Dynamic expression of microRNAs during the differentiation of human embryonic stem cells into insulin-producing cells

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Abstract

Human embryonic stem (hES) cells with the capacity of self-renewal and multilineage differentiation are promising sources for generation of pancreatic islet cells for cell replacement therapy in diabetes. Here we induced hES cells into insulin-producing cells (IPCs) in a stepwise process which recapitulated islet organogenesis by directing cells through the stages resembling definitive endoderm, gut-tube endoderm, pancreatic precursor and cells that expressed pancreatic endocrine hormones. The dynamic expression of microRNAs (miRNAs) during the differentiation was analyzed and was compared with that in the development of human pancreatic islets. We found that the dynamic expression patterns of miR-375 and miR-7 were similar to those seen in the development of human fetal pancreas, whereas the dynamic expression of miR-146a and miR-34a showed specific patterns during the differentiation. Furthermore, the expression of Hnf1β and Pax6, the predicted target genes of miR-375 and miR-7, was reciprocal to that of miR-375 and miR-7. Over-expression of miR-375 down-regulated the expression of gut-endoderm/pancreatic progenitor specific markers Hnf1β and Sox9. Therefore, the miRNAs may directly or indirectly regulate the expression of pancreatic islet organogenesis-specific transcription factors to control the differentiation and maturation of pancreatic islet cells.

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1. Introduction

MicroRNAs (miRNAs), which are endogenous small non-coding RNAs, play important roles in embryogenesis, cell fate, growth control and cell apoptosis (Alvarez-Garcia and Miska, 2005). They are also required for stem cell maintenance and differentiation. Human embryonic stem (hES) cells derived from the inner cell mass of the blastocysts are characterized by self-renewal and pluripotency (Thomson et al., 1998). Thus, hES cells may serve as a model of human embryology and provide insights into developmental process (Dvash and Benvenisty, 2004).

Characterizations of miRNA expression in mouse (Houbaviy et al., 2003; Tang et al., 2006) and human (Lakshmipathy et al., 2007; Laurent et al., 2008; Suh et al., 2004) ES cells and ES-derived embryoid bodies (EB, containing cells of all three germ layers) (Tripathi et al., 2011) have already been described. In recent years, the patterns of miRNA expression in the models of directing hES cell differentiation towards extraembryonic endoderm (Laurent et al., 2008) and endoderm (Tzur et al., 2008) have also been reported. Furthermore, specific miRNAs were proposed to modulate the specified differentiation of both mouse and human ES cells (Ivey et al., 2008; Krichevsky et al., 2006). However, the expression profiles and the roles of miRNAs in the stem cell differentiation towards pancreatic islet cells are poorly understood.

In this investigation, the expression of miRNAs associated with islet development and function was analyzed during the differentiation of hES cells into insulin-producing cells (IPCs), the expression patterns were compared with those in the development of human pancreatic islets, and the relationship of the expression of these miRNAs with that of their potential target miRNAs was also analyzed. Furthermore, a representative miRNA miR-375 was over-expressed to observe its effects on the expression of islet cell differentiation specific transcription factors. To our knowledge, this is the first report to describe the dynamic expression of miRNAs during the differentiation of hES cells towards IPCs.

2. Materials and methods

2.1. hES cell culture and differentiation

The hES cell line PKU1.1, established by the Reproductive Medical Center of Peking University Third Hospital, exhibits normal female
karyotype (46, XX) (Hong-mei and Gui-an, 2006). The cells were cultured on γ-ray irradiated mouse embryonic fibroblast feeder layers in hES medium under 5% CO2 in air at 37 °C. The hES medium contained KnockOut™ Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 20% (v/v) KnockOut™ serum replacement (SR, Gibco), 1% (v/v) nonessential amino acids (Gibco), 2 mM GlutaMax (Gibco), 4 ng/ml basic fibroblast growth factor (bFGF, Peprotech) and 0.1 mM β-mercaptoethanol (Gibco). The cell colonies were passaged every 5–7 days by incubation in 1 mg/ml collagenase IV (Gibco).

