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miR-7 Modulates hESC Differentiation into Insulin-Producing Beta-like Cells and Contributes to Cell Maturation

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Human pluripotent stem cells retain the extraordinary capacity to differentiate into pancreatic beta cells. For this particular lineage, more effort is still required to stress the importance of developing an efficient, reproducible, easy, and cost-effective differentiation protocol to obtain more mature, homogeneous, and functional insulin-secreting cells. In addition, microRNAs (miRNAs) have emerged as a class of small non-coding RNAs that regulate many cellular processes, including pancreatic differentiation. Some miRNAs are known to be preferentially expressed in islets. Of note, miR-375 and miR-7 are two of the most abundant pancreatic miRNAs, and they are necessary for proper pancreatic islet development. Here we provide new insight into specific miRNAs involved in pancreatic differentiation. We found that miR-7 is differentially expressed during the differentiation of human embryonic stem cells (hESCs) into a beta cell-like phenotype and that its modulation plays an important role in generating mature pancreatic beta cells. This strategy may be exploited to optimize the potential for *in vitro* differentiation of hESCs into insulin-producing beta-like cells for use in preclinical studies and future clinical applications as well as the prospective uses of miRNAs to improve this process.

INTRODUCTION

MicroRNAs (miRNAs) were identified in *Caenorhabditis elegans* as essential regulators of development,[1] and the first miRNAs described in animals were lin-4 and let-7.[2–6] To control the expression of protein-coding genes, miRNA genes are primarily transcribed by RNA polymerase II into long precursor molecules that are processed via RNase III enzymes Drosha and Dicer into mature miRNAs (~22 nt).[7,8]

These small non-coding RNAs are critical for translational regulation within the cell, and they play a key role in regulating several cellular processes, including differentiation, proliferation, and signal transduction.[9–11] This type of regulation occurs through base pairing of miRNAs to target sites in the 3′ UTR of mammalian protein-coding genes; thus, miRNAs exert control as central regulators of development.[12–14] In embryonic stem cells (ESCs), miRNAs play a role in maintaining pluripotency and proliferation, as well as differentiation and cell fate determination.[15–18]

During pancreatic islet development, many gene expression changes related to efficient differentiation and function of the pancreas occur.[19,20] Although the molecular mechanisms underlying pancreatic development remain unclear, recent discoveries related to miRNA-dependent post-transcriptional gene regulation have opened a new area of research, such that miRNAs are very likely to have regulatory roles in the differentiation, maturation, and physiology of pancreatic islet cells.[21,22]

Proper pancreatic islet development is controlled not only by key transcription factors and specific signaling pathways but also by miRNAs, as evidenced by the generation of pancreas-specific Dicer1-knockout mice.[23] A few miRNAs are preferentially expressed in specific tissue, and, as such, some miRNAs were found to be preferentially expressed in islets, with miR-375 and miR-7 being the most abundant endocrine miRNAs in rat and human islets.[21–25] Several miRNAs are highly expressed during human pancreatic islet development, and they are known to play a functional role in pancreatic beta cell development and function: miR-15a induces insulin biosynthesis by inhibiting UCP-2 gene expression;[26] miR-30d has been described as a glucose-dependent regulator of insulin transcription;[27] miR-124a is a key regulator of beta cell physiology through Foxa2 and preproinsulin gene expression;[28] miR-9 is a key factor in modulating Sirt1 expression and, thus, in regulating exocytosis and insulin secretion;[29] miR-373 overexpression promotes human ESC (hESC) differentiation toward the mesendodermal lineage;[30] miR-24, miR-26,

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miR-182, and miR-148 are regulators of insulin transcription in cultured islet or beta cells;[31] miR-375 is required for normal glucose homeostasis and, thus, is implicated not only in pancreatic islet development but also in mature islet function;[21,22,32,33] and miR-7 is the most abundant endocrine miRNA and is expressed at high levels during human pancreatic islet development,[21,22,25] and inhibition of miR-7 results in decreased beta cell numbers and glucose intolerance in the developing pancreas.[34] Previous studies have shown that the overexpression of miR-375 promotes pancreatic endocrine differentiation of ESCs and provides evidence that constitutive miR-375 overexpression in hESCs leads to the expression of beta cell markers, as well as insulin release in response to glucose in islet-like clusters.[35] Furthermore, the expression of miR-7 in human fetal pancreas increases at weeks 14–18, coinciding with the induction of PDX-1 and other key genes required for endocrine cell fate specification,[21] and these data suggest that a novel mechanism controls endocrine cell differentiation.

Pancreatic beta cell specification depends on a succession of signaling and transcription factor-activating events that are coordinated in a spatial and temporal manner during pancreatic development. In this study, we induce *in vitro* pancreatic differentiation of hESCs through a multistep protocol by adding growth factors and/or chemical compounds that activate specific signaling pathways and induce the expression of transcription factors at the suitable stage of differentiation. However, because several reports implicated miRNAs in pancreatic differentiation, we examined the expression profiles of miRNAs in hESCs and the resulting differentiated cells, as well as the involvement of miR-7 in the different steps of the differentiation process.

RESULTS

miRNA Signature in hESC Samples during Differentiation

To understand the molecular basis of pancreatic induction, we performed microarray assay to further depict the molecular changes in HS181 hESCs during multi-staged pancreatic commitment. This array analysis aimed to evaluate miRNA expression during hESC differentiation into insulin-producing beta-like cells[36] and in human adult islets and pancreas. A total of 2,689 probes was used and 850 miRNAs were detected.

To characterize the expression of miRNAs possibly implicated in pancreatic development and to determine whether gene expression profiles differ among cells, we compared miRNA expression among undifferentiated (UN)-, differentiated (DD)-, and spontaneous differentiated (SD)-hESCs after culturing for 22 days (Figure 1A). At first, hierarchical clustering was used to display the differentially expressed miRNAs (rows) and differentiated states of hESCs (columns) using half-square Euclidean distance as a similarity measure. Based on the hierarchical clustering of our expression data, our results revealed distinct clusters with a diverse expression profile. We could also detect genes that were most dynamically changed in their expression patterns during sequential differentiation into insulin-producing beta-like cells. When hESCs committed to insulin-producing beta-like cells (DD-hESCs), miRNAs related to pancreatic development, such as miR-375 and miR-7, one of the most abundant miRNAs in the pancreas, were highly upregulated, whereas miRNAs related to pluripotency, such as the miR-302 family, one of the key maternal materials essential for maintenance and renewal of hESC pluripotency, were downregulated (Figure 1A).

To determine whether there is any link between human adult pancreatic lineage and our hESC pancreatic lineage differentiation, we compared our microarray data with miRNAs preferentially expressed in human pancreas using isolated human islets of Langerhans and DD-hESCs to identify distinct and/or similar miRNAs (Figure 1B). Probes were separated into seven clusters by hierarchical clustering analysis. Assuming that miRNA abundance is an indicator of their biological function, our results suggest that the preferential expression of certain miRNAs in hESCs indicates a potential role in self-renewal and maintenance of pluripotency (clusters 5 and 6), whereas the preferential expression of other miRNAs in islets possibly indicates involvement in pancreatic islet development and function (cluster 1). The clustering result showed that pancreas and islets shared a slight similarity in their expression profiles with hESC-derived insulin-producing cells (clusters 2, 3, 4, and 7), suggesting acquired properties similar to pancreatic polypeptide (PP) cells.

To determine whether miRNAs might contribute to the switch in gene expression programs, we compared the level of these small non-coding RNAs in DD-hESCs and SD-hESCs: the level scores were converted into fold changes with log2 base. We detected 850 miRNAs and found that 61 of these miRNAs displayed expression changes larger than 0.58-fold ($p < 0.05$) during the differentiation of hESCs into insulin-producing beta-like cells. Of these, 19 were upregulated and 42 were downregulated in the DD-hESC group (Figure 1C; Table S1).

We found that several downregulated miRNAs belonged to the ESC-specific cell cycle-regulating (ESCC) family of miRNAs (i.e., miR-302 family), which is essential for hESC maintenance and renewal. In contrast, upregulation of a characteristic set of miRNAs constitutes a small group of miRNAs and is, therefore, ideally suited to coordinate modifications in gene expression programs that occur during hESC differentiation into insulin-producing beta-like cells. Based on these observations, we selected miRNAs belonging to this group as well as a group of individual miRNAs showing dramatic changes between the pancreas and islets for further analysis.

