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MiR-205 is up-regulated in islets of diabetes-susceptible mice and targets the diabetes gene *Tcf7l2*

Meriem Ouni[1,2] | **Pascal Gottmann**[1,2] | **Efraim Westholm**[1,3] | **Kristin Schwerbel**[1,2] | **Markus Jähnert**[1,2] | **Mandy Stadion**[1,2] | **Kilian Rittig**[4,5] | **Heike Vogel**[1,2,6,7] | **Annette Schürmann**[1,2,5]

[1]Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE), Nuthetal, Germany

[2]German Center for Diabetes Research (DZD), München-Neuherberg, Germany

[3]Unit of Islet Cell Exocytosis, Department of Clinical Sciences Malmö, Lund University Diabetes Centre, Lund University, Malmö, Sweden

[4]Clinic for Angiology and Diabetology, Frankfurt (Oder), Germany

[5]Institute of Nutritional Science, University of Potsdam, Brandenburg, Germany

[6]Research Group Genetics of Obesity, German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE), Nuthetal, Germany

[7]Research Group Molecular and Clinical Life Science of Metabolic Diseases, Faculty of Health Sciences Brandenburg, University of Potsdam, Brandenburg, Germany

Correspondence
Annette Schürmann, Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE), Arthur-Scheunert-Allee 114-116, Nuthetal 14558, Germany.
Email: schuermann@dife.de

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Abstract

Aim: MicroRNAs play an important role in the maintenance of cellular functions by fine-tuning gene expression levels. The aim of the current study was to identify genetically caused changes in microRNA expression which associate with islet dysfunction in diabetic mice.

Methods: To identify novel microRNAs involved in islet dysfunction, transcriptome and miRNome analyses were performed in islets of obese, diabetes-susceptible NZO and diabetes-resistant B6-*ob/ob* mice and results combined with quantitative trait loci (QTL) and functional in vitro analysis.

Results: In islets of NZO and B6-*ob/ob* mice, 94 differentially expressed microRNAs were detected, of which 11 are located in diabetes QTL. Focusing on conserved microRNAs exhibiting the strongest expression difference and which have not been linked to islet function, miR-205-5p was selected for further analysis. According to transcriptome data and target prediction analyses, miR-205-5p affects genes involved in Wnt and calcium signalling as well as insulin secretion. Over-expression of miR-205-5p in the insulinoma cell line INS-1 increased insulin expression, left-shifted the glucose-dependence of insulin secretion and supressed the expression of the diabetes gene *TCF7L2*. The interaction between miR-205-5p and *TCF7L2* was confirmed by luciferase reporter assay.

Conclusion: MiR-205-5p was identified as relevant microRNA involved in islet dysfunction by interacting with *TCF7L2*.

KEYWORDS

GSIS, islets of Langerhans, microRNA, T2D, *Tcf7l2*

1 | INTRODUCTION

Type 2 diabetes (T2D) is a polygenic disease with a strong heritable component, which is also influenced by environmental factors. Genetic alterations were intensively explored in humans and mouse models by using genome-wide association studies (GWAS) and positional cloning strategies respectively.[1] The latter procedure was successfully applied for the identification of novel diabetes genes. The New Zealand Obese (NZO) mouse is a well-studied model for the metabolic syndrome and for polygenetically driven T2D that closely resembles the human disease.[2] In previous studies, we performed a genome-wide linkage analysis with the NZO and C57BL/6 (B6) strains and detected six quantitative trait loci (QTL)[3] that participate in the development of insulin resistance and T2D. The investigation of these QTL allowed not only the identification of genes (*Gjb4, Zfp69, Tbc1d1*)[4-6] involved in this complex trait, but also led to the discovery of small non-coding RNAs, for example, microRNAs (miRNA) that participate in the disease progression. Expression of miR-31, located in the obesity QTL *Nob6*, was elevated in adipose tissue of obese mice and humans and its manipulation in a human adipocyte cell line further confirmed that miR-31 targets genes involved in adipogenesis and insulin signalling.[7]

MiRNAs are small (~19-24 nucleotides) non-coding RNAs involved in post-transcriptional gene regulation. The main mode of regulation occurs through mRNA degradation; however, miRNAs can also affect protein translation. Nowadays, it is well known that miRNAs are responsible for fine-tuning the gene expression of important targets in the metabolic syndrome and T2D. Hence, miRNAs have emerged as powerful regulators in the function of islets of Langerhans as they play a role in proliferation, beta-cell survival and insulin synthesis.[8,9] For example, it has been shown that the expression levels of the (miR)-200 family are strongly induced in islets of diabetic mice and its overexpression in mice is sufficient to induce beta-cell apoptosis and T2D.[10] Other miRNAs (eg, miR-7, -9, -29, -30, -33, -96, -124 and -145) target components of the secretory machinery and thereby control insulin secretion.[9] In some cases, a miRNA can be implicated in multiple functions in islets such as miR-375, which was described to affect islet proliferation, the regulation of alpha- and beta-cell mass and insulin secretion.[11,12] In humans, high-throughput sequencing allowed the identification of novel miRNAs involved in islet-cell function (eg, miR-148, -124a) and revealed similarities in miRNA expression patterns between diabetic subjects and mice.

While several studies in mice and humans have suggested a role for miRNAs to regulate pancreatic islet and beta-cell function,[13,14] little is known about the genetic determinants of altered miRNAs expression in T2D. Here, we compared islet transcriptome of diabetes-susceptible New Zealand Obese (NZO) mice with that of diabetes-resistant B6-*ob/ob* mice. Both obese mice were first kept on a specific feeding regimen of initial carbohydrate restriction for 15 weeks (−CH) to avoid hyperglycaemia. This was followed by a 2-day feeding of a diet containing carbohydrates (+CH). We have previously shown that the +CH intervention increases blood glucose levels and induces islet-cell apoptosis in NZO mice within a period of 2-3 weeks. By contrast, B6-*ob/ob* mice, which carry a leptin mutation on the B6 background, do not develop hyperglycaemia under +CH conditions because of an induction of beta-cell proliferation.[2,15] We screened for miRNAs which are (a) located in diabetes QTL and (b) exhibit a differential expression in islets of diabetes-prone and -resistant mice 2 days after carbohydrate feeding (Figure 1A).

2 | RESULTS

2.1 | Identification of miRNAs in pancreatic islets linked to islet-cell dysfunction

To identify novel miRNAs associated with islet dysfunction and the pathogenesis of T2D, transcriptome and miRNome analysis were assessed in islets of two obese mouse strains differing in their diabetes susceptibility. As mentioned above, the leptin-deficient B6-*ob/ob* and NZO mice were fed with a carbohydrate-free (−CH) diet for 15 weeks, followed by a carbohydrate containing diet (+CH) for 2 days (Figure 1A, left panel). Islets were collected for sequencing of total RNA (RNA-seq) and miRNA (miRNA-seq). As described previously,[15,16] after 2-day feeding of a carbohydrate-containing diet, NZO mice displayed significantly higher blood glucose levels than B6-*ob/ob* (Figure 1A right panel), without showing differences in body weight (Figure S1).

RNA-seq analysis identified 2497 differentially expressed genes in islets of B6-*ob/ob* and NZO with a fold change higher than 1.5 (n = 1072 up, n = 1425 down in NZO, uncorrected *P* value <.05) (Table S1). We confirmed that genes down-regulated in NZO islets were enriched in pathways involved in cell cycle, DNA replication, insulin secretion and glutathione metabolism[16] whereas the up-regulated genes were rather linked to focal adhesion and extracellular matrix (ECM) receptor interaction and others (Table S2). MiRNA-seq analysis detected 309 mature miRNAs in islets and among these, the highest expression levels were found for miR-148a-3p, miR-26a-5p and miR-141-3p (Table S3). Of note, several of the highly expressed miRNAs, listed in Table S3, (including those mentioned above) have been previously described in rodent or human islets,[9,13,17] which further supports the relevance of the current study design and the quality of the miRNA-seq data.

Comparative analysis of samples from B6-*ob/ob* and NZO mice identified 94 differentially expressed miRNAs.