The five-stage procedure (Fig. 1A) for in vitro differentiation of hES cells into IPCs modified from previously published protocols (D’Amour et al., 2006; Zhang et al., 2009) was as follows:

Stage I Expansion of undifferentiated hES cells.
Stage II Formation of definitive endoderm. The hES cells were dissociated into small clumps by collagenase IV and were collected by sedimentation. The dissociated colonies were plated on matrigel (1:50, BD Biosciences)-coated dishes (Corning) and incubated with DMEM/F12 supplemented with 100 ng/ml activin A (Peprotech) and 1 μM wortmannin (Sigma), 1% N2 (Gibco) and 1% B27 (Gibco) for 4 days.
Stage III Induction of pancreatic progenitor cells. The Stage II-cells were cultured in IMDM/F12 with 50 ng/ml Noggin (Peprotech), 0.25 μM KAAD-cyclopamine (CYC, Calbiochem) and 1% B27 for 4 days.
Stage IV Expansion of pancreatic progenitor cells. The Stage III-cells were cultured in DMEM (high glucose, Gibco) with 50 ng/ml endothelial growth factor (EGF, Peprotech), 1% insulin-transferrin-selenium (ITS, Gibco) and 1% N2 for 5 days.
Stage V Formation of IPCs. The Stage IV-cells were incubated in DMEM/F12 (low glucose, Gibco) with 1% ITS, 10 ng/ml bFGF, 10 mM nicotinamide (Sigma), 50 ng/ml exendin-4 (Sigma) for 7–9 days. For further maturity, the cells were digested by 0.05% trypsin (Gibco) and were transferred to ultra-low attachment 6-well plates (Corning) for 3 day-suspension culture to form clusters.

2.2. Lentivirus-mediated transfection

miR-375 (MIMAT0000728) sequence was obtained from the miRBase database. miR-375 lentiviral vector construction, identification, packaging and titration were completed by Invitrogen. A lentiviral vector containing only green fluorescent protein (GFP) acted as the negative control vector. Lentiviral vectors were stored at −80 °C. The stage II (definitive endoderm)-cells during the hES cell differentiation were infected with miR-375 or negative control lentiviral vector at a multiplicity of infection (MOI) of 10, with optimal infection efficiency occurring at 30%–60% confluence. The GFP expression in the miR-375 infection and negative control groups was observed under a fluorescence microscope at 24, 48 and 72 h after infection to assess infection efficiency. The cells were further induced along the differentiation protocol. All experiments were performed at a minimum of three times.

2.3. Reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR

RNA samples were prepared from the cells of different stages with RNaseasy Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesized from total RNA using reverse transcriptase by First Strand cDNA synthesis Kit (Fermentas). Primers were designed using Primer 5.0 software. The cDNA was then amplified by PCR using Taq Plus PCR Master Mix (Tiangen, Beijing, China) or by real-time PCR using iQ™ SYBR Green Supermix (BioRad) with an iQ5 real-time PCR detection system (BioRad). In real-time PCR analysis, all experiments were performed in triplicate. The sample input was normalized against the Ct (critical threshold) value of the housekeeping gene GAPDH. The expression level of each gene at every checkpoint was normalized to the maximal level observed, which was set as 100% (Livak and...
and stained with DAPI. Images were captured under a 4 °C, and with secondary antibodies for 1 h at RT, followed by washing PBS. The cells were incubated with primary antibodies for 24 h at 80 °C (Sigma). Primary and secondary antibodies were diluted in corresponding isotypic sera to replace the primary antibodies. The following antibodies and dilutions were used: goat anti-Sox17 (R&D Systems), 10 μg/ml; mouse anti-Foxa2/Hnf3β (Santa Cruz), 1:400; rabbit anti-Sox9 (Santa Cruz), 1:200; rabbit anti-Hnf1β (Santa Cruz), 1:400; goat-anti-Nkx6.1 (Santa Cruz), 1:200; goat anti-Pdx1 (Santa Cruz), 1:400; rabbit anti-C-peptide (Cell Signaling Technology), 1:200; and rabbit anti-pancreatic polypeptide (PP, Chemicon), 1:200. FITC-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG and TRITC-conjugated rabbit anti-goat IgG (Beijing Zhongshan Biotechnology, China) were used at 1:200 dilutions.