Identification of miRNAs Enriched in the Pancreas

We used microarray expression results from a subset of miRNAs to identify those that were exclusively expressed and most abundant in pancreatic tissue and islets (Figure 2A). We identified several miRNAs preferentially expressed in human pancreatic islets, and we generated miRNA expression profiles of the pancreas. miRNA profiling from pancreatic samples identified the most abundant miRNAs, indicating that these miRNAs play a significant role in pancreatic development and function.

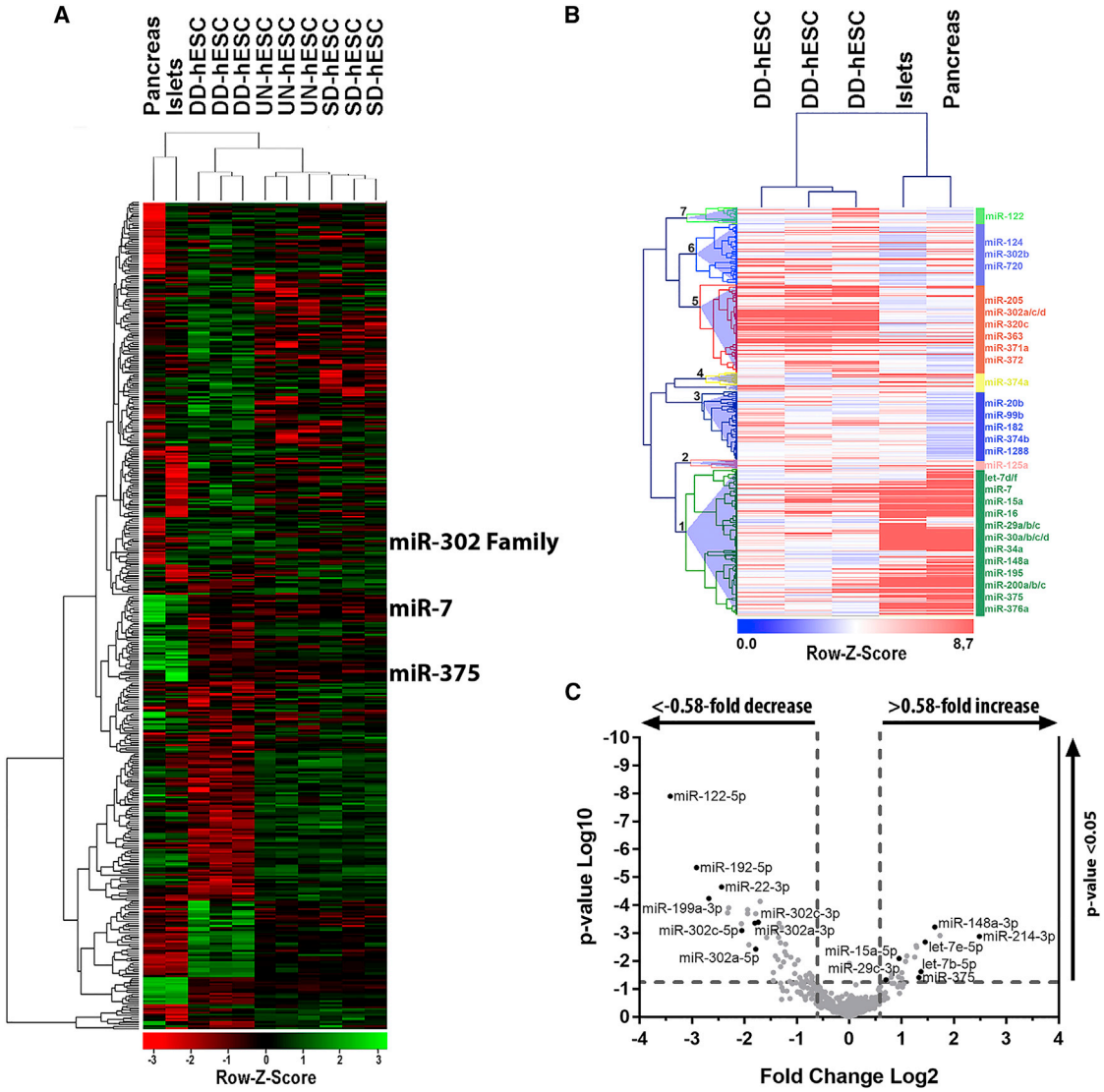

Figure 1. Microarray Analysis of hESC Differentiation into Insulin-Producing Beta-like Cells

(A) Heatmap summarizing patterns of expression for miRNAs loci that were differentially expressed in all samples of HS181 and adult human pancreas (n = 2–3 per group). Scaled expression value, denoted as the row *Z* score, is plotted in a red-green color scale with green indicating increased expression and red indicating decreased expression. (B) Heatmap comparing differentially expressed miRNAs among DD-hESCs, adult human pancreas, and pancreatic islets (n = 2–3 per group). Scaled expression value, denoted as the row *Z* score, is plotted in a red-blue color scale with red indicating increased expression and blue indicating decreased expression. Hierarchical clustering of miRNAs (rows) and samples (columns) is based on their expression profiles using the open-source data analysis Multiple Experiment Viewer (MeV) software package (average linkage and Euclidean distance as similarity measure). Expression data are shown for miRNAs with log2(fold change) differences of expression >0.58 ($p < 0.05$). (C) Scatterplot shows differentially expressed miRNAs log2(fold change) between DD- and SD-hESCs ($p < 0.05$). Selected miRNAs up- and downregulated in HS181 are highlighted (n = 2–3 per group).

A total of 29 miRNAs were found to be expressed in the primary screening (e.g., miR-148a, miR-7, miR-375, miR-16, miR-26a, miR-30d, miR-15b, miR-376a, and miR-195) and 21 miRNAs were found to be downregulated (e.g., miR-29a/b/c, miR-15a, and miR-200a/c). We investigated all miRNAs that were upregulated and downregulated in pancreatic islets by qRT-PCR analysis in order to confirm the differentially and commonly expressed miRNAs in the pancreas (Figure 2B). We confirmed high expression levels of miRNAs that were previously known to be abundant in pancreatic tissue, such as miR-15a, miR-16, and the miR-29 family, and we identified miRNAs differentially expressed in islets; 3 miRNAs were highly expressed in islets with a fold change in expression of islets versus pancreas ranging from 1.6- to 3.8-fold (relative expression is indicated by the log2 value). Five miRNAs were considered equally expressed with a fold change ranging from 0.03- to 0.3-fold, and 7 miRNAs were less expressed in islets with a fold change ranging from 0.6- to 4.8-fold.

