-3p, mmu-miR-26a-5p, hsa-miR-100-5p, hsa-miR-146a-5p, hsa-miR-148a-3p, has-miR-150-5p, hsa-miR-181a-5p, hsa-miR-210-5p, hsa-miR-342-3p, hsa-miR-375 and hsa-miR-1275) consistently dysregulated in T1DM-related tissues. Species prefixes were used in miRNA identifiers to clearly designate the species under consideration while reporting these results. First, we searched for targets of these miRNAs using 6 distinct resources, including experimentally validated databases and prediction programs (Supplementary Fig. 1). A total of 5867 validated and 2979 predicted miRNA–target interactions were retrieved for human miRNAs, while 573 validated and 453 predicted interactions were retrieved for the mmu-miR-26a-5p (Table 3; Supplementary Table 2).

After target prediction, we performed functional enrichment analysis of miRNA target genes using pathway maps from the KEGG Pathway Database, aiming to better understand the biological pathways affected by the selected miRNAs. Out of 518 pathways annotated in KEGG Database (accessed in August 2017), a total of 127 pathways were significantly overrepresented (q-value <0.05) in the putative target lists analyzed, and 77 KEGG terms were enriched for more than one miRNA. Targets of hsa-miR-21-5p, hsa-miR-24-3p, mmu-miR-26a-5p, hsa-miR-100-5p, hsa-miR-146a-5p, hsa-miR-148a-3p, hsa-miR-150-5p hsa-miR-181a-5p, hsa-miR-342-5p and hsa-miR-375 are involved in several pathways (Supplementary Table 3), many of them having a recognized role in T1DM pathogenesis, such as TNF, MAPK, Jak-STAT, PI3K-Akt, apoptosis, insulin, toll-like receptors (TLRs) and T cell receptor (TCR) signaling pathways (Supplementary Table 3). No significantly enriched KEGG terms were found for hsa-miR-210-5p and hsa-miR-1275, probably due to the small number of retrieved targets (40 and 121, respectively), as well as for hsa-miR-150-5p despite its broad regulatory action.

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Table 3 Number of miRNA-target interactions for each analyzed miRNA considered individually and grouped by tissue related to T1DM.

	miRNA/tissue	Validated interactions	Predicted interactions
Analysis by miRNA	hsa-miR-1275	121	–
	has-miR-100-5p	279	14
	hsa-miR-146a-5p	300	409
	hsa-miR-148a-3p	621	375
	has-miR-150-5p	637	433
	hsa-miR-181a-5p	1159	361
	hsa-miR-21-5p	725	198
	has-miR-24-3p	1052	450
	hsa-miR-210-5p	40	–
	hsa-miR-342-3p	433	436
	hsa-miR-375	500	303
	mmu-miR-26a-5p	573	453
Analysis by tissue related to T1DM	PBMCs	1491	1278
	Serum/Plasma	4376	1701
	Pancreas	573	453

PBMCs, peripheral blood mononuclear cells.

Considering different T1DM-related tissues, results indicated 2539 targets in PBMCs, 4665 targets in serum/plasma and 1026 targets in pancreas for the selected miRNAs (Table 3), where numbers reflect the size of the non-redundant set of target genes found for the group of miRNAs differentially expressed in each T1DM-related tissue. Forty-five significant KEGG pathways were found for targets of miRNAs dysregulated in PBMCs, which included NF-KB, apoptosis and neurotrophin signaling pathways. Similarly, 17 KEGG terms were found in pancreas, including Wnt and phosphatidylinositol signaling pathways. For serum and plasma, 94 significant KEGG terms were found, comprising signaling pathways by TNF, Jak-STAT, MAPK, TCR and insulin as well as pathways associated to protein processing in endoplasmic reticulum and apoptosis, which have key roles in T1DM pathogenesis (Supplementary Table 4; Fig. 3).