2.5. Dithizone staining

Dithizone (DTZ) staining was performed as described previously (Shiroi et al., 2002). Briefly, a DTZ stock solution was prepared with 50 mg DTZ (Sigma) in 5 ml dimethyl sulfoxide (DMSO) and stored at −20 °C. Staining solution was freshly made by adding 10 μl of the stock solution to 1 ml PBS. The staining solution was filtered through a 0.2-μm nylon filter and then used as the DTZ working solution. The stage V-cell clusters in suspension culture in ultra-low adherent dishes were incubated at 37 °C for 15 min in the DTZ solution. After the dishes were rinsed three times with PBS, the clusters stained crimson red were examined with a stereomicroscope (Nikon). The embryoid body formed by hES cell colony in suspension was used as control.

2.6. Insulin secretion

The stage V-cell clusters in suspension culture were rinsed twice in Krebs-Ringer Bicarbonate HEPES (KRBB) buffer (120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.1 mM NaHCO3, 0.5% bovine serum albumin and 10 mM HEPES) and pre-incubated for 1 h with KRBB buffer. The clusters were then incubated for 1 h in KRBB buffer with either 2.5 (low

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**Table 1**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequence</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
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<td>60</td>
<td>331</td>
</tr>
<tr>
<td>Insulin</td>
<td>Reverse: 5′-GCTCTTGAACCAACACCTG-3′</td>
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<td>261</td>
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<tr>
<td>GAPDH</td>
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Schmittgen, 2001). The primer sequences and PCR conditions used in this study are listed in Table 1.

For miRNA detection, total RNA samples were prepared from the cells of different stages with Trizol (Gibco). Total RNA including miRNAs was then polyadenylated with poly(A) polymerase and was reversely transcribed using miRcute miRNA first-strand cDNA synthesis kit (Tiangen). The poly(A)-tailed cDNA was amplified by miRcute SYBR miRNA qPCR detection kit (Tiangen) with an iQ5 real-time PCR detection system (Benes and Castoldi, 2010). The sample input was normalized against the Ct value of the gene U6. The forward primer sequence of miR-223 was obtained from GeneCopoeia, and the forward primer sequences of miR-146a, miR-34a, miR-150 and let-7e were from Tiangen. The other forward primer sequences synthesized by AuGCT DNA-SYN Biotechnology company (Beijing, China) were as follows: miR-7, 5′-CGGCGGTGGAAGACTAGTGATT-3′; miR-375, 5′-GCGTTGGTGCGCTC-3′; and miR-145, 5′-GTTCAATCTTTCAGGA-3′. The reverse primer sequence was universal (Tiangen). All of the annealing temperatures were 60 °C. The ID and accession of miRNAs in miRBase are shown in Table 2.

2.4. Immunofluorescence

Cells were fixed for 20 min in 4% (w/v) paraformaldehyde in phosphate buffer saline (PBS) at room temperature (RT), washed several times in PBS and blocked for 30 min with 10% (v/v) normal serum (isotypic with the secondary antibodies) in PBS with 0.1% (v/v) Triton X-100 (Sigma). Primary and secondary antibodies were diluted in PBS. The cells were incubated with primary antibodies for 24 h at 4 °C, and with secondary antibodies for 1 h at RT, followed by washing and stained with DAPI. Images were captured under a fluorescent microscope (Nikon). The embryoid body formed by hES cell colony in suspension was used as control.
Taken together, the speciﬁc and their positive percentage reached approximately 90% (Fig. 3). The mRNA and protein levels of pancreatic hormones (insulin, C-peptide and PP) were also detectable in the cells, indicating their gene expression at both mRNA and protein levels. The mRNA expression of islet-speciﬁc markers Pdx1, Isl1, Pax6 and insulin was clearly detected by PCR (Figs. 2A and C). The proteins of key transcription factors for pancreatic β cells (Pdx1 and Nkx6.1) and pancreatic hormones (insulin, C-peptide and PP) were also detectable in the cells, and their positive percentage reached approximately 90% (Fig. 3).