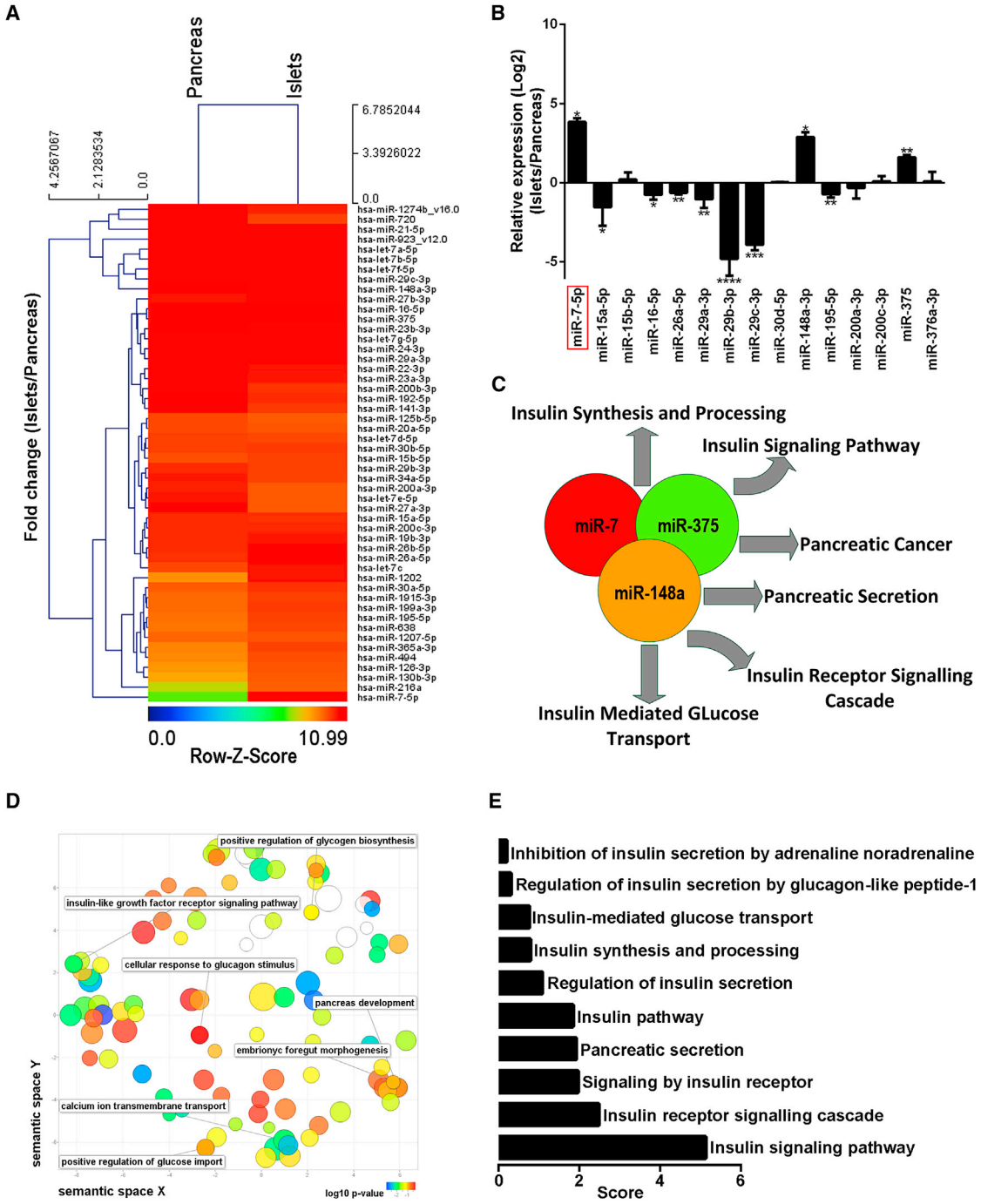

Figure 2. Differentially Expressed miRNAs in the Human Islets and Pancreas

(A) Heatmap summarizing differential expression of microarray data for the most highly expressed set of 50 miRNAs in adult human pancreas and pancreatic islets. Scaled expression value, denoted as the row *Z* score, is plotted in a rainbow color scale with red indicating increased expression and blue indicating decreased expression. Hierarchical clustering of miRNAs (rows) and samples (columns) is based on their expression profiles using the open-source data analysis Multiple Experiment Viewer (MeV) software package (average linkage and Euclidean distance as similarity measure). (B) qRT-PCR validation of differentially expressed miRNAs identified using Human miRNA Microarray Agilent 60-mer SurePrint technology in human adult pancreas and pancreatic islets. miRNA levels were normalized to 60S acidic ribosomal protein P0 (RPLP; endogenous control) and expressed in fold change (n = 3 per group). Values are shown (mean ± SD). Significant difference between human adult pancreas and pancreatic islets RNA levels was assessed by one-way ANOVA with Holm-Šídák test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$, and ****$p < 0.00001$. (C) miRSystem pathway analysis for upregulated miRNAs in pancreatic islets. Pathways targeted by our candidate miRNAs (miR-7, miR-148, and miR-375) were predicted using the web-based tool miRSystem,

(legend continued on next page)

Between miR-375 and miR-7, which are miRNAs preferentially expressed in beta cells and known as islet miRNAs, miR-7 was the most abundant (3.8-fold) in human pancreatic islets (Figure 2B).

Based on the data obtained from the qRT-PCR-based miRNA-profiling analysis, we determined 3 miRNAs were upregulated in islets, miR-7, miR-148a, and miR-375, and, using the miRSystem database, bioinformatics analysis with target genes of these miRNAs revealed a set of networks closely related to pancreatic development, as well as multiple signaling pathways participating in the regulation of insulin synthesis and secretion (Figure 2C; Figure S1). The Database for Annotation, Visualization and Integrated Discovery (DAVID) tools were used to identify potentially enriched biological processes (BPs) among the putative gene targets of the differentially expressed miRNAs using gene ontology (GO) terms, and to identify the related pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Accordingly, GO enrichment analysis revealed functional clusters significantly enriched for genes involved in pancreas development, transport, and secretory processes (Figure 2D). Besides, KEGG pathway analysis based on these 3 upregulated miRNAs identified 10 signaling pathways predicted to be mainly related to the insulin-signaling pathway (Figure 2E), which was largely consistent with the data from the GO analysis indicated above.

Characterization of Pancreas-Specific miRNA Expression during *In Vitro* Differentiation

Based on our microarray analysis of SD-hESCs, in comparison to DD-hESCs, we identified various upregulated miRNAs that may function as regulators of pancreatic differentiation. To define the expression pattern of miRNAs in our protocol for pancreatic differentiation, the expression profiles of pancreas-specific miRNAs from hESCs during differentiation were quantitated using qRT-PCR, and the expression level of each miRNA was indicated as fold change over UN-hESCs.

To confirm the sequential differentiation of cultured hESCs, identify potential novel miRNAs for pancreatic differentiation, and determine the expression of these miRNAs in hESCs, the expression of pancreas-specific miRNAs was analyzed by qRT-PCR at days 0, 5, 14, and 22, and these cells were then induced to differentiate into hESC-derived insulin-producing cells for 22 days. Differentially expressed miRNAs with a p value of 0.05 or lower and at least a 3-fold differential expression value were defined as differentially expressed between the two groups (Figure 3A). Consistent with our microarray data, qRT-PCR analysis indicated that, after hESCs became committed to definitive endoderm (DE) *in vitro*, 3 miRNAs (miR-7, miR-375, and miR-373) were progressively upregulated. Moreover, we found that these miRNAs were dramatically increased in the early stages of the protocol and coincided with factors that should induce DE and that these miRNAs then decreased during endocrine proliferation at day 14. These hESCs were directed to sequentially differentiate into PP cells through definitive and posterior foregut endoderm stages, and the expression of 3 miRNAs (miR-15a/b and miR-16a) appeared to be increased during endocrine induction. In the late stages, compared to that in SD-hESCs, the expression of 2 miRNAs (miR-29b and miR-148a) reached a peak in DD-hESCs following endocrine proliferation at day 22. Furthermore, our expression profiling analyses in hESC-derived lineage displayed similar expression profiles for human induced pluripotent stem cell (hiPSC)-derived insulin-producing cells (Figure S2). Taken together, these data suggest that these miRNAs are likely to be associated with the beta cell-like phenotype and to be functionally involved in pancreatic differentiation. According to the results obtained by qRT-PCR, miRNA fluorescence *in situ* hybridization (FISH) showed that miR-7 and miR-375 were expressed across a population of cells during the first steps of DE establishment (5 and 11 days) and that their expression decreased gradually in the next steps (11 and 22 days); however, miR-148a showed a different expression pattern with increasing expression levels preferentially in the last steps (11 and 22 days) (Figure 3B).

Modulation of Specific miRNAs in hESCs

miR-7 is a candidate miRNA selected for this study because it is highly expressed in the developing human pancreas and because its expression coincides with an exponential increase in pancreatic endocrine hormones. Assuming that the abundance of miRNAs is an indicator of their biological function, these findings indicated a potential role for miR-7 in endocrine cell differentiation and/or function. However, our DD protocol reported herein shows very high expression and then progressively decreased expression of miR-7 during differentiation of the pancreatic endocrine precursors, which supports the establishment and DE differentiation of hESCs. Here we investigated the effects of miR-7 modulation during differentiation of endocrine progenitors and insulin-expressing cells in DD-hESCs and their further maturation into glucose-responsive beta-like cells.

Therefore, we examined whether miR-7 is able to enhance pancreatic differentiation in hESCs. Using miRNA mimics (Dharmacon, miRIDIAN microRNA Mimics), HS181 cells were transiently transfected with a control mimic or miR-7 mimic and incubated for 24 hr in pancreatic differentiation medium (Figure 4A). To ensure effectiveness of the miR-7 mimic throughout the differentiation process, we verified its persistence after transfection by qRT-PCR from

which integrates several databases (KEGG, BIOCARTA, Pathway Interaction Database, Reactome, and GO molecular function) to predict gene targets and targeted pathways. (D) Data of 3 differentially upregulated miRNAs were processed using miRSystem (version 20160513) and DAVID online bioinformatics resource version 6.8. Gene ontology scatterplots were generated using the open-source online tool REViGO. Bubble color indicates the p value of gene ontology terms (expressed as log10 p value), where blue and green bubbles are gene ontology terms with more significant p values than the orange and red bubbles. Bubble size indicates the frequency of the gene ontology term in the underlying gene ontology database. (E) The predicted KEGG signal pathways to be regulated by the differentially expressed miR-7, miR-148a, and miR-375. The ranking score was obtained by summation of the weight of its miRNA times its enrichment −log (p value) from the predicted target genes. The x axis represents score and the y axis represents the top 10 enriched KEGG pathways.