Next, we searched for KEGG terms linked to T1DM pathogenesis regardless of the functional enrichment analysis of miRNA targets and found 5 significant signaling pathways associated with this disease: type 1 diabetes (KEGG hsa04949), TCR (KEGG hsa04660), cytokine–cytokine receptor interaction (KEGG hsa04060), Jak-STAT (KEGG hsa04630) and neurotrophin (KEGG hsa04722). Then, TCR and Jak-STAT pathways were selected for further detailed analysis since they are targeted by most of the miRNAs in the list of interest (11 miRNAs each).

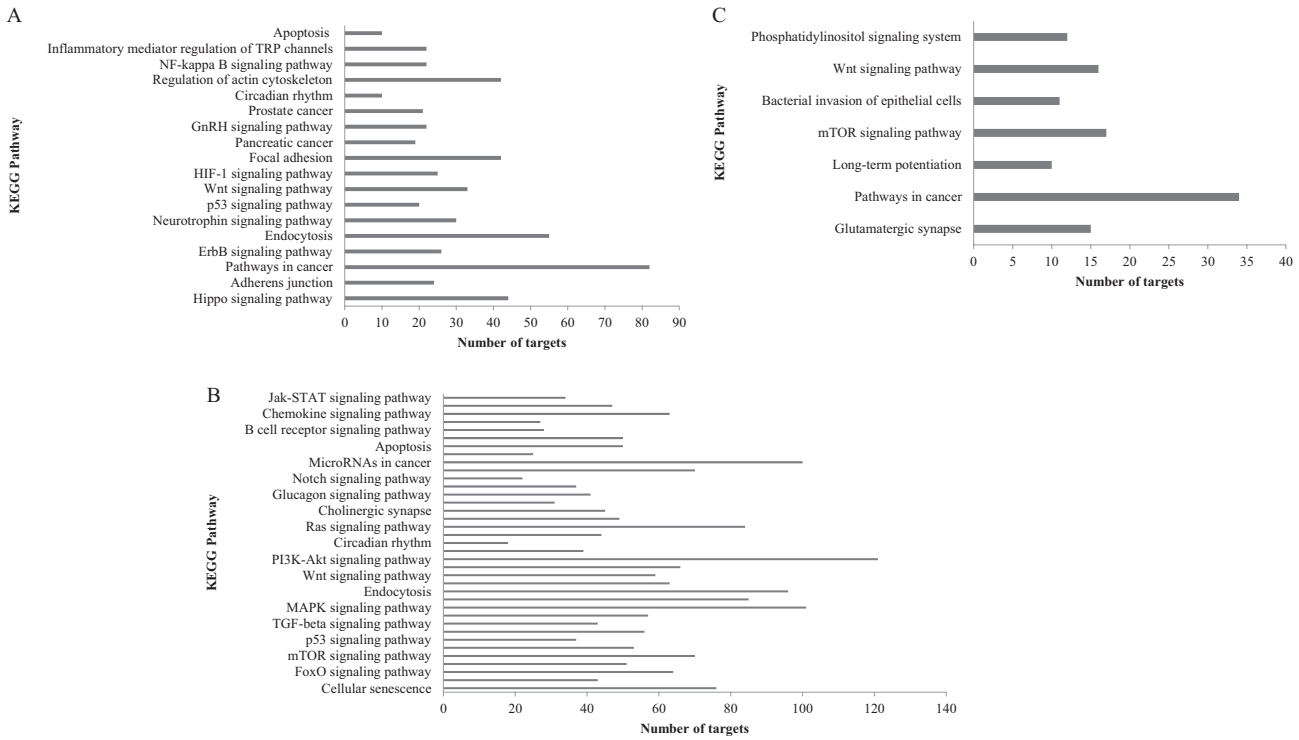

Figure 3
KEGG pathway functional annotation of the differentially miRNAs expressed in PBMCs (A), serum/plasma (B) and pancreas (C). Enrichment scores corresponding to each pathway are displayed as number of targets.