### 2.7. Western blot

The cells were lysed in lysis buffer containing a cocktail of protease inhibitors, and the cell lysate was collected to perform protein quantiﬁcation using the BCA™ Protein Assay Kit. Equal amounts of protein (20 μg) from each cell sample were separated by SDS-PAGE on a 10% polyacrylamide gel and electrically transferred to nitrocellulose ﬁlter membrane (Millipore). Membranes were blocked with 5% bovine serum albumin in TBST (Tris-buffered saline with 0.05% (v/v) Tween-20) and then incubated at 4 °C overnight with primary antibodies including rabbit anti-Hnf1β (Santa Cruz), 1:400; rabbit anti-Sox9 (Santa Cruz), 1:200; and mouse anti-GAPDH (Beijing Zhongshan Biotechnology), 1:1000. Thereafter, the membranes were incubated with secondary antibodies IRDye 800CW-conjugated goat anti-rabbit and goat anti-mouse IgGs (1:10,000, LI-COR Biosciences) for 1 h, and protein bands were visualized with the Odyssey infrared imaging system (LI-COR). GAPDH was used as a loading control.

### 2.8. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was assessed by SPSS statistical package (SYSTAT Software Inc.) by Student’s t test. A P<0.05 was considered statistically signiﬁcant.

### 3. Results

#### 3.1. hES-derived IPCs were successfully induced by the stepwise protocol in vitro

We used a modiﬁed stepwise protocol to direct pancreatic differentiation from the hES cell line PDK1.1. The stage V-cells in suspension culture could aggregate into islet-like clusters. DTZ is a zinc-binding substance and pancreatic islets can be stabilized in the red by its treatment because of their higher zinc contents compared with other tissues (Latif et al., 1988). In this study, the stage V-cells both in monolayer and in cluster could be stained by DTZ (Fig. 1B), suggesting the presence of IPCs. To further characterize the IPCs at the mRNA level, we examined their gene expression at both mRNA and protein levels. The mRNA expression of islet cell speciﬁc markers Pdx1, Isl1, Pax6 and insulin was clearly detected by PCR (Figs. 2A and C). The proteins of key transcription factors for pancreatic β cells (Pdx1 and Nkx6.1) and pancreatic hormones (insulin, C-peptide and PP) were also detectable in the cells, and their positive percentage reached approximately 90% (Fig. 3). Taken together, the speciﬁc expression patterns of these important pancreatic hormones and transcription factors strongly indicated that IPCs were obtained at the ﬁnal stage of differentiated culture.

#### 3.2. The differentiation strategy mimicked the embryonic islet development in vivo

The character of differentiated cells induced by the stepwise protocol was similar to that of pancreatic islet cell development in vivo. The expression patterns of speciﬁc genes and proteins were monitored by PCR and immunofluorescence during the differentiation. First, deﬁnitive endoderm was induced by activin A and wortmannin. The expression of Sox17 (one of speciﬁc markers of deﬁnitive endoderm) was peaked on day 4, a time when the expression level of Oct4 (one of speciﬁc markers of ES cells) was rapidly reduced (Fig. 2A). At the same time, the expression of Sox17 and Foxa2 proteins could be found in the stage II-cells as detected by immunofluorescence (Fig. 3). Second, the deﬁnitive endoderm cells were further differentiated. After treated by RA, Noggin and FGF7, the cells with characteristics of pancreatic progenitors could be acquired. The gut-endoderm speciﬁc markers Hnf1β and Sox9 were detected in the stage III-cells (Figs. 2A and 3), and the markers of pancreatic progenitors Pdx1 and Nkx6.1 were then visible in the stage IV-cells (Fig. 3). The up-regulated gene expression of Ngn3, Pdx1 and Isl1 was determined by real-time PCR (Fig. 2A), which suggested pancreatic specialization. Finally, the progenitors were differentiated towards pancreatic islet cells. The expression of insulin emerged and reached a maximum level on day 15 (Fig. 2A), indicating the appearance of IPCs. Pdx1 was co-expressed with C-peptide (Fig. S1), which conﬁrmed their pancreatic β cell characteristics. In addition, co-expression of insulin with C-peptide (Fig. S1) validated the insulin synthesis de novo. Therefore, the speciﬁc expression patterns of the crucial pancreatic hormones and transcription factors strongly indicated that islet-like cells were obtained in vitro at the ﬁnal stage. Overall, the expression dynamics during the in vitro differentiation of IPCs from hES cells were similar to those of pancreatic β cell development in vivo (Murtaugh, 2007; Oliver-Krasinski and Stoffers, 2008), suggesting the differentiation protocol used in this study recapitulates key developmental stages in human pancreatic β cell specialization.