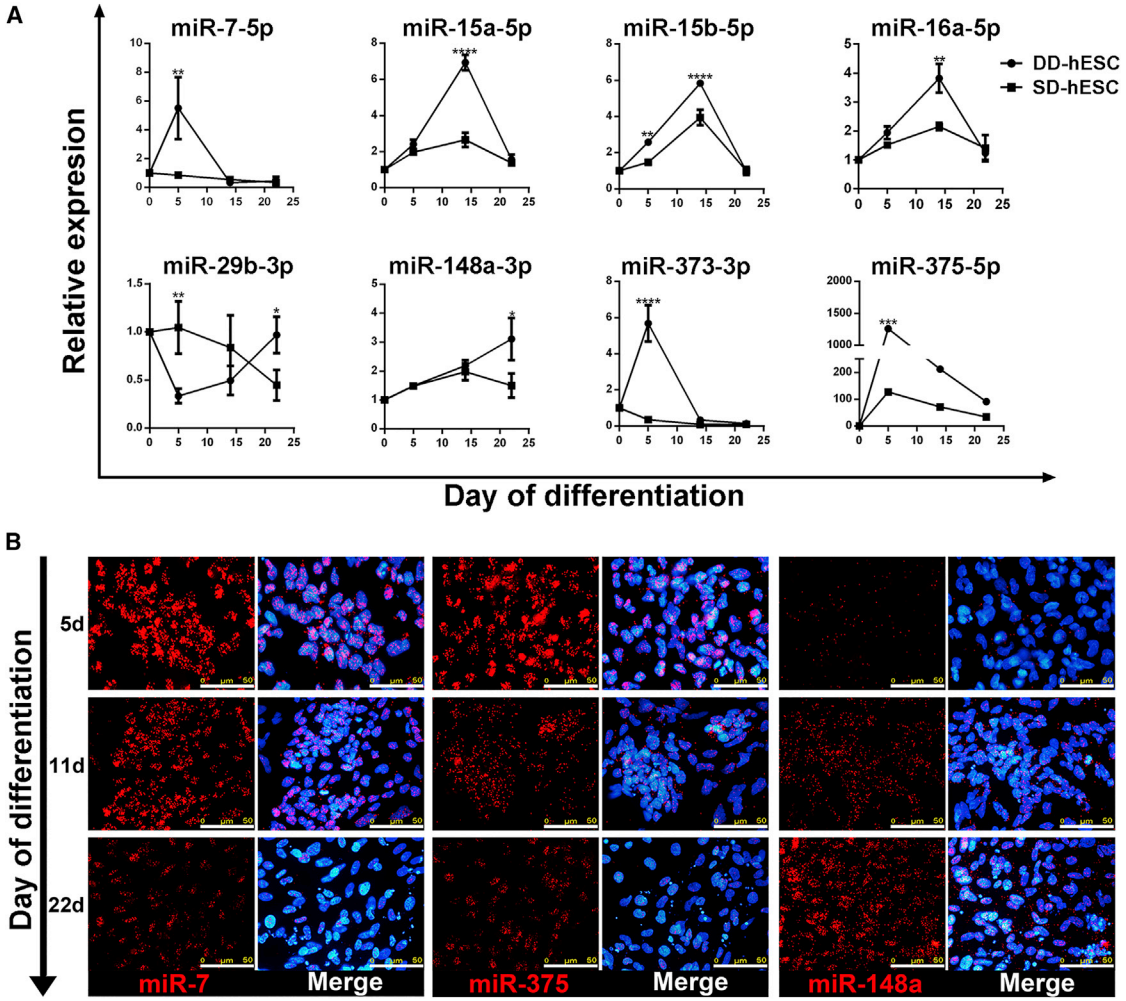

Figure 3. Differences in miRNA Expression during Differentiation of hESCs

(A) Verification of expression of representative miRNAs by qRT-PCR analysis. RNA was extracted from hESC-derived insulin-producing cells at 0, 5, 14, and 22 days after various treatments (n = 3–6 per group). miRNA levels were normalized to U48 and expressed in fold change (n = 4–6 per group). Values are shown (mean ± SD). Significant difference in HS181 miRNA levels between groups was assessed by one-way ANOVA with Holm-Šídák test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (B) FISH was used to visualize the expression of a subset of critical miRNAs at defined stages of differentiation at days 5, 11, and 22: miR-7, miR-375, and miR-148 (red). Nuclei were counterstained with Hoechst (blue).

isolated RNA of transfected and control hESCs. The results confirmed the significant expression levels of miR-7 in transfected hESCs compared to those in control hESCs. The fold change in the expression of miR-7 in hESCs transfected for 24 hr was 702-fold, which was dramatically higher than that of the control group (Figure 4B). miRNA expression levels were stable after at least 4 days, and, thus, we hypothesized that the transfected miRNA mimic was valid to evaluate its effect in hESC differentiation.

We next wanted to determine how the miR-7 level increases might affect pancreatic gene expression in differentiating hESCs. The optimal concentration of mimic will vary depending on the potency of the mimic used and the efficiency of mimic delivery into the cells. To determine the optimal concentration that coincides with the beginning symptoms of adverse effects that have a negative impact on the phenotype, the expression level of miR-7 was examined after transfection with several concentrations of miR-7 mimic ranging from 20 to 200 nM (Figure 4D). Analysis of the relationship between miRNA expression levels and miRNA mimic transfection suggested that overexpression depends on a threshold miRNA mimic concentration. On one hand, the amount of miRNA mimic introduced into cultured cells can be controlled simply by adjusting the transfection concentration; on the other hand, the miRNA mimic concentration should be sufficient to achieve specific changes in pancreatic gene expression. Thus, within our multi-step differentiation protocol (DD-hESCs), cells were transfected with an miRNA mimic, and then qRT-PCR was used to determine pancreatic gene changes on day 22. Results showed that the minimum concentration significantly