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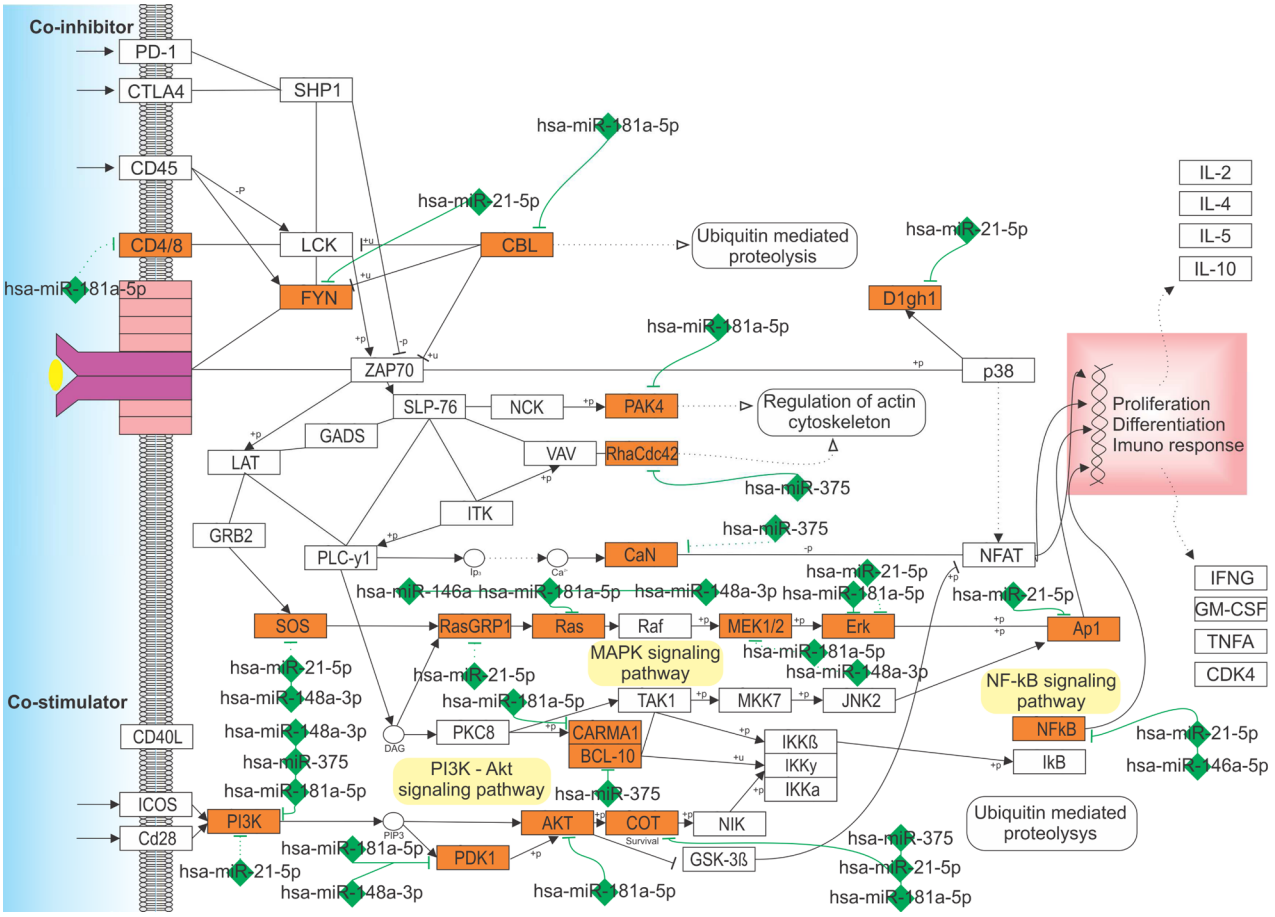

Figure 4
Schematic diagram of the selected miRNA-mRNA interaction networks involved in T cell receptor signaling pathway. The network was built based on KEGG pathway map (KEGG: hsa04660). The miRNAs are indicated by rhombus. The predicted miRNA-mRNA interactions are indicated by dotted lines and the thick lines indicate validated miRNA-mRNA interactions.