#### 3.3. The IPCs were immature

Although the IPCs at the ﬁnal stage were somewhat similar to the adult human islet cells, they were far less mature. The quantitative PCR analysis showed that the expression levels of islet speciﬁc makers Pdx1, insulin and MafA in the IPCs were much lower than those of adult human islets, whereas the level of Isl1 was comparable (Fig. 2B). The immunofluorescent staining revealed that the several pancreatic hormones such as insulin, glucagon and PP could be detected in the stage V-cells (Figs. 3 and S1). Furthermore, these pancreatic hormones secreted by different pancreatic endocrine cell types were co-immunostained with each other in the cells (Fig. S1), suggesting that the IPCs co-expressed several pancreatic hormones which is never shown in adult human islets. Moreover, the glucose-stimulated insulin release response of the stage V-cell clusters was poor and highly variable. The insulin release at low and high levels of glucose challenge was 100.1±36.7 vs. 139.6±45.3, 73.9±16.8 vs. 63±11.9 and 223±69.8 vs. 273±86.6 fmol/mg protein in three different batches of studies (Fig. 5). Therefore, we need to investigate the mechanism of the islet development and create new differentiation strategies to promote the maturation of the IPCs.

#### 3.4. The dynamic expression of miRNAs during the differentiation

During the differentiation induced by stepwise protocol, the dynamic expression levels of the miRNAs involved in the development of islet cells, such as miR-7 and miR-375 (Correa-Medina et al., 2009; Joglekar et al., 2009), and insulin secretion, such as miR-375, miR-
146a and miR-34a (Lovis et al., 2008; Poy et al., 2004), were detected by real-time PCR. The miRNAs which are specific for the differentiation of hES cells, such as miR-145 (Xu et al., 2009), and the miRNAs which are associated with the diabetes complication or show significant differences in the expression levels between diabetic patients and healthy adults, such as miR-223 and let-7e (Zampetaki et al., 2010), were also analyzed in this study. The expression of miR-375 and miR-7 increased from day 4, peaked on day 8, and then began to decline until the end of the differentiation. In contrast, the levels of miR-146a, miR-145 and let-7e were dramatically decreased from day 4, reached nadir on day 6, and maintained at this level to day 21. Interestingly, the miR-34a level was reduced to the minimum on day 6 to 10, and then gradually ascended and reached the maximum level on day 21. Notably, the dynamic expression of miR-146a and miR-34a showed specific W-shaped patterns during the differentiation. Moreover, the expression levels of miR-150 and miR-223 fluctuated during the differentiation, and maintained at the similar levels at different stages of differentiation (Fig. 4).

Based on the fact that miRNAs negatively regulate their target genes, we showed the expression of miRNAs as their reciprocal values (1/miRNA expression) and found the results very inspiring. The 1/miR-7 expression ascended rapidly on day 10 when the pancreatic progenitors were induced initially and successfully, whereas those of 1/miR-146a emerged on day 13 and 19 when the cells that expressed endocrine hormones began to form and became close to terminal differentiation (Fig. S2).