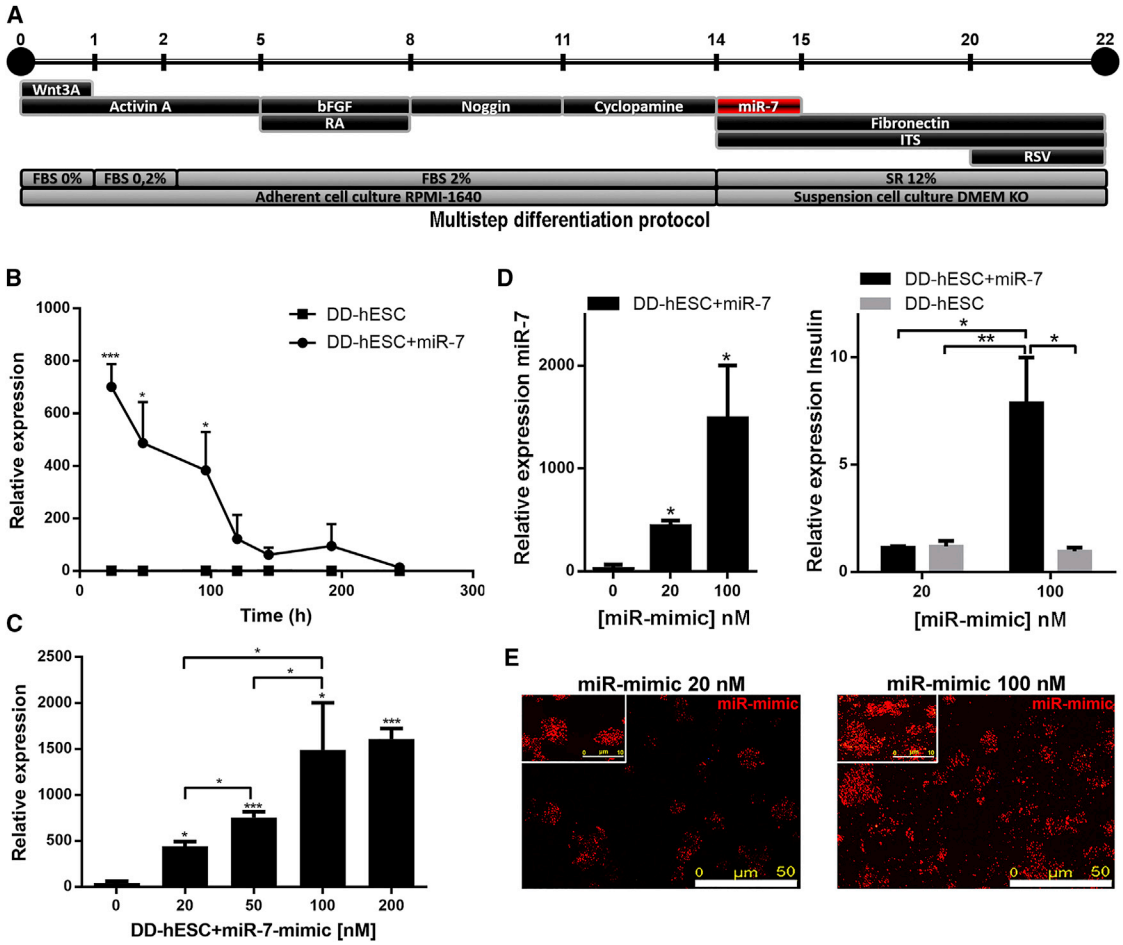

Figure 4. Integration of miR-7 in the hESC Differentiation Process

(A) Schematic representation of the steps involved in hESC differentiation toward a beta cell fate and the factors involved in this process. (B) Expression time course of high-stability miR-7 expression after a transient transfection with miRNA miR-7 mimic. Concentration of measured miR-7 by qRT-PCR in hESCs tends to gradually decline over time, but it occurs with a statistical significance only after day 4. (C) Bar graph of the dose of miRNA transfection ranging from 0 to 200 nM. (D) Effects of upregulated miR-7 on insulin gene expression in hESC-derived insulin-producing beta-like cells, as determined by qRT-PCR. In each case experiments, n = 6. Values are shown (mean ± SD). Significant difference in HS181 RNA levels between groups was assessed by one-way ANOVA with Holm-Šídák test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (E) Successful transfection was observed by fluorescence microscopy, and representative images shown are of hESCs 48 hr after transfection with Pierce Dy547-labeled miRIDIAN Mimic Transfection Controls.

associated with the upregulation of miR-7 was 20 nM, but we observed evidence of saturation at 200 nM, the highest concentration tested (Figure 4C). Interestingly, we noted significant upregulation of late markers of pancreatic endocrine differentiation, such as insulin, relative to optimal concentration of mimic from 100 nM (Figure 4D). Therefore, we decided to focus on 100 nM miR-7 mimic, which is required for the activation of beta cell genes and for DE markers, suggesting that miR-7 may be an important factor in differentiating hESCs. These results suggest that use of the miR-7 mimic is feasible and suitable for further study at 100 nM (Figure 4E).

miR-7 Induces Pancreatic Cell Differentiation in hESCs

To detect alterations in expression of pancreatic-related genes after miR-7 transfection, hESCs were prepared as described above and stimulated to differentiate for 22 days (Figure 4A). To determine the functional role of miR-7 in the endocrine lineage and verify whether miR-7 level increases by transfection could induce differentiation into pancreatic endocrine cells, hESCs were transfected for 24 hr with control mimic, miR-7 mimic, miR-7 inhibitor (anti-miRs (amiR-7), or transfection reagent alone (mock) in the last stage of the protocol and cultured in suspension with pancreatic differentiation medium for 7 days.

To examine the effect of miR-7 for the last stage of hESC differentiation *in vitro*, we tested the cell population for expression of islet hormones and key transcription factors. We observed that the expression of a pancreatic beta cell marker such as insulin was markedly increased 6.5-fold in hESCs transfected with a high concentration

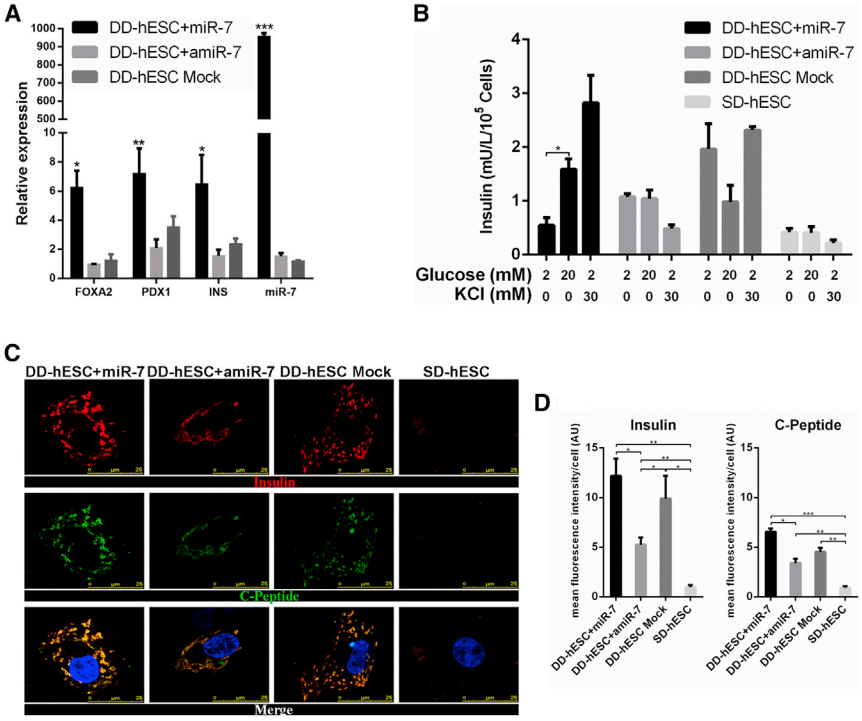

Figure 5. Influence of miR-7 on Insulin Secretion in Response to Glucose Challenge in hESC-Derived Insulin-Producing Beta-like Cells

(A) hESC-derived insulin-producing beta-like cells were transiently transfected with miRNA mimic with homologous sequence to miR-7 or a control. The cells were harvested, and the expression profiles of the FOXA2, PDX1, and insulin during pancreatic differentiation induction were analyzed by qRT-PCR using RPLP expression as a loading control. Mock denotes transfection reagent. miR-7 mimic was transfected using DharmaFECT, and endogenous miR-7 and mimics were quantified also by qRT-PCR using RNU48 expression as a loading control (n = 3 per group). (B) ELISA measurements of insulin secretion at various glucose concentrations from SD-hESC, DD-hESC + miR-7, DD-hESC + amiR-7, or DD-hESC + mock. Cultures were exposed to Krebs solution for 1 hr containing either 2 or 20 mM glucose. After incubation with low or high glucose, cells were depolarized with 30 mM KCl, as indicated. Cell medium from each group was harvested after incubation, and insulin concentration was measured using a Mercodia ELISA kit (n = 3 per group). (C) Representative images from confocal immunofluorescence of cells stained for insulin (red), C-peptide (green), and nuclei (Hoechst, blue) in SD-hESCs, DD-hESC + miR-7, DD-hESC + amiR-7, or DD-hESC + mock. (D) Graphs show the quantification by MetaMorph analysis of average intensity of insulin- and C-peptide-positive cells. Values are shown (mean ± SD). Statistical difference was assessed by one-way ANOVA with Holm-Šídák test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

of miR-7 mimic (50–100 nM), when miR-7 is dramatically upregulated 1,490-fold. However, if levels of miR-7 were reduced using low concentrations of miR-7 mimic (<20 nM), no significant change in insulin expression level was found in the transfected group, even after 22 days (Figure 4D). To assess the developmental changes in the insulin-producing beta-like cells resulting from miR-7 modulation, we evaluated the expression of various genes by qRT-PCR. Importantly, islet cell-specific marker genes in miR-7 mimic-treated cells were upregulated, including PDX1 and insulin, which were respectively increased 7.2- and 6.5-fold compared to their expression in differentiated hESCs without miR-7 mimic ($p < 0.05$); moreover, compared to non-transfected (mock) or control mimic-transfected hESCs (control mimic), transfection with the miR-7 mimic increased the expression of pancreatic marker genes. Interestingly, FOXA2, a major upstream regulator of PDX1, which is highly expressed during DE differentiation of hESCs, was re-expressed 6.2-fold higher after miR-7 upregulation in the last stages of the protocol than with the control or mock treatment (Figure 5A).