In the TCR signaling pathway (Fig. 4), miR-181a-5p directly targets mRNAs for CD4⁺ and CD8⁺ cell receptors. Moreover, this miRNA post-transcriptionally regulates genes associated with PI3K-Akt (*PI3K*, *Akt*, *COT*), actin cytoskeleton (*PAK4*) and ubiquitin mediated proteolysis (*CBL*) pathways, which are triggered after activation of T cell and co-stimulatory receptors. miR-21-5p targets mRNAs from MAPK (*Ras*, *ErkSOS* and *RasGRP1*) and ubiquitin-mediated proteolysis (*FYN*) pathways. Furthermore, miR-146a-5p targets mRNAs from MAPK (*Ras*) and NF-κB signaling pathways. miR-148a-5p, miR-100-5p, miR-24-3p and miR-150-5p target mRNAs from ubiquitin-mediated proteolysis (*CBL*), PI3K-Akt, NF-κB and MAPK pathways (Fig. 4).

In the Jak-STAT signaling pathway (Fig. 5), miR-21-5p, miR-24-3p, miR-181a-5p and miR-210-5p target mRNAs for different cytokine and hormone receptors, such as IL6R, LIFR, IL2RB and IFNLR1. miR-375 targets *JAK2* mRNA while miR-181a-5p and miR-21-5p bind to *STAT3* mRNA. In addition, miR-375, miR-181a-5p, miR-146a-5p, miR-148a-3p, miR-100-5p, miR-150-5p and miR-21-5p target different mRNAs codifying proteins related to apoptosis (BCL2, SOCS, PIAS and MCL1), cell cycle progression (cMyc), cell cycle inhibition (p21), proliferation and differentiation (SHP2, SOS and Ras) and cell survival (PI3K and Akt) (Fig. 5).

Discussion

Since the exact origin of T1DM remains uncertain, the discovery of novel biomarkers and their implications in T1DM pathogenesis may contribute to a better understanding of the mechanisms involved in this disease. Clinically, new biomarkers might enable an earlier T1DM diagnosis as well as a more adequate treatment of T1DM

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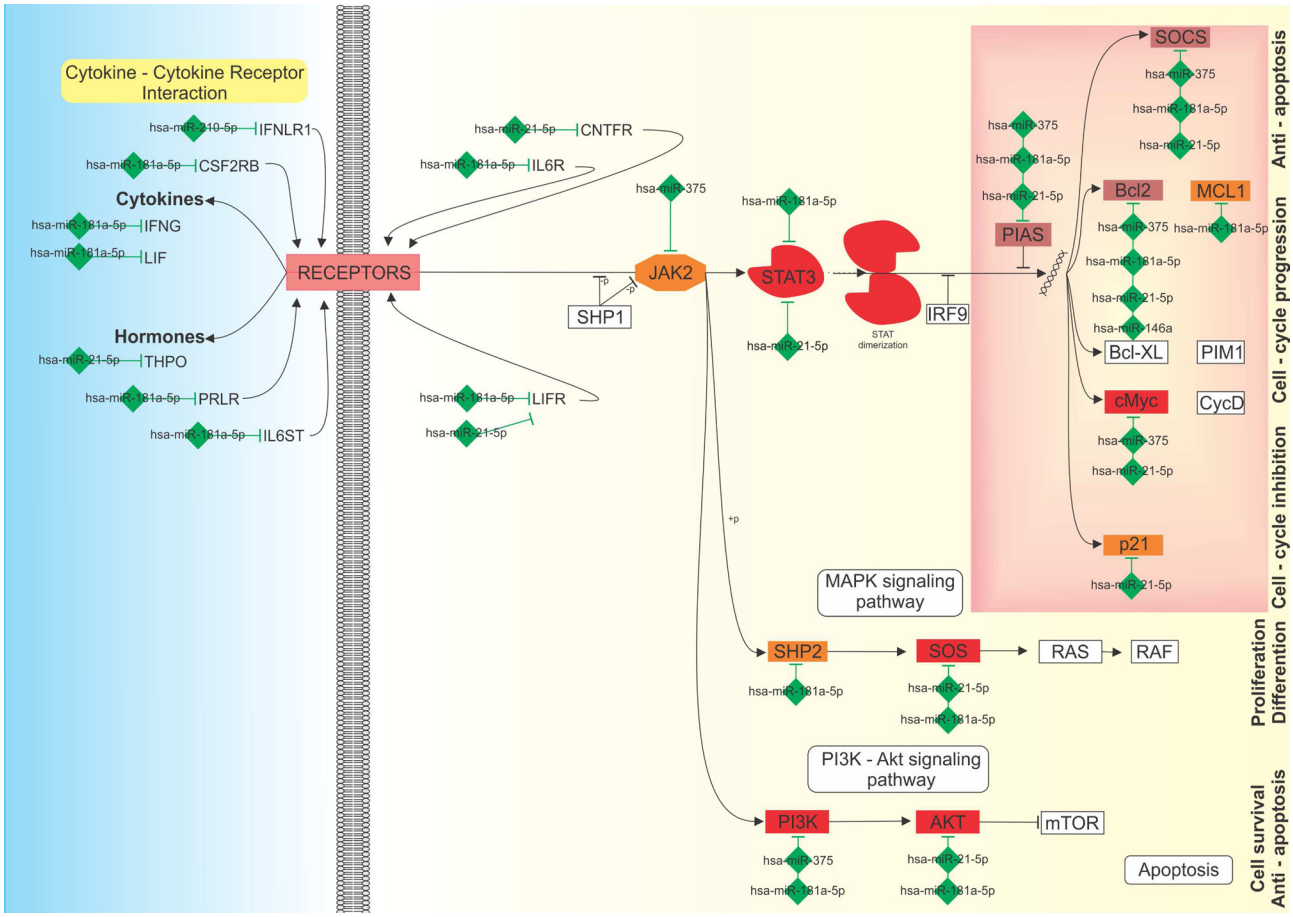