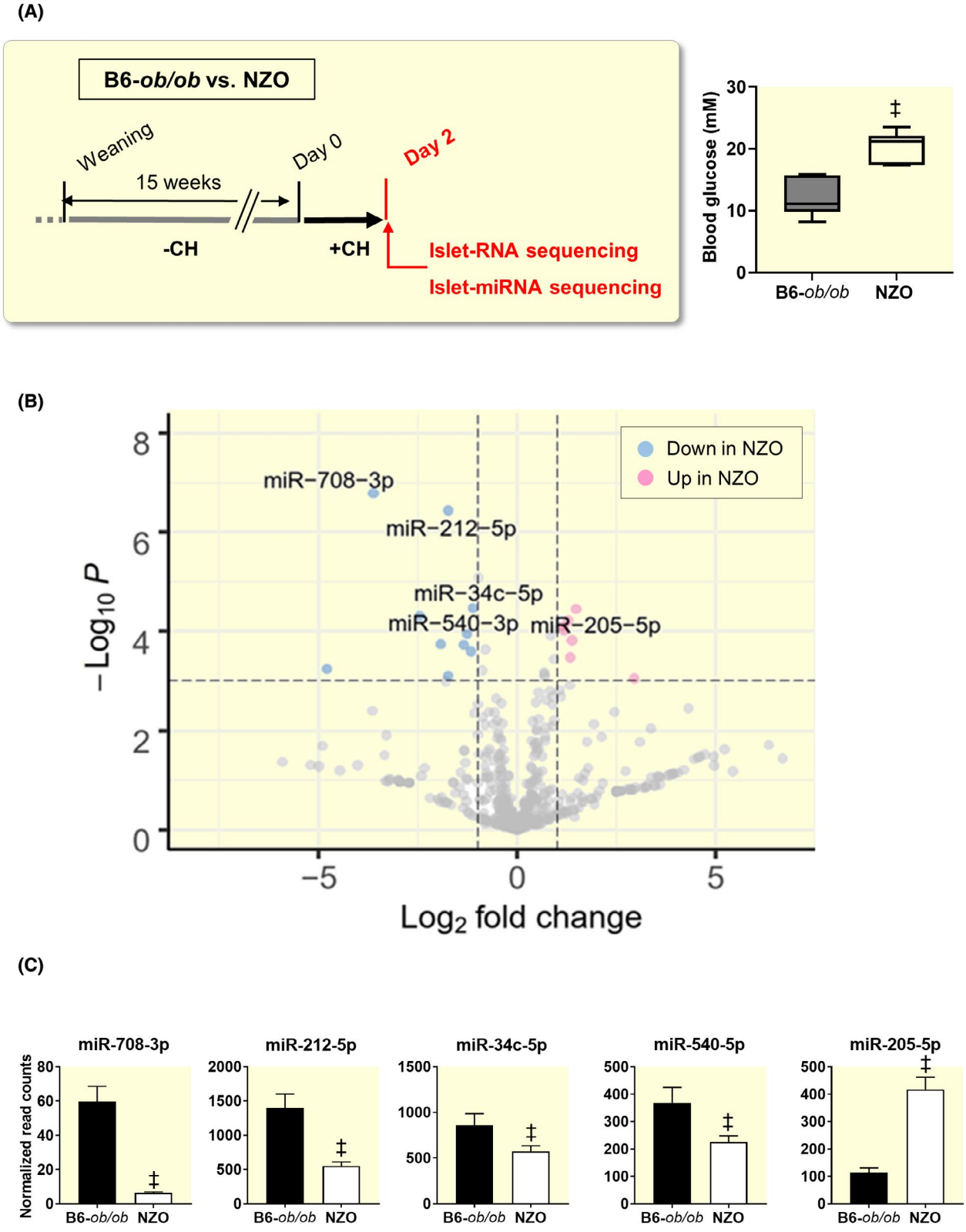


FIGURE 1 Identification of miRNAs associated with diabetes susceptibility. Left panel (A): Study design. Male B6-ob/ob and NZO mice were fed a carbohydrate-free, fat-enriched diet (−CH) until the age of 18 ± 1 weeks followed by a diabetogenic carbohydrate-enriched diet (+CH) for 2 days and islet RNA and miRNA sequencing were performed. Right panel (A): NZO exhibited higher blood glucose levels than B6-ob/ob. Unpaired two-tailed student *t*-test with Welch correction ***$P < .001$ (B) Volcano plot showing the changes in the miRNA profiles in B6-ob/ob vs. NZO islets. Pink and blue dots indicate miRNAs that are up or down-regulated respectively, in islets of diabetes-susceptible NZO in comparison with those of diabetes-resistant B6-ob/ob mice. The horizontal dashed line indicates the negative logarithm 10 of *P* value threshold and the vertical lines refer to the logarithm 2 of fold changes threshold between B6-ob/ob vs. NZO. C, Expression of the top five miRNAs ranked by the smallest *P* value (n = 5, unpaired two-tailed student test with Welch correction *P* value <.05) in islets of B6-ob/ob and NZO mice

As depicted in the volcano plot (Figure 1B), the number of up- and down-regulated miRNAs in islets of NZO mice was 48 and 46 respectively. The candidates which exhibited the most significant differences between the strains were miR-708-3p, -212-5p, -34c-5p, -540-5p and -205-5p (Tables S3 and S4, Figure 1B,C).

To identify novel miRNAs associated with islet dysfunction and link them to the genetic predisposition, the differentially expressed miRNAs were mapped to the six QTL associated with elevated blood glucose and/or reduced total pancreatic insulin identified in the NZOxB6 cross.[3] As shown in the Circos plot, 11 out of the 94 differentially expressed miRNAs were located in diabetes QTL (Figure 2A,B). Although some miRNAs were already described to play a role in insulin production and/or secretion or apoptosis (eg, miR-212, -204),[9,18] the majority of the miRNAs located in these QTL have not been linked to islet function yet (Table S4). To select the most reliable novel miRNA candidate, stringent criteria were applied: (a) miRNAs with unknown function in pancreatic islets, (b) most significantly altered miRNAs, (c) highest absolute fold change between B6-*ob/ob* and NZO and (d) a mature miRNA sequence conserved between mouse and human were chosen for further analysis. Among the 11 miRNAs located in QTL, miR-205-5p on chromosome one, was the only candidate fulfilling all criteria and was therefore selected for further investigations (Figure 2B, Table S4). Moreover, our analysis indicated that pancreatic islets, beside gWAT and kidney, exhibited the highest expression of miR-205-5p (Figure S2).

2.2 | Polymorphic *cis*-regulatory elements of *Mir205hg*

It is well accepted that genetic variances in enhancer and promoter regions play an important role in altered gene expression by changing the affinity of transcription factors (TFs) to bind to the DNA and/or affect the accessibility of the chromatin towards the transcriptional machinery.[19,20] To evaluate if *cis*-regulatory elements of the miR205 host gene (*Mir205hg*) carry genetic variants that could explain the expression difference, the publicly available histone modification marks H3K4me3, H3K27ac (for the characterization of promoter regions) and H3K4me1 and H3K27ac (for enhancer regions)[21] were used for evaluating the upstream and downstream sequences of *Mir205hg* (Figure 3A). In total, 542 single nucleotide polymorphisms (SNPs), insertion and deletion (InDel) events were identified in the *cis*-regulatory elements spanning from chr1:193 457 500 to 193 557 500. Next, we tested which transcription factor-binding sites (TFBS) could be affected by the SNPs in the promoter and enhancer regions and applied two approaches (Figure 3A). In the first approach (approach 1), all putative TFs[22] which could theoretically bind to *cis*-regulatory elements of *Mir205hg* and carry SNPs were filtered and the affinity score based on the position weight matrix was calculated. Prediction analysis revealed four SNPs, depicted in black lines in Figure 3B, which potentially enhance the binding of six TFs in the promoter region (Figure 3B, upper panel) and 7 SNPs that might affect binding of 14 TFs to the enhancer regions of the NZO sequence (Figure 3B, lower panel, Table 1). As shown in Figure 3C, the replacement of A with T in the NZO sequence could theoretically enhance the binding of cyclic AMP-responsive element-binding protein 3 like 2 (CREB3L2) in the promoter. Another SNP was detected in the enhancer region, which may increase the binding affinity of the motor neuron and pancreas homeobox 1 transcription factor (MNX1) (Figure 3D).

Beside the core sequence, the surrounding nucleotides can play a role in the chromatin accessibility and the affinity of a TF to the DNA. Therefore, in the second approach (approach 2, Figure 3A), we mapped all SNPs in near proximity of the binding sites of six key islet-specific TFs (FOXA2, MAFA1, NKX2.2, NKX6.1, NEUROD1, PAX6 and PDX1) within enhancer and promoter regions of *Mir205hg*. In contrast to the first approach that was based on predicted binding of TFs, the second utilized ChIP-Seq data which determine the molecular interaction between the selected TFs and DNA. The highest number of SNPs (16) and InDels (3) (indicated in red lines) were detected in proximity to the genomic area highly enriched in binding of NKX2.2 however, the binding sites of NEUROD1, MAFA1, PDX1 and FOXA2 were surrounded by six SNPs and two InDels (Figure 3B, see details in Table 2).

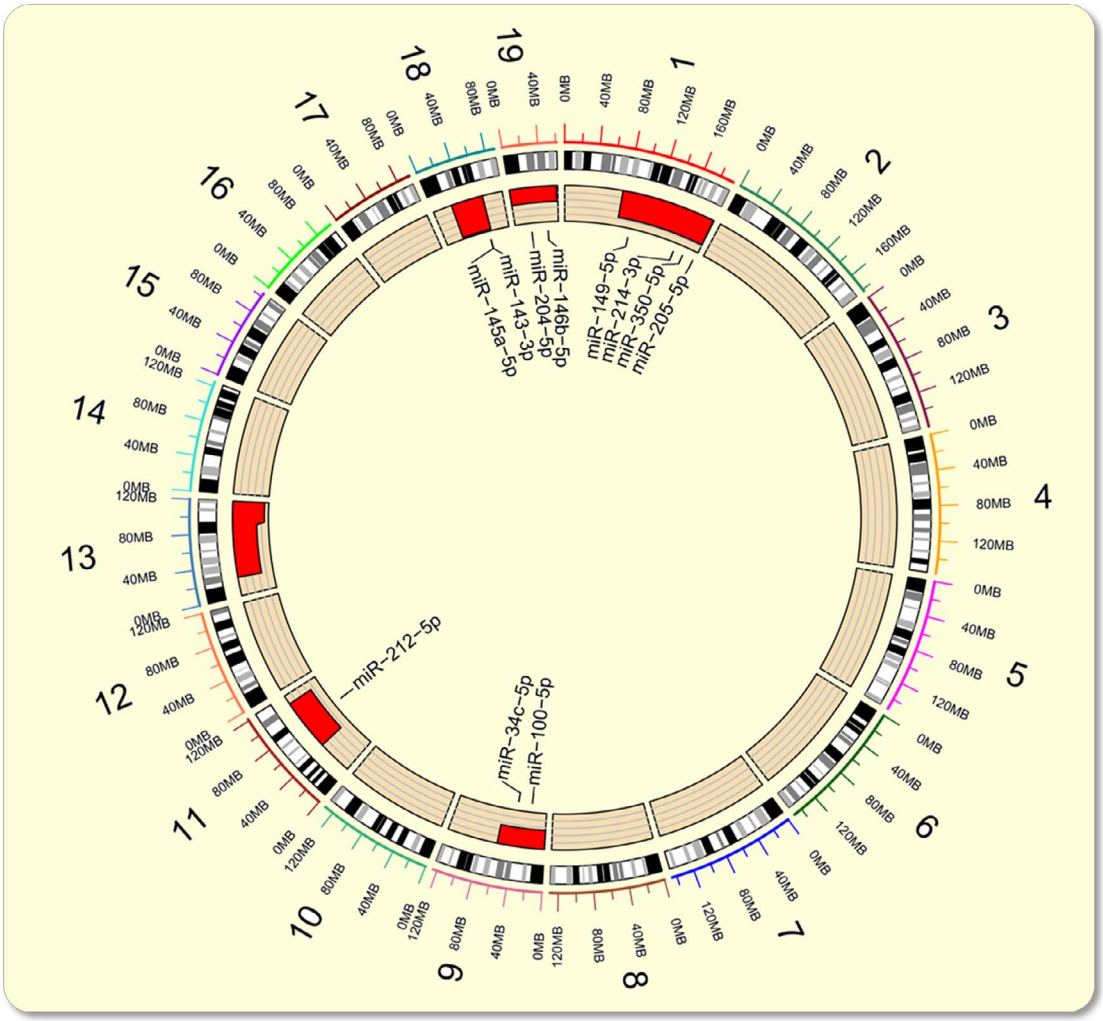
Taken together, the screen for altered TFBS identified genetic variants in the NZO sequence, which could explain the elevated miR-205-5p expression in islets.