3.5. Identification and dynamic expression of miRNA target genes during the differentiation

The potential target genes of miRNAs were predicted using the TargetCombo open source software (http://www.diana.pcbi.upenn.edu/cgi-bin/mirGen/v3/Targets.cgi), which predicts targets by the union of DIANA-microT, miRanda (microrna.org), miRanda (miRBase), PicTar (4-way), PicTar (5-way) and TargetScanS. The targets of miRNAs were identified by inverse relationships (highly negative correlations) between expression levels of miRNAs and their predicted target mRNAs. For example, the target genes of miR-375 contain tens of mRNAs, some of which play prominent roles during the pancreas development, including Hnf1β, connective tissue growth factor, Sox17, GATA6 and Pax6. The target genes of miR-7 involve GATA6, insulin receptor substrate 2 (IRS-2), IRS-1, Pax6, NeuroD1 and Nkx2.2. These results suggested that miRNAs probably play regulatory roles in the differentiation of pancreatic islet cells. Two of the potential target genes (Hnf1β and Pax6) were selected for further analysis. The expression of Hnf1β increased on day 12 and reached a maximum level on day 15 and the expression of Pax6 acquired the highest expression level on day 21, which were consistent with the expression of 1/miR-375 and 1/miR-7 (Figs. 2A and S2). Therefore, miRNAs may directly or indirectly
regulate the expression of pancreatic islet organogenesis specific transcription factors to control the cell fate.

3.6. The effects of miR-375 over-expression during the differentiation

The stage II (definitive endoderm)-cells during the differentiation were infected, and the expression of GFP was used to determine the efficiency of lentiviral infection. Fluorescence was apparent 24 h after infection, with infection efficiency increasing gradually with time. There were similar infection efficiency between negative control and miR-375 infected groups (Fig. 6A). Real-time PCR was used to determine the level of miR-375 expression. The level of miR-375 was two-fold higher in miR-375-infected group than that in the negative control group, while there was no difference between the negative control and the non-transfected groups (Fig. 6A).

To study the effects of miR-375 on the pancreatic islet cell differentiation of hES cells, we compared the expression of islet-specific transcription factors using PCR and western blot. One of the predicted targets Hnf1β and one of pancreatic progenitor cell markers Sox9 were analyzed. The mRNA level of Hnf1β was unchanged and its protein level was significantly decreased in the miR-375-infected group compared with two other groups. Moreover, both mRNA and protein levels of Sox9 were markedly reduced in the miR-375-infected group (Figs. 6B–C).

4. Discussion

It has been reported that hES cells have the ability of spontaneous differentiation towards IPCs (Assady et al., 2001). In order to obtain sufficient functional IPCs, many differentiation protocols have been developed. So far, there are two main strategies to induce the differentiation of ES cells into IPCs. One is based on the selection of nestin-positive progenitor cells (Blyszczuk et al., 2003; Chen et al., 2011; Hori et al., 2002; Lumelsky et al., 2001), and the other is through the generation of definitive endoderm (D’Amour et al., 2006; Jiang et al., 2007; Shim et al., 2007; Zhang et al., 2009). In recent years, more studies supported the viewpoint that hES cell-derived endoderm and pancreatic lineages may have better therapeutic potential. However, it is challenging to reproduce the differentiation results because of the variability between hES cell lines (D’Amour et al., 2006; Osafune et al., 2008; Rezania et al., 2012). In the present study, therefore, we differentiated the hES cell line PKU1.1 through the definitive endoderm, and the protocol was modified from Zhang’s (D’Amour et al., 2006; Zhang et al., 2009). At the final stage of our differentiated culture, IPCs could be successfully induced in vitro. These cells were characterized by analogous gene expression profiles to pancreatic islet cells, positive staining with islet β cell specific dye DTZ, and positive immunolabeling for both islet specific transcription factors and pancreatic hormones. Based on our observations, the dynamic expression patterns of mRNAs during the IPC differentiation of hES cells were similar to those of pancreatic β
miRNAs are small non-coding RNAs that play critical roles in post-transcriptional regulation of gene expression by interaction with specific sites located in the 3’ untranslated region of target mRNAs. They are involved in stem cell maintenance and differentiation (Bartel, 2004). It has been reported that specific miRNAs modulate ES-derived neurogenesis and cardiogenesis (Ivey et al., 2008; Krchikovsky et al., 2006). miRNAs do participate in the pancreatic development (Correa-Medina et al., 2009; Joglekar et al., 2009; Kloosterman et al., 2007; Lynn et al., 2007; Poy et al., 2009). However, the expression profiles and the roles of miRNAs in the process are poorly understood. Meanwhile, whether miRNAs regulate stem cell differentiation towards islet cells and how miRNAs work need to be further studied. In a recent publication, the miRNAs expression profiles of islet-like cell clusters were quantified using Taqman MicroRNA assays and were compared with hES cells and hES cell-derived EBs (Chen et al., 2011). However, the time kinetic changes of miRNA expression were not documented, and the protocol used in that study was based on the selection of nestin-positive progenitors. In the present study, we used a strategy which mimicked the development of human pancreatic islet in vivo, and detected the dynamic expression of specific miRNAs. We found that several miRNAs showed time-dependent expression patterns during the differentiation.