To assess the hormone release capacity of hESC-derived insulin-producing beta-like cells, we next examined medium samples collected on day 22 for insulin levels. The potential for improved stimulus-coupled hormone secretion in hESCs was examined by testing day 22-differentiated cells using a sequential secretion assay including low and high glucose levels and potassium chloride. Differentiated cells were examined for their insulin secretion potential (Figure 5B). The increase in insulin production by hESC-derived insulin-producing beta-like cells transfected with miR-7 (DD-hESC + miR7) that occurred when glucose was added to the medium at a concentration of 20 mM was significantly greater than that observed at a lower glucose concentration of 2 mM (1.6 mU/L/10^5 versus 0.5 mU/L/10^5, respectively). Furthermore, mock-transfected cells exhibited an increase in insulin secretion, but no significant difference in the insulin concentration was observed between the low and high glucose conditions (1.9 mU/L/10^5 and 0.9 mU/L/10^5, respectively). However, overall, hESC-derived insulin-producing beta-like cells either with or without miR-7 upregulation responded to depolarization with KCl (2.8 mU/L/10^5 and 2.3 mU/L/10^5, respectively). In contrast, SD-hESCs secreted a very low amount of insulin in response to low and high glucose challenges (0.4 mU/L/10^5 in either condition). In addition, under the same conditions, the inhibition of miR-7 (DD-hESC + amiR-7) partially inhibited insulin secretion and blocked glucose-dependent insulin secretion and depolarization with KCl (1.1 mU/L/10^5, 1.03 mU/L/10^5, and 0.5 mU/L/10^5, respectively) (Figure 5B).

We performed confocal double immunofluorescence of hESC-derived beta cell-like cells with miR-7 increased (DD-hESC + miR-7), miR-7 inhibited (DD-hESC + amiR-7), transfection reagent alone (DD-hESC + mock), and SD-hESCs. Colocalization of insulin and C-peptide was observed in only hESC-derived insulin-producing beta-like cells (Figure 5C). However, MetaMorph-based quantitative

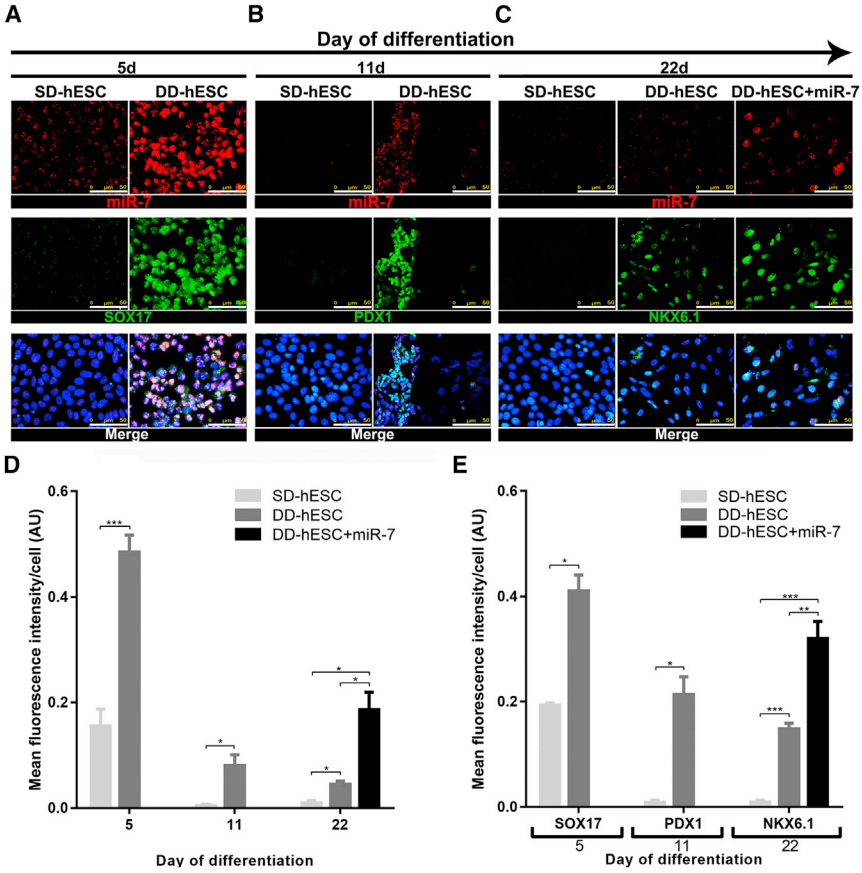

Figure 6. Effect of miR-7 Upregulation on Pancreatic Differentiation in ESCs

(A) Endoderm differentiation in hESCs at day 5: immunofluorescence of SOX17 (green), FISH of miR-7 (red), and counterstain of nuclei (Hoechst, blue). (B) At 11 days, DD-hESCs showed colocalization of PDX1 protein and miR-7, counterstained with Hoechst (blue). (C) At 22 days, NKX6.1 (green) and miR-7 (red) were expressed in the hESC-derived insulin-producing beta-like cells and were counterstained with Hoechst (blue). (D) Graph shows the quantification by MetaMorph analysis of average intensity of miR-7 expression. (E) Graph shows the quantification by MetaMorph analysis of SOX17-, PDX1-, and NKX6.1-positive cells.

analysis confirmed that miR-7 inhibition resulted in significantly fewer insulin- and C-peptide-positive cells than that of the other treatments (Figure 5D). These results correspond with the observation that DD-hESCs + amiR-7 secrete lower amounts of insulin than the other cells and are not glucose responsive, as shown in Figure 5B.

Finally, we tested whether miR-7 could be detected by FISH at days 5, 11, and 22 during differentiation protocol. On a morphological level, FISH revealed that miR-7 was expressed at substantially higher levels in the earlier stages of the protocol at day 5 in DD-hESCs than in SD-hESCs (Figure 6A), and we observed a gradual decrease in miR-7 expression from day 5 to day 22 during the differentiation protocol (Figures 6B and 6C). We evaluated miR-7 expression level after transfection, and we confirmed miR-7 upregulation in hESC-derived insulin-producing beta-like cells at day 22 by FISH.

To identify the nature of the cells expressing miR-7, we counterstained the FISH sections with antibodies specific for critical transcription factors involved in pancreas formation and beta cell differentiation in a time course study, and we examined three different time points during the pancreatic differentiation protocol: 5, 11, and 22 days. Ratio immunofluorescence levels were analyzed using MetaMorph software (Figures 6D and 6E), and we quantified the DE marker SOX17 as well as the miR-7 co-staining with nuclear marker Hoechst 33342 in SD-hESCs and DD-hESCs. There was a significant increase in the number of SOX17- and miR-7-expressing cells at day 5 (Figures 6D and 6E). We repeated this experiment at day 11 with PDX1, which plays an important role in the induction of insulin-producing cells and miR-7 expression; colocalization experiments showed that miR-7-positive cells (red, FISH with a locked nucleic acid locked nucleic acid [LNA] probe) were PDX1 positive, and the PDX1-negative cells (green, immunohistochemistry) were miR-7 negative, thus supporting a direct role for miR-7 in PDX1 expression (Figures 6B and 6E). In the last stages of the differentiation protocol at day 22, hESC-derived insulin-producing beta-like cells containing populations of Nkx6.1-positive and miR-7-positive cells could be generated, and, furthermore, we found that the modulation of miR-7 significantly upregulated Nkx6.1, an important transcription factor in pancreatic development (Figures 6C and 6E). Thus, these results highlight the ability of specific miRNAs, such as miR-7, to alter the expression of genes involved in the pancreatic specification in hESCs.

DISCUSSION

Currently, several studies have established different protocols to efficiently generate PP cells from hESCs. *In vitro* differentiation protocols are intended to mimic conditions in pancreatic development, and these methods require the addition of expensive growth factors and generated heterogeneous cell mixtures with low reproducibility. Our lab has established a new cell culture protocol that induces the differentiation of hESCs into a beta cell-like phenotype.[36] To advance research, it is essential to be able to control hESC differentiation to endoderm lineages with high efficiency and reproducibility and in an inexpensive manner. Moreover, understanding the function of pancreatic islet-specific miRNAs can help to define a universal protocol for pancreatic differentiation that can be applicable for several cell lines. In this study, we enumerate certain miRNAs, such as miR-7, miR-375, miR-148, and others, which might improve further

differentiation of the progenitor cells into the functional beta cells needed in diabetes cell therapy. miRNAs play critical roles in normal stem cell functions during developmental events,[37,38] and they are also important regulators of pancreatic function.[25] To further characterize these cells, RNA was extracted and used to perform microarray analysis to unveil the expression profile of miRNAs during differentiation.