2.3 | Over-expression of miR-205-5p alters insulin synthesis and secretion in INS-1 cells

To evaluate if miR-205 affects insulin synthesis, *Ins1* expression levels and intracellular insulin content were measured in INS-1 cells treated with miR-205-mimic and non-targeting control (Con). Here, 72 hours after transfection, *Ins1* mRNA as well as the protein levels were significantly higher in cells over-expressing miR-205-5p in comparison with Con (Figure 4A).

To test the functional role of miR-205-5p in pancreatic islets, insulin secretion in miR-205-mimic-treated INS-1 cells was measured and compared with Con. No significant differences were detected between Con and miR-205 expressing cells after treatment with 1.0 mM glucose when the data were normalized to the DNA content of the cells. At a glucose concentration of 2.8 mM, cells over-expressing the miR-205-5p released about 40% more insulin than cells transfected with

(A)



(B)

miRNA symbol	p-value	Log fold change	Human conservation
miR-205-5p	$6x10^{-3}$	1.4	Yes
miR-350-5p	$3x10^{-2}$	-0.7	Not annotated in humans
miR-214-3p	$9x10^{-3}$	4.0	Yes
miR-149-5p	$1x10^{-2}$	1.6	Yes
miR-34c-5p	$9x10^{-4}$	-0.9	Yes
miR-100-5p	$3x10^{-3}$	1.2	Yes
miR-212-5p	$7x10^{-4}$	-1.6	Yes
miR-143-3p	$2x10^{-2}$	0.4	Yes
miR-145a-5p	$1x10^{-3}$	1.1	Hsa-miR-145-5p
miR-146b-5p	$3x10^{-3}$	0.4	Additional G
miR-204-5p	$8x10^{-4}$	0.6	Yes

FIGURE 2 MiRNAs located in diabetes-related QTL. A, Circos plot of miRNAs located in diabetes-related QTL. Red rectangles correspond to diabetes QTL of the NZOxB6 cross and black dashes in the inner part of the Circos plot indicate all miRNAs located in these diabetes QTL. B, Detailed list of the 11 miRNAs located in the diabetes-related QTLs, n = 5 per group, unpaired two-tailed *t*-test with Welch correction *P* value <.05

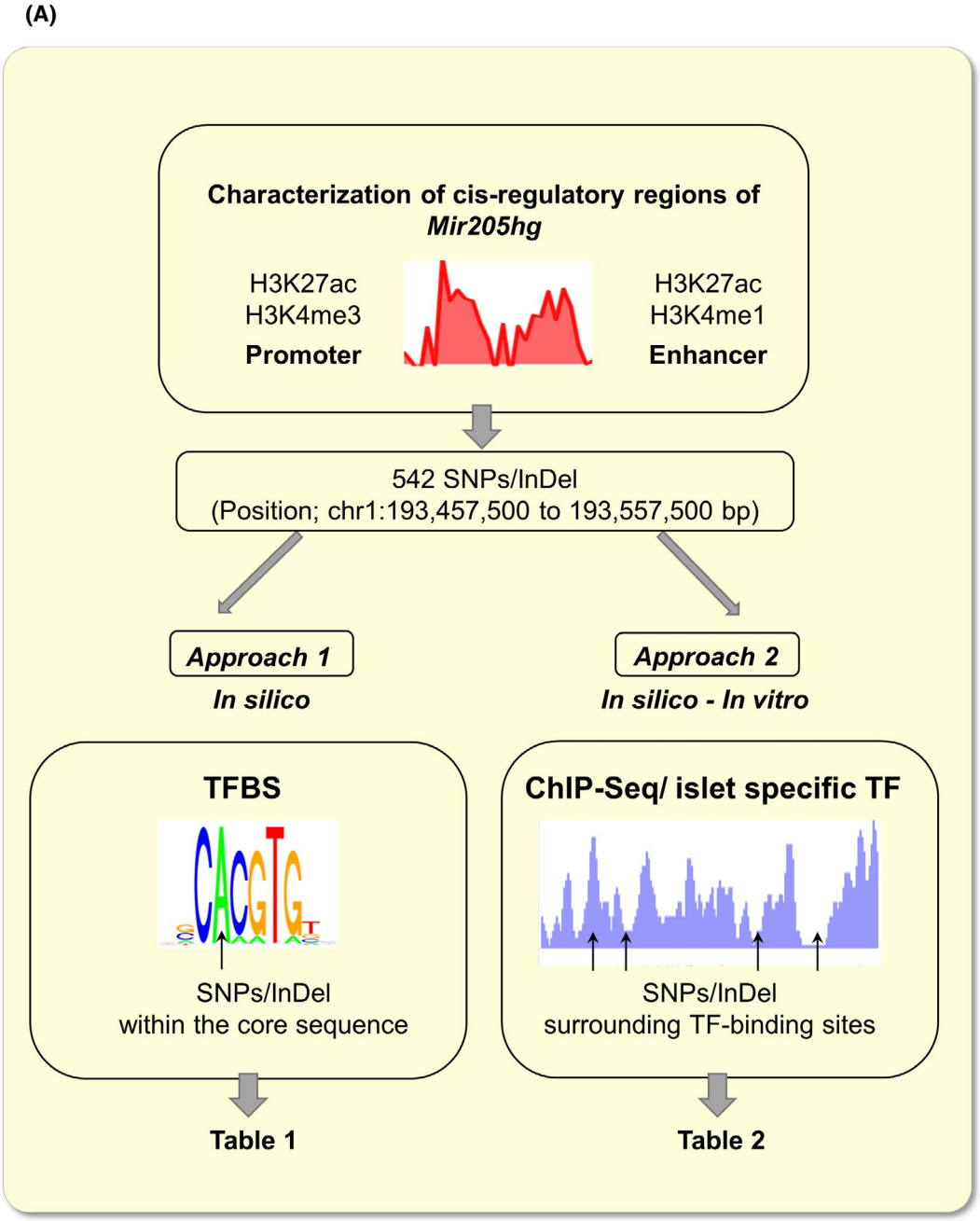


FIGURE 3 Polymorphic *cis*-regulatory elements of *Mir205hg* gene. A, A diagram summarizing the two different in silico approaches used to identify genetic variants responsible for altered miR-205-5p expression. B, Schematic representation of *cis*-regulatory elements of *Mir205hg* gene. *Mir205hg* (in the middle) is illustrated in black, each rectangle corresponds to exonic regions and the corresponding mature miR-205 is shown as small black rectangles below the corresponding exonic sequence. The promoter is depicted in the upper panel and the enhancer region in the lower panel. ENCODE data for histone marks H3K4me1, H3K4me3 and H3K27ac was used to characterize the promoter (upper part) and enhancer regions (lower part). H3K4me1, H3K4me3 and H3K27ac are shown in black, dark grey and light grey respectively. ChIP-seq data of islet-specific transcription factors MAFA1, NEUROD1, NKX22, FOXA2 and PDX1 were shown for both enhancer and promoter regions. The latter TFs were illustrated in blue. Vertical black lines refer to SNPs predicted to alter the binding sites of the transcription factors listed in Table 1A. SNPs located in the near proximity (500 bp) of islet-specific transcription factors were coloured in vertical red lines. C, Core sequence of the CREB3L2-binding site, which carries an SNP (highlighted in red) in the promoter region of *Mir205hg* in the NZO genome. D, Core sequence of the MNX1-binding site, which carries an SNP (highlighted in red) in the enhancer region of *Mir205hg* in the NZO genome; the affinity score is calculated with the underlined letters the nucleotides; the affinity score calculation is based on the underlined letters the nucleotides

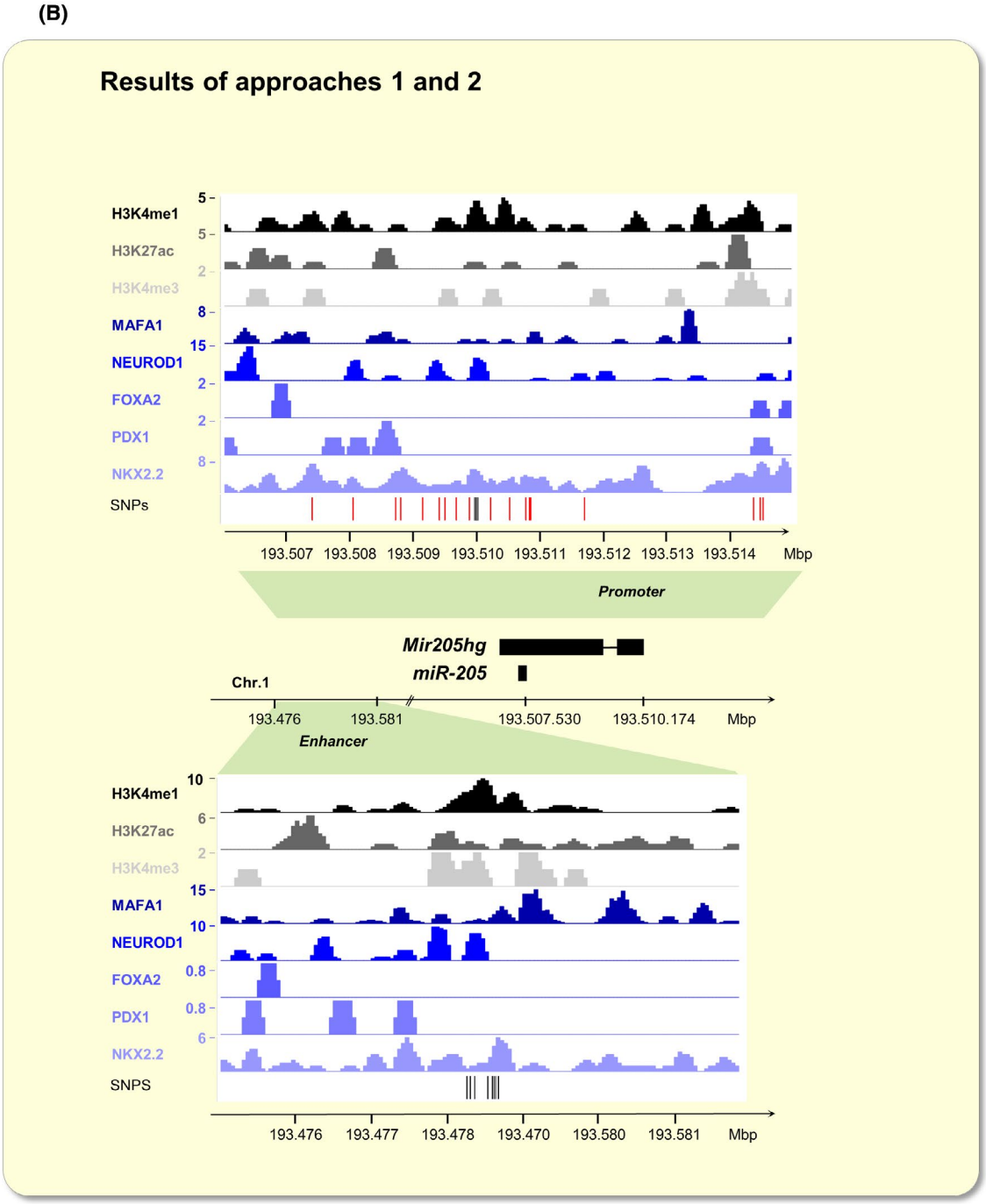


FIGURE 3 (Continued)

the non-targeting control construct (Figure 4B). In contrast to Con cells, we did not detect an increase in insulin secretion in response to 20 mM glucose in miR-205-5p cells. Presumably because of the higher insulin content in miR-205-5p-expressing cells, their KCl-induced insulin secretion was higher than in Con cells (Figure 4B). To evaluate insulin secretion in proportion to its availability, we normalized the data to residual insulin of the INS-1 cells. As shown in Figure 4C, miR-205 mimic-treated cells showed an elevated insulin secretion at 2.8 mM glucose in comparison with Con. These levels did not further increase at 20 mM glucose. The KCl-mediated insulin secretion was comparable between control and miR-205 expressing cells (Figure 4C).