A recent study showed that four islet specific miRNAs, miR-7, miR-9, miR-375 and miR-376, were expressed at high levels during human pancreatic islet development (Joglekar et al., 2009). It has been demonstrated that miR-375 and miR-7 are essential for pancreatic β-cell development and function (Bravo-Egana et al., 2008; Kloosterman et al., 2007; Lynn et al., 2007; Poy et al., 2009). Our study showed that the expression of miR-375 and miR-7 increased from day 4 and peaked on day 8, and then began to decline until the end of the differentiation. Meanwhile, the expression of Pdx1, Isl1 and insulin began to ascend on day 12. The peak of the miR-375 and miR-7 expression levels coincided with the time at which the expression levels of pancreatic hormone encoding genes began to rise dramatically, which is in agreement with the previous study (Correa-Medina et al., 2009). On the other hand, miR-375 has been shown to negatively regulate insulin secretion (El Ouaamari et al., 2008; Poy et al., 2004; Xia et al., 2011), and its up-regulation is associated with type 2 diabetes and apoptosis of pancreatic islet β cells (Li et al., 2010; Zhao et al., 2010). It is conceivable, therefore, that the increase of miR-375 is important for the generation of islet cells, whereas its decrease is essential for the maturity and function of islet β cells. Surprisingly, the time-course change of 1/miR-375 and 1/miR-7 levels resembled the expression levels of islet specific genes such as Hnf1β, Pax6, Isl1, Pdx1 and insulin, which may reflect the negative regulation of miR-375 and miR-7 on these genes and may indicate that miR-375 and miR-7 function during the differentiation of pancreatic progenitors into IPCs. Furthermore, the dynamic expression patterns of miR-375 and miR-7 were similar to those of developing human fetal pancreas (Correa-Medina et al., 2009), which further supported that the differentiated protocol used in this study mimicked the development of human pancreas.

miR-146a and miR-34a also play reciprocal roles in the function of pancreatic β cells and are involved in the development of diabetes (Li et al., 2009; Lovis et al., 2008; Roggli et al., 2010). Therefore, the low level of miR-146a and miR-34a is substantial for the function of islet β cells. Our results showed that the level of miR-146a declined along with the differentiation process, while the miR-34a level was reduced to the minimum on day 6 to day 10 and then gradually ascended and reached maximum on day 21. In a recent report, miR-34a is required for proper neuronal differentiation (Aranha et al., 2011). It is well known that neuron and pancreas have some common mechanisms to control development (Lumelsky et al., 2001) and share lots of transcription factors including Isl1, Ngn3 and Pax6. Thus, our result
supposed that miR-150 was not associated with islet development (Cordes et al., 2009). In the protocol of this study, we needed to inhibit heart and smooth muscle cell (SMC) and modulate SMC plasticity. As we previously demonstrated that miR-145 are highly expressed in the differentiated cells of stage III in this study. Therefore, we presume that down-regulation of miR-145 may be critically involved in the development of B lymphocytes (Xiao et al., 2007). We have previously shown that miR-375 targets and down-regulates insulin-like growth factor-1 receptor (Shi et al., 2011) which further illustrated that miR-375 could regulate the differentiation of pancreatic progenitors into IPCs.