In this study, profiling pancreatic miRNA expression led to the identification of a large fraction of differentially expressed miRNAs during hESC differentiation into beta-like cells, as previously reported by other groups.[39,40] In addition, our findings bring an overview of specific miRNAs acting on the maturation and functionality of insulin-producing cells. Analysis of the signal intensities revealed that the global miRNA expression patterns with the most considerable differences were detected in the pancreas and human islets, and the expression of several miRNAs was more similar between hESC-differentiated cells (DD-hESCs) and human islets. By there being a high degree of similarity in the overall analysis, these findings suggest that DD-hESCs may be more mature.[36] However, there are still differences between the expression profiles of adult pancreatic samples and DD-hESCs, suggesting that we probably obtained a neonatal cell population.[41,42]

In our study, miR-7 and miR-375 were found to be upregulated during differentiation into the beta cell-like phenotype. Interestingly, miR-7 has been reported to be predominantly expressed in the endocrine cells of the developing and adult pancreas in humans.[21,24] miR-375 has been reported to be exclusively expressed in pancreatic islets[23,24] and to play an important role in mature islet cell function.[43] A former report has indicated that miR-148a is a positive transcriptional regulator of insulin signaling.[31] Our results showed elevated expression of three islet-specific miRNAs (i.e., miR-7, miR-375, and miR-148a). Overall, miR-302 was found to be downregulated during hESC differentiation. Moreover, the downregulation of the miR-302 family in hESCs has been previously reported.[44] These miRNAs constitute a family highly expressed in ESCs, and their expression levels decline as cells begin to differentiate.[45] However, the sustained expression of some pluripotency-associated miRNA clusters in hESC-derived insulin-producing beta-like cells indicates that these miRNAs are not only necessary in the maintenance of pluripotency but also act as timing regulators of several differentiation processes.[46–48] Even the miR-302 family promotes early events of lineage commitment[49–51] and may participate in cell reprogramming and specialization.[47]

Previous reports suggest that miRNAs play essentials role in the developing pancreas.[31,52,53] Therefore, we examined different pancreatic tissues and identified several miRNAs, some of which are highly expressed in a tissue-specific manner. This study shows that some of the most abundant miRNAs in the endocrine pancreas are miR-375, miR-376, miR-200a/c, miR-214, miR-148, miR-29a/b/c, miR-195, miR-26a, miR-30d, miR-7, miR-15a/b, miR-16, miR-96, and miR-663, which is consistent with previous reports.[54] An interesting study of human islets showed that miR-7 is the most abundant endocrine miRNA in islets, while miR-375 is the most abundant intra-islet miRNA.[24] Our miRNA-profiling data also showed a higher level of miR-7 in human islets than in the exocrine pancreas. A recent study showed the role of miRNAs during pancreatic development by islet-specific miRNAs, miR-7, miR-9, miR-375, and miR-376.[25] miRNAs have been shown to be essential in the processes of growth and differentiation in pancreas organogenesis. miR-375 and miR-7 have been shown to play key roles in pancreatic beta cell development and function, and both miRNAs exhibit increased expression during pancreatic islet development in humans.[25] Microarray analysis revealed the dynamic expression of miRNAs during hESC differentiation, and several miRNAs are highly and specifically upregulated during DE formation. Our study showed that the expression levels of a characteristic set of miRNAs, miR-7, miR-375, and miR-373, peaked at day 5 and then began to decrease until the end of differentiation, which suggests that these miRNAs play a role that supports the highly efficient differentiation of hESCs to DE. Interestingly, a member of the miR-371-miR-373 cluster, which is part of a large family of miRNAs highly expressed in hESCs (termed ESCC miRNAs),[55,56] such as miR-373, was upregulated in DE, thus also implicating a potential role for this family in the differentiation of the endoderm lineage.

While these miRNAs seem to be essential for regulating the induction of events in DE, other miRNAs have been recognized as playing a role in regulating specific transcription factors involved in pancreatic differentiation. Among these miRNAs, miR-29a/b are highly expressed and enriched in islets, and the maintenance of their expression in beta cells may be implicated in regulating insulin secretion, in islet development,[57] and in the inhibition of miR-148-dependent downregulation of insulin mRNA in primary cultured islets.[31] Therefore, miR-29a/b and miR-148a could regulate insulin synthesis and its secretion. The miR-200 family is abundantly expressed in the mouse and human pancreas, and this family regulates the epithelial-to-mesenchymal transition and has been implicated in pancreatic carcinogenesis.[58,59] Our miRNA-profiling data showed high levels of miR-29b, miR-148a, and miR-200a in the later stages of differentiation.

It has been recently reported that the overexpression of miR-375 represents a new strategy to generate insulin-producing cells *in vitro*.[35,60] We found that miR-7, miR-375, and miR-373 were rapidly increased to establish the endoderm lineage and then decreased again as differentiation progressed toward a beta cell-like phenotype. Furthermore, it is important to note that miR-7 is present in the developing endoderm lineage and has been suggested as a critical regulator of insulin and adult beta cell proliferation.[61–63] Kredo-Russo et al.[64] demonstrated a potential role of miR-7 in inhibiting endocrine cell differentiation in pancreas explants and transgenic mice, which is mediated by Pax6 downregulation. The inhibition of miR-7 disrupts pancreatic differentiation during early developmental stages of mouse, and it seems to have a beta cell-specific effect causing an overall downregulation of insulin production;[34] besides, miR-7 inhibition could promote adult beta cell proliferation via mTOR signaling.[63] All together, the data suggest a specific effect of miR-7 on endocrine cell differentiation and function, and they demonstrate the dual regulatory

function of miR-7. Mature miR-7 is highly conserved among mammals, such as human, rat, and mouse, among which miR-7 shares the same seed sequence. This might result in a similar function in these species.

Gene expression analysis revealed that miR-7 is upregulated during pancreatic differentiation of hESCs, and, in the final stages of differentiation, miR-7 was overexpressed in hESCs. This system provides the opportunity to examine the miRNAs expressed in a cell population that are capable of inducing lineage-specific cell differentiation. It was observed that, with the increase in miR-7 expression, there was a significant increase in the expression of pancreatic markers, including FOXA2, PDX1, and insulin. Notably, miR-7 may have a positive effect on the stimulated release of insulin. Taken together, these data suggest that overexpression of miR-7 has dose-dependent effects on the expression of insulin and that high concentrations of miR-7 result in an increased insulin release during a glucose challenge, which suggests that miR-7 improves the maturation of hESC-derived insulin-producing beta-like cells to functionally respond to elevated glucose levels. In contrast, the inhibition of miR-7 is unfavorable for insulin secretion. We note that the levels of insulin secreted in response to glucose challenges in miR-7-inhibited cells *in vitro* was generally lower than that of cells expressing either basal or overexpressed levels of miR-7. Our findings indicate that the upregulation of miR-7 improves beta cell function, while the inhibition of miR-7 is deleterious for beta cell function and blocks glucose-induced insulin secretion. Therefore, while miR-7 is beneficial and critical for the generation and specification of DE and PP cells, late-stage increases in the expression of miR-7 are critical to regulate functional aspects of the beta cell phenotype.

In conclusion, our results suggest a pivotal role of miR-7, miR-375, and miR-373 in the establishment of the endoderm lineage as well as its terminal differentiation, and they provide compelling evidence to support our finding that miR-7 positively regulates markers for pancreatic differentiation and improves beta cell function. We are aware that much work remains to be done, but these findings may increase our understanding of the molecular mechanisms that control pancreatic development.

MATERIALS AND METHODS

Cell Culture

The hESC line HS181 was derived in the Fertility Unit of Karolinska University Hospital, Huddinge at the Karolinska Institute, after approval of a project entitled "Derivation and early differentiation and characterization of hESC lines" by the Karolinska Institute Research Ethics Board South, Drno 454/02. HS181 was derived from an embryo that could not be used for infertility treatment of a couple. Both partners of the couple signed a consent form to donate the embryo for the derivation of a possibly permanent stem cell line to be used in stem cell research. HS181 is included in the European Union hESC registry (https://hpscreg.eu/).[65] The hiPSC line MSUH-001 was obtained from the ISCIII National Biobank (Spanish Ministry of Health). HS181 and MSUH-001 were cultured, according to previous reports.[65,66]

ESC Culture and Differentiation

The UN-hESC HS181 line was maintained and passaged as previously described.[65,66] The protocol of hESC directed differentiation into insulin-producing beta-like cells (DD-hESCs) and the protocol of spontaneous differentiation (SD-hESCs) were achieved, as described by Pezzolla et al.[36] Cells generated at the final stage were then transfected with miR-7 mimic (10–100 nM) and cultured in differentiation medium to facilitate the formation of cell clusters and to mature the beta cell-like phenotype.

RNA Extraction and qRT-PCR

Total RNA from cells was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Synthesis of cDNA from mRNA was obtained by using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI, USA) and random hexamers according to the manufacturer's instructions. Analysis (qRT-PCR) was performed in triplicate using a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), ViiA 7 Software (Applied Biosystems), and SYBR Green SuperMix Low ROX (BIOLINE, Luckenwalde, Germany), using the standard instrument protocol. mRNA levels were normalized to the expression of RPLP (TATAA-Biocenter, Gothenburg, Sweden).