2.4 | Identification of predicted target genes of miR-205-5p

To define putative target genes of miR-205-5p, we first applied an in silico method based on five different prediction

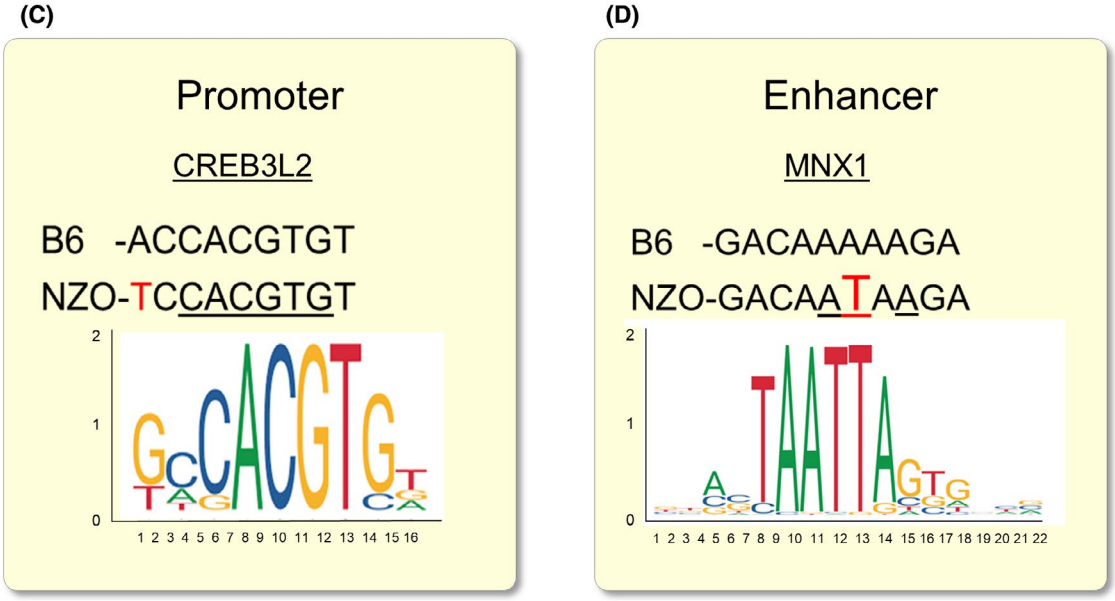


FIGURE 3 (Continued)

tools, DIANA-microT, miRDB, TarPmiR, TargetScan7.1 and RNA22, whereas optimal targets were given when at least three tools resulted in overlapping target results.[7] Comparing predicted targets with genes exhibiting a lower expression in pancreatic islets of NZO versus B6-*ob/ob* resulted in 121 genes involved in pathways of thyroid hormone synthesis, inositol phosphate metabolism, insulin secretion, calcium and Wnt signalling (Figure 5A, Table S5). The connection between miR-205-5p and its putative target genes as well as their enrichment in relevant pathways for islet function is depicted in Figure 5B,C. The graph also indicates that as expected, several target genes (eg, *Kcnma1*, *Prkca, Adcy9*) are linked to more than one of the designated pathways (Figure 5B).

The fact that the mature sequence of miR-205-5p is well conserved in mice, rats and humans, suggests that this miRNA targets the same genes in those species. Therefore, mouse-binding sites of miR-205-5p located in *Plcb1*, *Tcf7l2*, *Prkca*, *Nphp1, Plcb4, Kcnma1*, *Gsk3b*, *Cxxc4* and *Adcy9*, which exhibited a differential expression in islets of B6-*ob/ob* and NZO mice and (Figure 5C) were aligned with the rat and human orthologues. The alignment revealed that only *Plcb1*, *Tcf7l2*, *Prcka*, *Nphp1* and *Cxxc4* carried conserved binding sites across the different species (Figure 5D, Figure S3).

To address the question whether conserved genes related to insulin secretion and Wnt signalling pathways are targeted by miR-205-5p, its expression was manipulated in INS-1 cells. MiR-205-mimic and the corresponding non-targeting control were transiently transfected and the endogenous expression of the selected genes were evaluated 72 hours later (Figure 6A). *Plcb1* and *Tcf7l2* were significantly down-regulated in miR-205-mimic transfected cells by 50% and 40% respectively. Expression of *Prcka*, *Cxxc4* and *Nphp1* showed a tendency towards lower levels in miR-205-mimic transfected cells; however, these effects were not significant after multiple testing correction (Figure 6A). To test the specificity of the observed down-regulation, the mRNA levels of *Trim25*, which does not carry a miR-205-5p targeting sequence, were measured. *Trim25* gene expression was not affected by miR-205-mimic (Figure 6A). Next, the miR-205-mediated down-regulation of *Plcb1* and *Tcf7l2* expression was further confirmed by western blotting. The over-expression of miR-205-5p led to a significant decrease in PLCB1 and TCF7L2 protein levels in INS-1 cells (Figure 6B). This effect was specific for miR-205-5p as the over-expression of miR-27b-3p did not affect PLCB1 and TCF7L2 (Figure 6B). Accordingly, the inhibition of miR-205-5p led to an increase in *Plcb1* and *Tcf7l2* mRNA levels in INS-1 cells (Figure S4). Taken together, our results provide evidence that *Plcb1*and *Tcf7l2* are targeted by miR-205-5p. It was already described that the over-expression of miR-205-5p down-regulates *Plcb1*,[23] therefore we focussed on *Tcf7l2*, a transcription factor known to play a role in the Wnt signalling pathway and in beta-cell function.[24] As *Tcf7l2* was less abundant in islets of diabetes-prone animals, we tested whether downstream targets of this transcription factor exhibited lower expression levels as well. Of note, *Lrp5*, *Lrp6*, *Myc*, *Isl1*, *Axin2* and *Cpt1a* known to be regulated by TCF7L2[25-27] exhibited low mRNA levels in islets of NZO mice compared with B6-*ob/ob* (Figure S5).

2.5 | Polymorphic enhancer region of *MIR205HG* in humans

In humans, miR-205-5p is encoded as an individual gene (*MIR205HG*). To estimate whether SNPs in

TABLE 1 List of SNPs located in promoter and enhancer of *Mir205hg* and potentially enhance the binding of the listed transcription factors

Chr.	Genomic position	RS.ID	B6	NZO	Transcription factor	Score change
Promoter						
1	193 509 945	rs33174655	A	G	THAP1	0.61
1	193 509 945	rs33174655	A	G	THAP1	2.86
1	193 510 003	rs33174658	T	C	HINFP	0.56
1	193 510 012	rs51632649	C	T	SOX18	0.65
1	193 510 012	rs51632649	C	T	SOX18	1.45
1	**193 510 055**	**rs33174661**	**T**	**A**	**CREB3L2**	**6.83**
1	193 510 055	rs33174661	T	A	HEY1	0.58
1	193 510 055	rs33174661	T	A	NPAS2	0.56
Enhancer						
1	193 478 275	rs222315750	T	A	FOXO4	4.44
1	193 478 275	rs222315751	T	A	FOXO4	4.04
1	193 478 275	rs222315752	T	A	ID1	0.96
1	193 478 275	rs222315753	T	A	ID1	2.66
1	193 478 275	rs222315754	T	A	ID1	3.8
1	193 478 275	rs222315755	T	A	ID1	3.6
1	193 478 275	rs222315756	T	A	ID1	3.6
1	**193 478 275**	**rs222315757**	**T**	**A**	**MNX1**	**6.22**
1	193 478 319	rs47989799	A	G	CREB3L2	2.3
1	193 478 319	rs47989800	A	G	HEY1	0.19
1	193 478 319	rs47989801	A	G	NPAS2	1.34
1	193 478 319	rs47989802	A	G	SOX18	0.71
1	193 478 377	rs50597002	T	G	SOX18	0.49
1	193 478 550	rs49423083	C	T	BARHL1	4.1
1	193 478 550	rs49423084	C	T	CUX2	1.97
1	193 478 550	rs49423085	C	T	HHEX	1.09
1	193 478 550	rs49423086	C	T	ID1	0.45
1	193 478 550	rs49423087	C	T	NKX6-1	1.52
1	193 478 550	rs49423088	C	T	NKX6-1	4.06
1	193 478 550	rs49423089	C	T	SOX13	0.14
1	193 478 638	rs45722551	A	G	BARHL1	1.23
1	193 478 644	rs48252554	A	T	BARHL1	1.16
1	193 478 644	rs48252554	A	T	LHX1	2.54
1	193 478 646	rs48072392	T	C	BARHL1	0.82
1	193 478 646	rs48072392	T	C	SOX12	0.622

Note: The affinity of the listed transcription factors was altered by the corresponding SNPs by the indicated score change. SNPs with the strongest Score change in the enhancer and promoter regions are presented in bold and depocted in Figure 3C,D.

regulatory elements of *MIR205HG* could affect its expression, we performed comparable analysis as described earlier (Figure 3A) and found 10 SNPs that theoretically alter the binding of 9 TFs in the enhancer. Interestingly, similar to our finding in mice, rs1218275375 could theoretically enhance the binding of MNX1 in the enhancer region of *MIR205HG* (Table 3).