Figure 5
Schematic diagram of the selected miRNA-mRNA interaction networks involved in Jak-STAT signaling pathway. The network was built based on KEGG pathway map (KEGG: hsa04630). The miRNAs are indicated by rhombus. The predicted miRNA-mRNA interactions are indicated by dotted lines and the thick lines indicate validated miRNA-mRNA interactions.

patients, improving their quality of life (23). Circulating miRNAs are ideal biomarkers because they are stable and resistant to degradation by ribonucleases or repeated freezing/thawing cycles and can be easily detected in body fluids by highly sensitive and specific quantitative RT-PCR (2). Thus, as part of the ongoing effort to identify a profile of circulating miRNAs as biomarkers of T1DM, we performed a systematic review of studies that evaluated miRNA expressions in tissues from T1DM patients and non-diabetic controls. Eleven circulating miRNAs (miR-21-5p, miR-24-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-375 and miR-1275) were consistently dysregulated in T1DM patients, suggesting that they may be potential minimally invasive biomarkers of this disease.

miR-21-5p, miR-24-3p, miR-148a-3p, miR-181a-5p, miR-210-5p and miR-375 seem to be upregulated in serum/plasma or PBMCs from T1DM compared to controls (Table 2). Emerging studies have indicated diverse roles of miR-21-5p in immunity (75). Particularly, this miRNA acts in TCR signaling transduction, augmenting T cell proliferation (76); regulates Th1 vs Th2 responses (77) and Treg development (78) and is a key mediator of the anti-inflammatory response in macrophages (79). miR-21-5p also appears to have anti-inflammatory and anti-apoptotic effects since it inhibits the proinflammatory tumor suppressor programed cell death protein 4 (PDCD4), which promotes the activation of NF-KB and suppresses IL-10 (80). Several studies have reported increased miR-21-5p expression in diseases characterized by impaired immune responses, including asthma, cancer, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), chronic bacterial and viral infections (14, 79, 81, 82) and T1DM (reviewed here).