All reactions were performed in a 20-μL reaction mixture volume with 400 nM forward primer and 400 nM reverse primer for mRNA. Primer sequences are described in Table S2. miRNA reverse transcription reactions were performed using a Universal cDNA Synthesis kit (Exiqon Vedbaek, Denmark), in accordance with the manufacturer's protocols. Prior to qRT-PCR reactions, cDNA was diluted 1 in 80 for Exiqon assays. miRCURY LNA Universal RT microRNA PCR assays were performed using SYBR Green MasterMix Universal RT (Exiqon), according to the manufacturer's instructions. Reactions were performed, according to the manufacturer's instructions, using a ViiA 7 Real-Time PCR System (Applied Biosystems). Table S3 gives details of the qRT-PCR primers used. The average expression levels of miRNAs in cells were normalized to U48 small nuclear RNA (snRNA). ViiA 7 Software version 1.2 (Life Technologies, Carlsbad, CA, USA) was used to calculate the quantification cycle (Ct) value, which is defined as the number of cycles at which the fluorescence signal is significantly above the threshold; expression of each mRNA and miRNA was defined from the threshold cycle (Ct), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization with reference to expression of internal control. Data are expressed as the means ± SD of three replicates of each experiment.

Microarray Analysis

Microarray image analysis was conducted using an Agilent's 60-mer SurePrint (Agilent Technologies, Santa Clara, CA, USA) scanner to acquire the microarray images, which were subsequently processed with GenePix Pro software 7.0 (Molecular Devices) to generate the

expression data in GPR (GenePix Result File) format, and all analyses were conducted according to the manufacturer's instructions. Background intensity values were corrected using an algorithm normexp[67,68] and were used for the analysis, and normalization was conducted using quantile normalization.[69] Similarly, expressed genes were filtered based on the SD among three biological replicates with >0.58-fold and $p < 0.05$ changes in upregulated miRNAs, and fold changes of <−0.58-fold and $p < 0.05$ of downregulated miRNAs were used for further analysis.

Detection of Insulin Secretion

Supernatants were collected at day 22 following differentiation induction in SD-hESCs and DD-hESCs with or without miR-7 transfection, and they were stored at −20°C until assay of insulin secretion. Insulin secretion was measured as previously described.[36] Briefly, clusters of cells were plated onto Matrigel-coated dishes and rinsed twice in Krebs solution (NaCl 129 mM, $NaHCO_3$ 5 mM, KCl 4.8 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1 mM, HEPES 10 mM, and BSA 0.1% [pH 7.4]). Cells were incubated for 60 min at 37°C with Krebs solution containing 2 or 20 mM glucose. This sequence was repeated one additional time (data not shown). Finally, cells were exposed to 2 mM glucose challenge and depolarized with 30 mM KCl. Insulin levels were measured in technical duplicate and biological triplicates using a commercially available human insulin ELISA kit (Mercodia AB, Uppsala, Sweden).

Immunofluorescence and FISH

Non-radioactive FISH was performed using a QuantiGene ViewRNA FFPE Assay (Affymetrix Panomics, Santa Clara, CA, USA) according to the manufacturer's instructions. A probe set was designed by Affymetrix that corresponded to human mature miR-7, miR-148a, and miR-375; the sequences were 5′-uggaagacuagugauuuuguugu-3′, 5′-ucagugcacuacagaacuuugu-3′, and 5′-uuuguucguucggcucgcguga-3′, respectively. As a negative control, a probe set was designed against a target that was absent in our cell line (5′-cagaacgaacaccacauuuugac-3′, DapB) and was applied instead of the miR-specific probes. Cells that are cultured in suspension were enzymatically dissociated with Accutase (STEMCELL Technologies, San Diego, CA, USA) and seeded into μ-Dish 35 mm (Ibidi, Martinsried, Germany) coated with BD Matrigel (BD Biosicences, San Diego, CA, USA) for 12 hr to promote adhesion. Adherent cells (at a different point of differentiation) were fixed for 20 min in fresh 4% paraformaldehyde. After hybridization, washing, preamplifier hybridization, amplifier hybridization, and alkaline phosphatase reactions, cells were washed thrice for 5 min in PBS.

Next, cells were blocked for 1 hr in PBS supplemented with 4% BSA, permeabilized with 0.5% Triton X-100 in PBS, and incubated with primary antibodies overnight at 4°C. After rewashing with PBS, cells were then incubated with fluorochrome-conjugated secondary antibodies (Invitrogen) for 1 hr at room temperature. Following PBS wash, cell nuclei were stained with Hoechst 33342 (1 μg/mL). Primary and secondary antibodies used in this study are listed in Table S4. Fluorescence images were captured with a Leica SP5 confocal microscope and Leica Application Suite software (Leica, Mannheim, Germany) or an inverted fluorescence microscope Olympus IX71 and DPManager software (Olympus, Tokyo, Japan). After acquisition, images were analyzed using Meta Imaging Software MetaMorph (MDS Analytical Technologies, Sunnyvale, CA, USA). Fluorescence quantification was conducted using MetaMorph software by measuring integrated fluorescence intensities in a defined region of 2 by 2 pixels and subtracting minimum background fluorescence from every value. Quantification was performed for at least 50 cells per experiment from three independent experiments (biological replicates).

Transfection of miRNA

2-'O-Methyl oligonucleotides complementary to miR-7 or miRNA negative control was chemically synthesized by Dharmacon (Dharmacon, Lafayette, CO, USA). The sequence of mature miR-7 is 5′-uggaagacuagugauuuuguugu-3′. For gain-of-function experiments using miR mimics or for inhibition experiments using anti-miRs (amiRs), hESC colonies were detached from culture dishes and grown in suspension culture in ultra-low attachment multi-well plates; cells were transfected with 100 nM miR-7, amiR-7, miRNA negative control, or transfection reagent alone (mock) using DharmaFECT (Dharmacon) in RPMI-1640 containing 12% knockout serum replacement (KSR) for 24 hr. To investigate any response of miR-7 upregulation, the medium was changed after 24 hr, and hESCs were cultured for an additional 7 days before being harvested.

Target Prediction of miRNA and Functional Annotation of the Predicted Target

To facilitate the interpretation of biological functions affected by miRNAs showing significantly differential expression by microarray and confirmed by qRT-PCR, target prediction and functional annotation were performed using the miRSystem database (version 20160513),[70] which is an integrated system used to characterize the enriched functions and pathways of miRNA targets. In target prediction, the miRSystem database integrates 2 experimentally validated databases, TarBase (version 7.0) and miRecords (April 27, 2013 release), and 7 target gene prediction algorithms, including DIANA-microT (version 5.0), miRanda (August 2010 release), miRBridge (April 2010 release), PicTar (March 2007 release), PITA (August 2008 release), RNA22 (version 2.0), and Targetscan (version 7.1). In functional annotation, the miRSystem database integrates 5 databases, including KEGG, BIOCARTA, Pathway Interaction Database (human only), Reactome (human only), and GO.

To identify significantly enriched pathways or biological function, several statistical approaches, including O:E (observed to expected) ratios of gene targets, hypergeometric and empirical value from permutation, and pathway ranking score from miRNA relative expression value (fold change), were also provided in the miRSystem database. In the present study, miRNAs and their fold changes were submitted to the miRSystem database. Only validated targets or miRNA-target interactions identified by at least 4 prediction programs were considered for annotation of the KEGG pathway and

GO molecular function. Moreover, parameter settings were 25–500 genes in biological functions/pathways, O:E ratios ≥ 1.8. Finally, target predictions of differentially expressed miRNAs were uploaded to the online DAVID tool (DAVID version 6.8, https://david.ncifcrf.gov/) to perform GO terms of the miRNA candidates putative target genes and REViGO to generate semantic similarity-based scatterplots.

Statistical Analysis

All experiments were performed at least three times. Data are expressed as the mean ± SD. Statistical analysis was performed by GraphPad Prism 6 (San Diego, CA, USA) using one-way ANOVA followed by Holm-Šídák test, as appropriate, to determine statistical significance for multiple comparisons. Student's t tests were performed for comparisons between two groups. p values < 0.05 were considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and four tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.06.002.

AUTHOR CONTRIBUTIONS

Conception and Design, J.L.-B., B.S., and A.H.; Development of Methodology, Analysis, and/or Interpretation of the Results, J.L.-B., V.C.-G., Y.A., N.M., C.C.L., F.M., T.S., B.S., and A.H. The manuscript was written by A.H. with input from all authors.

CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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