2.6 | MiR-205-5p targets human *TCF7L2*

As *TCF7L2* is the most prominent diabetes gene[28,29] and the results described above demonstrated that *Tcf7l2* is down-regulated in miR-205 over-expressing INS-1 cells (FDR <.05), we investigated the interaction of miR-205-5p with the *TCF7L2* mRNA. First, all putative

TABLE 2 List of SNPs located in near proximity to islet-specific transcription factors that bind to *cis*-regulatory elements of *Mir205hg*

Chr.	Genomic position Mm10	TF	RS.ID	B6	NZO
1	193 514 548	NKX2.2	rs33176263	A	T
1	193 514 504	NKX2.2	rs46582719	C	T
1	193 514 505	NKX2.2	rs33176260	A	G
1	193 514 397	NKX2.2	rs33176255	C	A
1	193 507 393	NKX2.2	rs33168372	A	G
1	193 509 145	NKX2.2	rs33172029	C	A
1	193 510 782	NKX2.2	rs52609635	C	A
1	193 510 839	NKX2.2	rs52636521	C	T
1	193 510 859	NKX2.2	rs52609307	T	C
1	193 510 528	NKX2.2	rs259431765/ rs237567586	ATG	A
1	193 509 410	NKX2.2	rs33172840	A	G
1	193 508 040	NKX2.2	rs33170286	C	G
1	193 510 225	NKX2.2	rs33174663	G	C
1	193 509 678	NKX2.2	rs217459842	T	C
1	193 509 978	NKX2.2	rs244130707	AGGT	A
1	193 508 798	NKX2.2	rs31480683	G	A
1	193 510 003	NKX2.2	rs33174658	T	C
1	193 510 012	NKX2.2	rs51632649	C	T
1	193 509 884	MAFA1	rs220905239	CA	C
1	193 514 548	FOXA2	rs33176263	A	T
1	193 511 713	NEUROD1	rs31526545	C	T
1	193 509 410	NEUROD1	rs33172840	A	G
1	193 509 497	NEUROD1	rs227936095	GT	G
1	193 510 003	NEUROD1	rs33174658	T	C
1	193 510 012	NEUROD1	rs51632649	C	T
1	193 508 716	PDX1	rs32044751	T	C

binding sites in each mRNA splice variant of the human *TCF7l2* gene were evaluated and then filtered for those transcripts that are highly expressed in pancreatic islets. This approach identified a 7-mer-binding site located in different coding regions exonic positions (exon 1, 2, 3, 8, 9 and 10; see Table S6). The binding site located in exon 10 was selected for functional analysis as it is conserved between mouse and human (Figure 7A). To investigate the putative interaction between miR-205 and the selected sequence in exon 10 of *TCF7L2*, a luciferase reporter assay was conducted in HeLa cells. As shown in Figure 7B, co-transfection experiments revealed that miR-205-mimic significantly decreased luciferase activity levels through the *TCF7L2*-exon10 (30%, *P* value = .01). In contrast, transfection of HeLa cells with a non-targeting miRNA mimic plus *TCF7L2*-exon10 did not suppress the luciferase activity. Moreover, neither miR-205-mimic, nor non-targeting miRNA mimic affected the luciferase activity of the control reporter vector (Con) (Figure 7B).

Taken together, the reporter assay confirmed (a) the interaction of miR-205-5p with sequences of *TCF7L2* as predicted by the initial in silico analysis, (b) the in vitro down-regulation of *Tcf7l2* in INS-1 cells by miR205-5p and thereby (c) provides functional evidence that miR-205-5p can mediate the repression of *TCF7L2* in a non-canonical mode by binding to the open reading frame located in exon 10.

3 | DISCUSSION

In the current study, transcriptome, computational, in silico and in vitro analyses were combined to identify miRNAs involved in islet function and T2D in mice. We identified miR-205-5p to regulate targets known to be involved in the development of T2D. RNA-seq data together with prediction analysis revealed that miR-205-5p affects target genes implicated in Wnt signalling and insulin secretion. Indeed, the over-expression of miR-205-5p

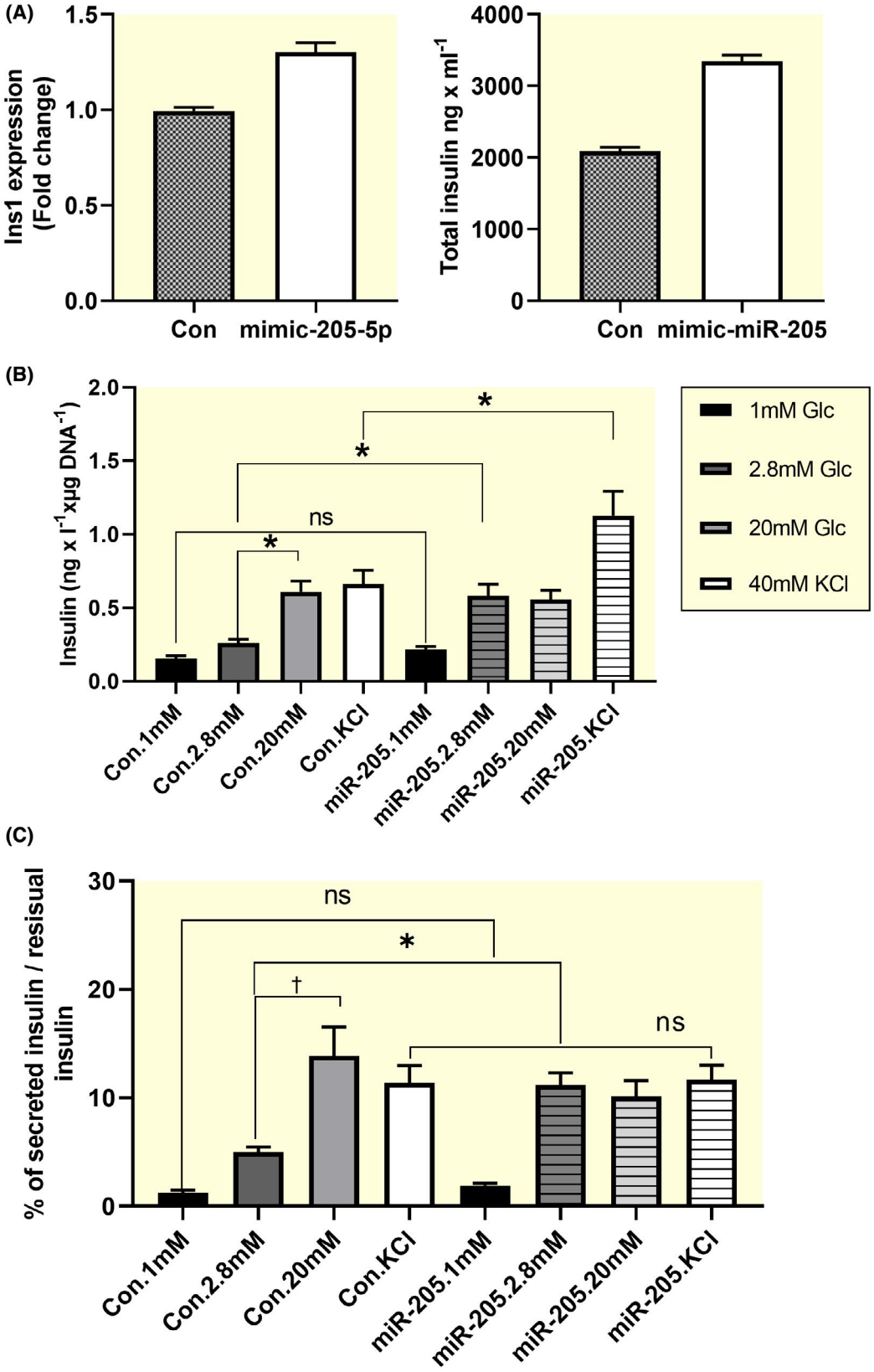


FIGURE 4 Over-expression of miR-205-5p increases insulin expression and basal insulin release. A, *Ins1* expression levels and intracellular insulin content in rat INS-1 cells after over-expression of miR-205-5p. B and C, Insulin secretion of INS-1 cells under low (1, 2.8 mM) and high (20 mM) glucose concentrations and in response to KCl (40 mM) (B) normalized to genomic DNA content and (C) to residual insulin. Data were obtained from at least three wells per condition from four independent experiments and analysed by unpaired two-tailed student *t*-test with Welch correction in (A) and one-way ANOVA and correction for multiple testing in (B and C) $*P < .05$, $***P < .001$ and $****P < 10^{-4}$