The role of miR-21-5p in beta-cells has not yet been clearly elucidated, but its function also seems to rely on the

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effect of cytokines via NF-KB (83). IL-1β and TNF strongly induce miR-21-5p expression in both insulin-secreting MIN6 cells and human islets (18). Moreover, miR-21-5p expression was increased during the development of pre-diabetic insulitis in islets from NOD mice, possibly as a protective response since miR-21-5p knockdown in MIN-6 cells promoted apoptosis (18). Accordingly, Ruan and coworkers (84) reported that NF-KB activated miR-21-5p in beta-cells, decreasing PDCD4 levels, inhibiting NF-KB activity in a negative regulatory loop, thus rendering beta-cells resistant to death. In contrast, Backe and coworkers (85) showed that miR-21-5p overexpression potentiated cell death after exposure to proinflammatory cytokines, leading to a reduced beta-cell number. Also, overexpression of miR-21-5p led to impaired glucose-stimulated insulin secretion through decreased *VAMP2* expression, a secretory granule protein that is essential for insulin exocytosis (18).

miR-148a-3p is a potent regulator of B cell tolerance and autoimmunity through suppression of *GADD45a*, *PTEN* and *BCL2l11* expressions (86). In agreement with this study, Pan and coworkers (81) showed elevated miR-148a-3p and miR-21-5p expression in $CD4^+$ T cells of SLE patients, which contributed to DNA hypomethylation by targeting the DNA methyltransferase 1 (DNMT1). Melkman-Zehavi and coworkers (87) reported that knockdown of miR-148a-3p in primary islets from mice or cultured beta-cells decreased insulin promoter activity and insulin mRNA levels.

miR-181a-5p also has a recognized role in the immune system (17, 88). This miRNA increases $CD19^+$ B populations and regulates T cell function (17, 89, 90, 91). miR-181a-5p seems to 'tune' TCR signal strength by targeting tyrosine phosphatases *SHP-2*, *PTPN22* and *DUSP5/6*, which enhances the basal activation of the TCR signaling molecules LcK and Erk, thus having an important role in thymic positive and negative selection (17, 88, 90). Xie and coworkers (92) reported that LPS and STZ strongly induced miR-181a-5p in macrophages from mice. Moreover, this miRNA was upregulated in patients with RA, which correlated with levels of proinflammatory cytokines (92). In this context, miR-181a-5p seems to have an anti-inflammatory function since it inhibited the increase of IL-1β, IL-6 and TNF in macrophages treated with LPS (93).

miR-210-5p is currently considered as a 'master miRNA' of hypoxic response as it was found upregulated by hypoxia in several cell types analyzed (94, 95). Consequently, miR-210-5p has been linked to various cancers and cardiovascular diseases (95, 96, 97, 98). Targets of this miRNA are involved in mitochondrial metabolism, angiogenesis, DNA repair and cell survival (95). Given that miR-210-5p targets many mitochondrial components, it is not surprising that manipulation of this miRNA leads to mitochondrial dysfunction and oxidative stress (94).

miR-375 is the most abundant miRNA detected in islets and is important for the development and maintenance of normal alpha- and beta-cell mass and insulin synthesis and secretion (16, 99, 100). Consequently, this miRNA has been proposed as a biomarker to detect beta-cell death and to predict the development of T1DM (2, 63, 66). Accordingly, massive beta-cell loss elicited by administration of STZ in C57BL/6 mice caused a dramatic increase in circulating levels of miR-375 (63). Moreover, plasma levels of this miRNA were increased in NOD mice 2 weeks before the onset of T1DM (63).

miR-24-3p seems to mark better preserved beta-cell function and/or insulin sensitivity 12 months after diagnosis (101). Moreover, this miRNA was elevated in serum of T1DM children (22), and its overexpression has been shown to inhibit beta-cell proliferation and insulin secretion (102).

miR-146a-5p, miR-150-5p, miR-342-3p, miR-1275 and miR-100-5p seem to be downregulated in serum/plasma or PBMCs from T1DM patients (Table 2). miR-146a-5p regulates Treg-mediated suppression of IFNγ-dependent Th1 responses and associated autoimmunity by directly targeting *STAT-1* (103), which was confirmed in our bioinformatic analysis (Supplementary Table 3). This miRNA has also a recognized role in innate immunity by negatively regulating the inflammatory response after recognition of bacterial components by TLRs on monocytes and macrophages (104). Upon activation by TNF and IL-1β (18), this miRNA downregulates *TRAF-6* and *IRAK-1* expressions, decreasing NF-KB activity, which seems to be a fine-tuning mechanism that prevents the overstimulation of the TLR pathway (104, 105). In disagreement with the downregulation of miR-146-5p in serum/PBMCs from T1DM patients, Roggli and coworkers (18) reported that this miRNA was increased in islets from NOD mice during development of insulitis. Blocking miR-146a-5p protected MIN6 cells from cytokine-induced apoptosis and also prevented the reduction in glucose-stimulated insulin secretion observed after IL-1β exposure (18).