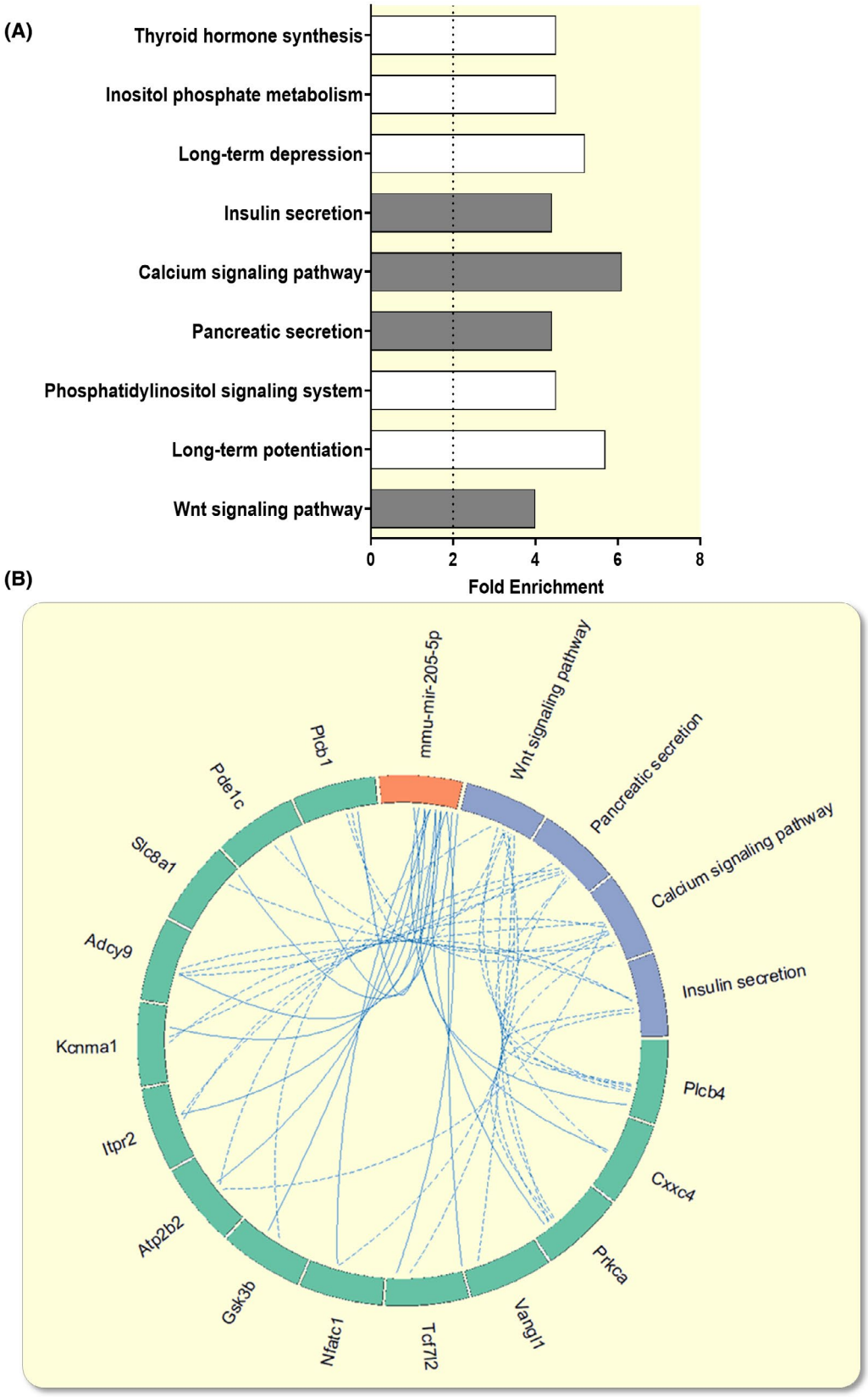


FIGURE 5 Pathway analysis of putative target genes of miR-205-5p. A, KEGG pathways of the identified 121 putative target genes of miR-205-5p. Pathways coloured in grey were selected for functional analysis in Figure 6. B, Circular representation of selected pathways. Solid blue lines refer to miR-205-5p-target gene interaction and dashed lines relate genes to indicated pathways. C, Expression levels of selected set of genes targeted by miR-205-5p and involved in insulin secretion, calcium signalling and Wnt signalling. Data extracted from RNA-seq (see Table S1), n = 5 per group (Benjamini–Hochberg adjusted *P* value <.1). D, Alignment of the miR-205-5p seed sequence with the target sequences of *Plcb1*, *Tcf7l2*, *Nphp1* and *Cxxc4* (red); non-aligned sequences are coloured in blue

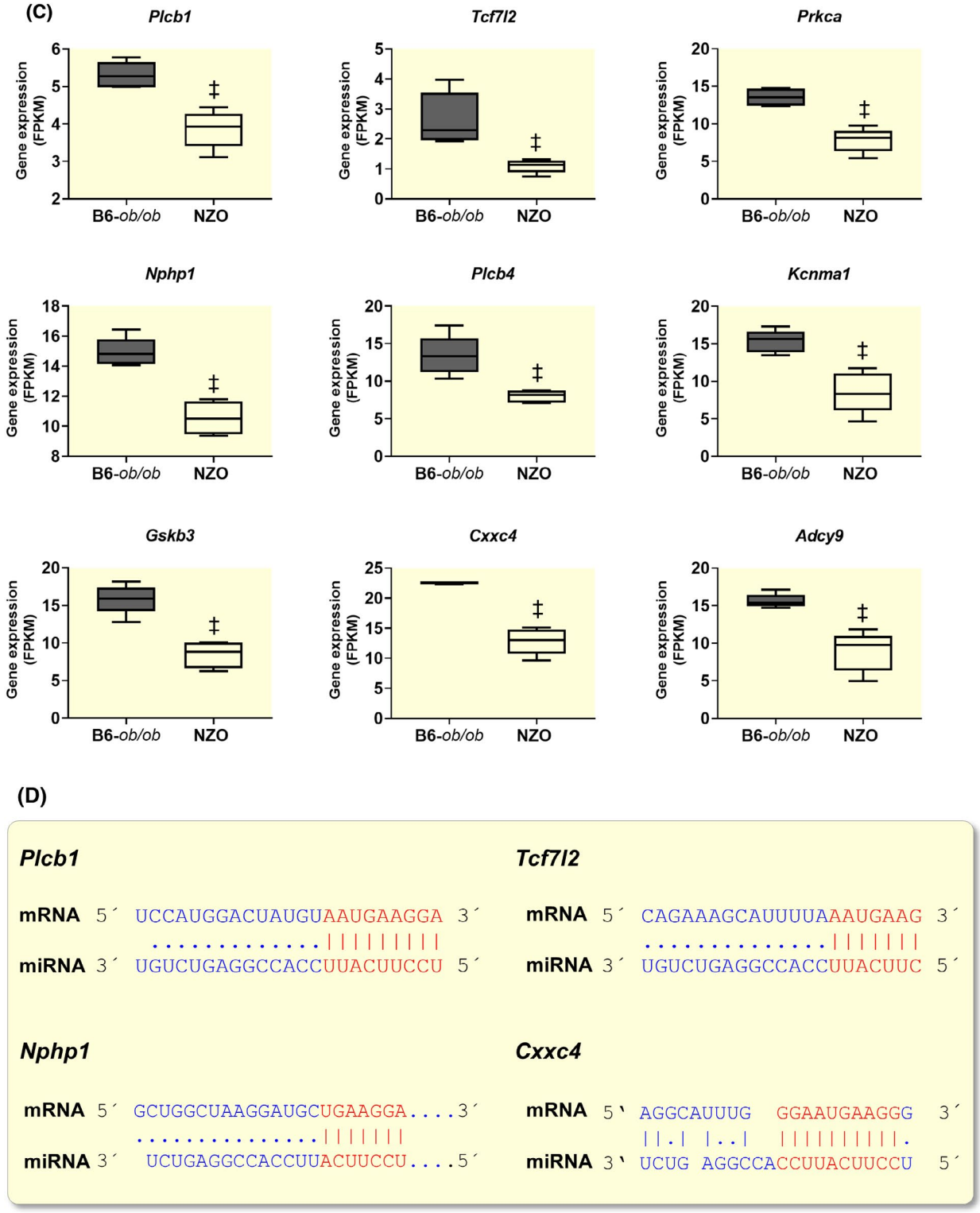


FIGURE 5 (Continued)

in INS-1 cells resulted in a higher expression of insulin and an elevated basal insulin secretion. In addition, miR-205-5p suppressed *Plcb1* and *Tcf7l2*, genes regulating beta-cell function and insulin secretion. Thus, this is the first study describing miR-205-5p to target the diabetes gene *TCF7L2*.

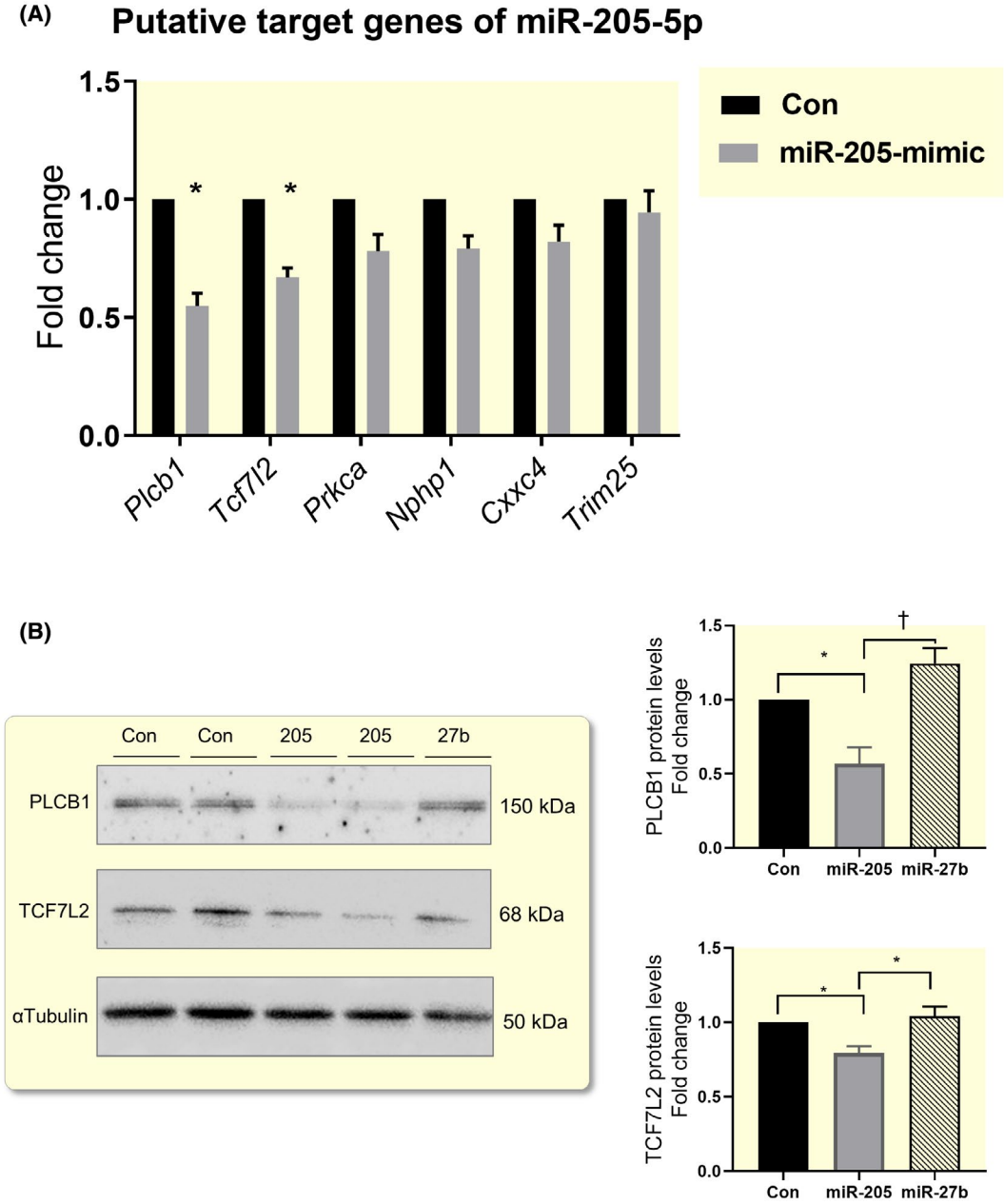


FIGURE 6 MiR-205-5p suppressed the expression of *Plcb1* and *Tcf7l2* in rat INS-1 cells. A, Rat INS-1 cells (832/13) were transfected with miR-205-mimic or non-targeting control (Con) and 72 h later, mRNA expression of indicated genes was assessed by qRT-PCR. Expression data were normalized to the average of negative control and represented as the fold change. *FDR <.05. B, TCF7L2 and PLCB1 protein levels were assessed by immunoblotting and quantified by ImageJ in INS-1 cells 72 h after transfection with miR-205 mimic (205), miR-27b (27b) or non-targeting control (Con). Data were obtained from at least three wells per condition from three independent experiments. Mean ± SEM, one-way ANOVA, *$P < .5$ and **$P < .01$

Islet dysfunction is one of the main aetiology in the development of T2D. However, the underlying molecular mechanisms are still incompletely defined. MiRNA-seq technology significantly enhanced our knowledge about miRNAs responsible for beta-cell failure and islet damage in T2D.[13,14] However, none of those previous studies utilized QTL data so far to identify genetic variants leading to altered miRNA expression. Here, the miR-QTL-Scan, a computational framework, previously described by our group,[7] was adapted to screen for novel miRNAs relevant for islet-cell function. In contrast to previous studies that focused on the most significantly altered miRNAs and/or miRNAs with the highest fold change,[4,30] the current work combined different information layers to select candidates.

Differentially expressed miRNAs were filtered for the criteria to be located in QTL for T2D, the degree of significance, fold change and the existence of a mature miRNA sequence conserved between mice and humans. The

TABLE 3 List of SNPs located in promoter and enhancer of *MIR205HG* and potentially enhance the binding of the listed transcription factors

Chr.	Genomic position	RS.ID	Reference	Mutation	Transcription factor	Score change
1	209 594 057	rs907740894	G	A	NKX6-1	0.86
1	209 594 954	rs932190015	A	G	ARNTL	1.48
1	209 595 047	rs1454335799	T	G	BARHL1	0.13
1	209 595 409	rs192617544	T	A, C, G	Arid3b	3.10
1	209 595 409	rs192617544	T	A, C, G	HHEX	0.98
1	209 595 546	rs958957512	T	C	THAP1	1.408
1	209 628 845	rs1015475485	C	T	LHX1	0.69
1	209 629 423	rs1558156886	A	G	TBP	0.62
1	209 630 162	rs1218275375	T	C	MNX1	2.88
1	209 630 162	rs1218275375	T	C	MNX1	0.21
1	209 630 162	rs1218275375	T	C	MNX1	0.16
1	209 630 589	rs757976599	G	C	THAP1	2.09

Note: The affinity of the listed transcription factors was altered by the corresponding SNPs by the indicated score change.

miR-205-5p fulfilled all these points and was also predicted to target genes related to Wnt signalling and insulin secretion. Hence, this is the first study reporting an altered miR-205-5p expression level in islets of Langerhans of diabetic mice so far. Computational approaches linked the high expression level of miR-205-5p to 4 and 7 genetic variants within the promoter and enhancer region of *Mir205hg*, respectively. Of note, SNPs rs222315757/rs1218275375 might enhance the binding of the transcription factor MNX1 within *cis*-regulatory sequences of both the mouse and human host gene *(Mir205hg/MIR205HG)*. MNX1 is described to be involved in pancreas development via orchestrating the beta- to delta-cell fate.[31] This enhanced interaction might be one mechanism among others that could explain the expression differences of miR-205-5p in islets of diabetes-prone and diabetes-resistant mice.

Although miR-205 was extensively discussed in cancer research,[32,33] the impact of this miRNA on beta-cell function and the development of T2D is limited. Langlet and colleagues investigated the miRNA expression pattern of FOXO-deficient mice and suggested miR-205-5p as an endogenous regulator of insulin sensitivity in the liver by targeting the insulin signalling cascade. The over-expression of miR-205-5p in primary hepatocytes increased the basal phosphorylation of Akt. These findings support the results of Cai and Peng[34,35] who demonstrated that miR-205-5p activates the PI3K/AKT axis by targeting two phosphatases, PTNE and SHIP2. In gain and loss function studies in mice, Langlet et al observed inconclusive results for the traits body weight and blood glucose.[36]

To identify the consequence of elevated miR-205-5p levels in islets, basal and glucose-stimulated insulin secretion was analysed in INS-1 cells. Over-expression of miR-205-5p resulted in a maximal insulin secretion at low glucose concentration (2.8mM), which might indicate a steeper dose-dependence on glucose compared with control cells. The insulin release induced by KCl was similar to that of control cells when data were normalized to residual insulin, demonstrating a well-functioning exocytosis machinery. As miR-205-5p expression does not increase insulin secretion at higher glucose levels, it can be speculated that these cells have a limited efficacy to stimulate insulin release at these conditions, presumably by a restricted glucose uptake or glucose metabolism.

As miRNA function is mediated through its effects on a specific set of target genes, we investigated all putative target genes of miR-205-5p that are involved in Wnt signalling and insulin secretion in vitro. As expected, not all predicted target genes, which showed a lower expression in islets of NZO animals, were also down-regulated upon miR-205-5p over-expression in INS-1 cells. Only *Tcf7l2* and *Plcb1* were significantly suppressed on both mRNA and protein levels. *Plcb1* was already described as a target of miR-205-5p in endometrial stromal cells.[23] We demonstrated that miR-205-5p binds to a 7-mer sequence within exon 10 of *TCF7L2*. Although the luciferase reporter assay experiment showed a moderate suppression, previous studies reported similar effect sizes for the miR-495/*TP53INP1* interaction.[13,37] One limitation of this experiment was the difficulty to test all the putative binding sites of miR-205-5p. Nevertheless, the most common binding site between all transcript variants and the most conserved between mouse and human was selected. In contrast to the majority of the previous studies, the identified binding site was rather in the coding sequence and not in the 3′-untranslated region (3′-UTR). Recently, the assumption that only the 3′-UTR is the principal recipients of miRNA activity was revised. Tay and colleagues described three miRNAs (miR-296, -470, -134) to target the transcription factors *Nanog*, *Oct4* and *Sox2* via binding to their amino acid coding region. In addition, it was demonstrated that

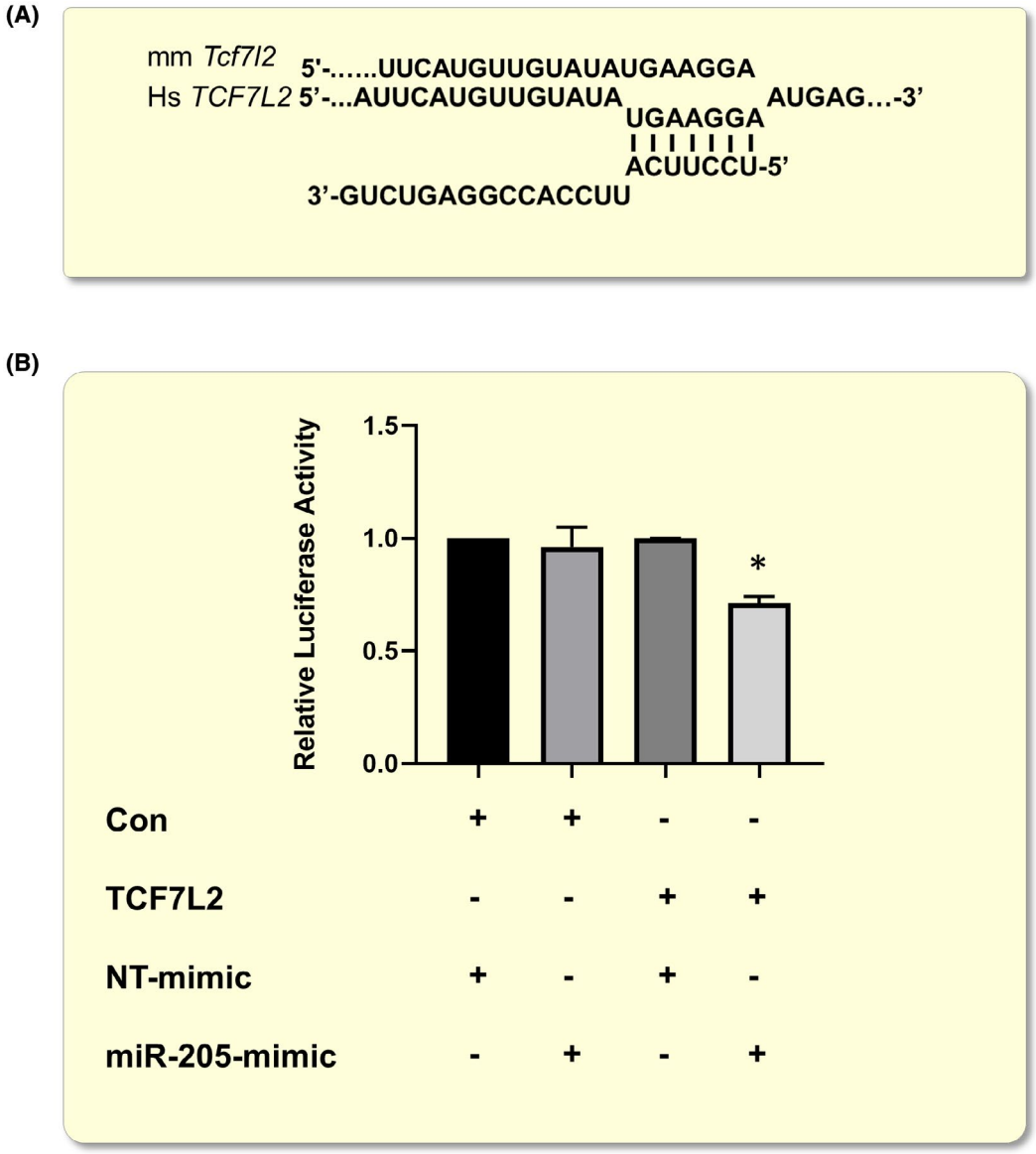


FIGURE 7 MiR-205 binds to exon 10 of the human TCF7L2 gene. A, Alignment of the miR-205-5p seed sequence with target sequence identified in exon 10 of the human *TCF7L2* gene. B, HeLa cells were co-transfected with miR-205-mimic (miR-205) or non-targeting mimic miRNA as negative control and a luciferase reporter gene vector containing a sequence of exon10 of TCF7L2 or with the empty luciferase vector (Con). Data were obtained from at least three wells per condition from three independent experiments. Bars represent means ± SEM, **P* < .05

Let-7 binds to the coding sequence of *Dicer* gene. Hausser et al used computational analysis based on different omics data and suggested that miRNAs may combine both mechanisms, targeting of coding sequence and 3′-UTR, to flexibly fine-tune the time and magnitude of their post-transcriptional regulatory effects.[38]

The verification whether the expression of a specific miRNA is genetically regulated in humans requires a sufficient number of islet donors with and without T2D. Therefore, expression levels of miR-205-5p in islets of humans were difficult to assess which represents the main limitation of our study. As miR-205-5p was not described yet in human islets, our results can be considered as the first indication of a novel player in islet function which might act via TCF7L2.