Although literature on miR-100 is sparse, it has been implicated in some types of cancer (106, 107, 108) and tissue differentiation (109, 110, 111). Expression of circulating miR-100-5p was significantly decreased

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in obese normoglycemic subjects and subjects with type 2 diabetes mellitus (T2DM) compared to healthy, lean individuals. Moreover, in visceral adipose tissue, expression of miR-100 was lower in obese subjects with T2DM compared to obese subjects without T2DM (112).

miR-342-3p has been found to be dysregulated in different cancers (113, 114, 115), SLE (14), obesity (116, 117), diabetic kidney disease (118), T2DM and gestational diabetes (119). It is involved in beta-cell differentiation and maturation by targeting *FOXA2* and *MAFB* (120). Furthermore, miR-342-3p was downregulated in human leukocytes in response to LPS (121) and in Tregs from T1DM patients (49), suggesting that this miRNA may be involved in the development of autoimmunity and inflammation in T1DM patients.

Only a few studies have analyzed miR-1275 expression in different diseases. Due to its downregulation in certain cancers, miR-1275 has been referred to as a tumor suppressor (122, 123, 124, 125). In a HuH-7 hepatocarcinome cell line, miR-1275 overexpression suppressed *IGF2BP* expression, effectively impairing tumor cell proliferation, migration, viability and colony formation (124). The role of this miRNA in autoimmunity and beta-cell function is unknown.

Bezman and coworkers (126) showed that miR-150 plays a critical role in the innate immune system, and decreased expression level of miR-150-5p was negatively associated with GADA autoantibody titers, independently of hyperglycemia and disease duration (73).

Our bioinformatic analysis suggest that miR-21-5p, miR-24-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-375 and miR-1275 significantly regulate several mRNAs from pathways related to immune system and T1DM pathogenesis, such as MAPK, Jak-STAT, NF-KB, PI3K-Akt, apoptosis, TNF, TLRs, insulin and TCR signaling pathways (Figs 3, and). This might help to raise hypothesis about genes and pathways under influence of these circulating miRNAs.

Regarding the pathway analysis methods, there are many tools in literature that provide support for this type of investigation. Nonetheless, they are very similar as they all calculate the enrichment *P* values of pathways for a user pre-selected list of genes using a statistical test and a database with functional annotation that links genes to biological pathways. In addition, the hypergeometric test, which we adopted in our paper, has been widely applied in literature and previous studies discuss that when identifying significant pathways, the differences among the statistical methods will not be dramatic (127). The functional annotation has been traditionally performed in literature using annotations derived either from Gene Ontology (GO) or KEGG Pathway. KEGG Pathway Database has less annotated terms compared to GO, but it covers a wide range of molecular mechanisms and diseases, providing a graphical description of pathways, which is an important resource in the interpretation of results. Thus, bioinformatic tools used in this study provide robust data, which might be very similar to those generated using different tools.

In conclusion, this systematic review and bioinformatic analysis suggest that 11 circulating miRNAs (miR-21-5p, miR-24-3p, miR-100-5p, miR-146a-5p, miR-148a-3p, miR-150-5p, miR-181a-5p, miR-210-5p, miR-342-3p, miR-375 and miR-1275) are consistently dysregulated in T1DM patients. Further studies aiming at clarifying the specific role of these 11 miRNAs in pancreatic islets and islet-infiltrating immune cells are needed to shed light if they are biomarkers of T1DM and which are their specific roles in beta-cell function.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/EC-17-0248.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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