This transcription factor is a member of the *TCF/LEF* (transcription factor/lymphoid enhancer factor) family, with all of them binding to β-catenin after its nuclear translocation to initiate transcription of several target genes. Thereby, TCF7L2 activates the Wnt signalling pathway and plays a role in beta-cell proliferation upon incretin signalling by glucagon like peptide 1 (GLP1).[24]

Although it is well accepted that *TCF7L2* harbours common genetic variants with a strong association to T2D risk, results from mouse models of *Tcf7l2* are conflicting and T2D phenotype was not reproduced in all studies. One explanation for this conflicting data could be the complex regulation of this gene. In vitro down-regulation of *Tcf7l2* in mouse islets, MIN6 and INS-1 cells resulted in impaired

glucose-stimulated insulin secretion,[39] whereby the underlying mechanism is still unclear. Xavier et al suggested that *Tcf7l2* is important for insulin processing by regulating genes of the exocytotic machinery,[39] whereas Zhou et al proposed that TCF7L2 is involved in the processing of proinsulin to insulin.[40] Moreover, TCF7L2 was described in an in vitro study to be a regulator of key beta-cell genes.[40]

Plcb1 plays a role in calcium signalling and thereby in insulin secretion.[41] Mice lacking *Plcb1* in beta cells exhibited a marked defect in glucose-stimulated insulin secretion, leading to glucose intolerance.[42] Thus, both genes, *Tcf7l2* and *Plcb1*, are important for the insulin secretion process and might contribute to the different phenotypes of diabetes-prone and -resistant mice.

As genetically caused changes in miRNA expression are rarely discussed in human studies, we believe that our mouse data provide a list of novel candidates to investigate in the future.

4 | METHODS

4.1 | Animals, diets and experimental design

A detailed description of animals and diets was previously given.[15] In brief, at 5 weeks of age, male NZO/HIBomDife and B6.V-*Lep*$^{ob/ob}$/JBomTac (B6-*ob/ob*) were placed on a carbohydrate-free diet (−CH, Altromin C105789). At the age of 18 ± 1 weeks, the animals received a carbohydrate-containing diet (+CH, self-made with 40% kcal carbohydrates) for 48 hours and were then killed for islet isolation.[15] Animal studies were approved by the Ethics Committee of the State Ministry of Agriculture, Nutrition and Forestry (State of Brandenburg, Germany).

4.2 | Blood glucose and body weight measurements

Body weight and blood glucose were measured from 7 to 9 AM at week 13, 17 and 18 by using CONTOUR® blood glucose meter (Bayer, Leverkusen, Germany).

4.3 | RNA isolation of pancreatic islets and INS-1 cells

Islet isolation was performed as previously described.[15] Total RNA from mouse islets and INS-1 cells was extracted using the miRNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, followed by DNase treatment. All RNA samples selected for miRNA and RNA sequencing displayed RNA integrity number (RIN) ≥8 (Bioanalyser, Agilent Technologies, Germany).

4.4 | RNA sequencing

For each group (B6-*ob/ob* and NZO), five samples (each containing islets of one to two mice) were sequenced by GATC (Konstanz, Germany) on an Illumina HiSeq platform. Quality control and bioinformatic analysis were described previously.[43] Only transcripts with an absolute fold change >0.7 were considered for further analysis. KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was performed using the David data base tool 7,[44] with cut-off fold enrichment set to >1.7 and enriched *P* value <.05.

4.5 | MiRNA sequencing analysis

MiRNA library was conducted with the same RNA samples used for RNA sequencing (RNA-seq) and carried out by Illumina HiSeq platform. The "seqclean" program was used to remove polyA/Ts, Ns, low-complexity regions and sequencing adapters. Clustering was done with "cd-hit" for 100% similarity. Scanning for known miRNAs from miRbase 21 was performed by "SSearch." Differences in miRNA expression were calculated according to the EdgeR-package. The dispersion of the data was estimated via maximum likelihood. Normalization was performed using the TMM-method. *P* values were calculated via unpaired two-tailed Student's test with Welch's correction.

4.6 | Quantitative real-time RT-PCR in INS-1

MiRNA was reverse transcribed using the miScript II RT Kit (Qiagen, Germany) and analysed by RT-qPCR, using the miScript SYBR Green PCR Kit (Qiagen, Germany). To investigate the expression of miR-205-5p target genes, total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, Fitchburg, WI, USA) as described.[45] Gene expression was calculated according to the ΔΔCt method.[46] Primers used for this analysis are given on request.

4.7 | Western blot analysis

Western blotting was performed as described previously[4,47] using a 12% polyacrylamide gel loaded with 10 μg protein. Primary antibodies against TCF7L2 (1:1000, ab76151, Abcam, Cambridge, UK), PLCB1 (1:1000, ab182359, EPR19085, Abcam) and α-tubulin as loading control (1:1000, Sigma, St. Louis, USA) were used, followed by application of a secondary horseradish 10 peroxidase-conjugated anti-mouse antibody (1:20 000, Dianova, Hamburg, Germany) and goat peroxidase-conjugated anti-rabbit antibody (1:20 000, Dianova).

As there is no overlap of predicted targets of miR-205-5p and miR-27b-3p (MSY0000798, Qiagen), protein lysates from cells transfected with miR-27b-3p mimic were included as a negative control.

4.8 | Over-expression of miR-205-5p in INS-1 cells

To over-express miR-205-5p, INS-1 (832/13) cells were seeded in 24-well plates and grown overnight to ~70% confluency. Cells were transfected with 33 nmol/L of mirscript miRNA mimics Rno-miR-205-5p (MSY0000878, Qiagen) or the corresponding non-targeting control (all star non-targeting control) using the lipofectamine transfection reagent (ThermoFisher Scientific: 11668027) according to the manufacturer's protocol. RNA for expression analysis was collected 72 hours after transfection. The down-regulation of miR-205-5p was achieved by using custom designed 2′-O-methyl miRNA inhibitors (Integrated DNA Technologies, Coralville, USA). By using lipofectamine reagent, cells were transfected either with 100 nmol/L of miR-205-5p inhibitor or 2′-O-methyl non-targeting control (Integrated DNA Technologies, Coralville, USA) in INS-1 cells. After 48 hours, cells were harvested for RNA isolation and RT-qPCR analysis.

4.9 | Luciferase assay in HeLa cells

Luciferase reporter assay was performed using LightSwitch luciferase assay kit (Switchgear Genomics, Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. LightSwitch-3UTR luciferase reporter plasmid containing a 247 bp sequence of the human exon10-*TCF7L2* was generated by Active Motive (Carlsbad, CA, USA). The empty vector was used as control vector (Con) as described previously by Kameswaran et al[13] HeLa cells were used because they show a higher transfections efficiency than INS-1 cells. HeLa cells were seeded in 96-well plates and grown overnight to ~70% confluency. Cells were transfected with either exon10-*TCF7L2* or Con plasmids together with hsa-miR-205 mimic or non-targeting miRNA mimic (LightSwitch, miRNA mimics), using transfection reagent (Viromer Red, Lipocalyx, Germany). Each condition (exon10-TCF7L2 + miR-205-mimic; exon10-TCF7L2 + non-targeting miRNA mimic; Con + miR-205-mimic; Con + non targeting miRNA mimic) was transfected in four replicate wells and repeated in three independent experiments.

4.10 | Glucose-stimulated insulin secretion of INS-1 cells

INS-1 cells were seeded in 96-well plates and 24 hours later transfected either with miR-205-5p mimic (MSY0000878, Qiagen) or Con (All Star non-targeting control, 1027281, Qiagen). 72 hours after transfection, insulin secretion was measured under low (1, 2.8 mM) or high glucose (20 mM) concentrations and after stimulation with KCl (40 mM). In the end of incubation with glucose or KCl, the cells were collected in a special lysis buffer (10 mM Tris-HCl, 1 mM EDTA and 1% Triton X-100) and next used for residual insulin measurments and DNA content. Secreted insulin levels were normalized to either residual insulin or total genomic DNA content as described.[4]

4.11 | In silico analysis

For the identification of putative TFBS within *Mir205hg/MIR205HG cis*-regulatory elements (enhancer and promoter regions), the JASPAR database[48] was used to download position weight matrices (PWM). Binding site prediction and score calculations were performed by using TFBSTools.[49]

4.12 | Statistical analyses

All statistical tests were conducted using R version 3.5.0 (2018-04-23). For animal experiments, Student's *t*-test with Welch's correction was performed for the comparison of two groups. RNA-seq analysis was analysed by Student's *t*-test with Welch's and Benjamini–Hochberg corrections. MiRNome analysis was done with the edgeR R-package.[50] *P* values were calculated via a likelihood ratio test. One-way ANOVA was included for in vitro experiments (Figure 4B and C, Figure 6) (see Figure legend for detailed informations). Significance levels were set for *P* values <.05 (*), <.01 (**) and <.001 (***).

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

Meriem Ouni and Annette Schürmann performed study conception and design. Meriem Ouni, Pascal Gottmann, Efraim Westholm, Kristin Schwerbel and Mandy Stadion performed data acquisition. Meriem Ouni, Pascal Gottmann and Markus Jähnert performed data analysis. Heike Vogel and Kilian Rittig provided expertise on study design and data interpretation. Meriem Ouni, Pascal Gottmann, Heike Vogel

and Annette Schürmann prepared the manuscript. Annette Schürmann has primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All materials used in the manuscript are detailed in the methods part. All datasets on which conclusions of the paper rely will be publicly available repositories upon acceptance.

ORCID

Meriem Ouni https://orcid.org/0000-0002-7781-8463
Efraim Westholm https://orcid.org/0000-0002-2383-8549
Heike Vogel https://orcid.org/0000-0002-5557-1009

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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