The expression level of miR-145 is low in self-renewing hES cells and is highly up-regulated during differentiation. Increased miR-145 expression inhibits hES cell self-renewal, represses expression of pluripotency genes and induces lineage-restricted differentiation (La Rocca et al., 2011; Xu et al., 2009). In addition, let-7 family members are notably low in mouse ES cells and are rapidly induced upon differentiation (Marson et al., 2008). In the present study, however, the expression levels of miR-145 and let-7e were dramatically decreased along with the differentiation. It has been shown that miR-145 targets and down-regulates insulin-like growth factor-1 receptor (Shi et al., 2007). Moreover, a recent report showed that miR-145 regulated cell differentiation by targeting Sox9 (Yang et al., 2011) which was strongly expressed in the differentiated cells of stage III in this study. Therefore, we presume that down-regulation of miR-145 may be essential for islet development. On the other hand, it has been convincingly demonstrated that miR-145 are highly expressed in the developing heart and smooth muscle cell (SMC) and modulate SMC plasticity (Cordes et al., 2009). In the protocol of this study, we needed to inhibit the differentiation of mesoderm for facilitating the derivation of endoderm, which may account for the decrease of miR-145.

It has been shown that miR-223 regulates cardiomyocyte glucose metabolism and is up-regulated in the insulin-resistant heart (Lu et al., 2010). miR-150, which is abundantly expressed in lymphocytes, is involved in the development of B lymphocytes (Xiao et al., 2007). We supposed that miR-150 was not associated with islet development and function, and therefore its dynamic expression could be used as a negative control for other miRNA expression. In this study, the expression levels of miR-223 and miR-150 during the differentiation were not varied so much, indicating that these two miRNAs may not be major regulating factors during the islet development.

The differentiation of IPCs from hES cells is varied between different hES cell lines and is usually fluctuated largely from batch to batch of experiments (D’Amour et al., 2006). To eliminate the deviations and to demonstrate the trend of miRNAs during the differentiation much clearer, we used the values of miRNA expression combined with those of 1/miRNA expression in this study. For example, the values of 1/miR-34a on day 10 and 1/miR-146 on day 17 showed sharp declines, but the expression of miR-34a and miR-146a only had the small peaks, indicating that the sharp declines of 1/miR-34a on day 8 and 1/miR-146 on day 17 might be caused by deviations. On the other hand, we used the combination of miRNA expression and its reciprocal values to infer the stage at which the miRNAs may have a given function. For example, miR-375 may have a role on day 21, miR-7 on day 15 and thereafter, miR-34a on day 4 to 12 and miR-146a on day 8 and 1/miR-146 on day 17 might be caused by deviations. On the other hand, we used the combination of miRNA expression and its reciprocal values to infer the stage at which the miRNAs may have a given function. For example, miR-375 may have a role on day 21, miR-7 on day 15 and thereafter, miR-34a on day 4 to 12 and miR-146a on day 8 and 21 (Fig. 7 as well as Figs. 4 and S2).

There are several predicted target mRNAs of miR-375 and miR-7, some of which play prominent roles during the pancreas development, including Hnf1β and Pax6. The expression levels of these two genes were consistent with the values of 1/miR-375 and 1/miR-7 (Fig. S2). Furthermore, over-expression of miR-375 down-regulated the protein level of Hnf1β but did not alter its mRNA level, which suggested a role of the miRNA in regulating post-transcriptional protein synthesis. Therefore, Hnf1β might be one of the transcription factors which directly interacted with miR-375. Moreover, the expression level of pancreatic progenitor cell marker Sox9 was significantly decreased when miR-375 was over-expressed, which further illustrated that miR-375 could regulate hES cell differentiation towards pancreatic islet cells. Nevertheless, the limitations of this study are that only a few miRNAs were analyzed...
during the differentiation and that the interaction of these miRNAs with their target genes was not analyzed in depth. Therefore, it is strongly needed to detect miRNA expression profiles extensively by using high-throughput screening technology and to identify specific miRNAs for further investigation of their function during the differentiation of pancreatic islet cells.

In summary, the hES cells were induced to differentiate into IPCs by a stepwise protocol which recapitulated organogenesis of pancreas in vivo. Interestingly, the dynamic expression patterns of miR-375 and miR-7 resembled those of developing human fetal pancreas, whereas the dynamic expression of miR-146a and miR-34a showed specific patterns during the differentiation. In addition, the expression of Hnf1β and Pdx6, which are the predicted target genes of miR-375 and miR-7 and play influential roles during the development of pancreas, was reciprocal to that of miR-375 and miR-7. Over-expression of miR-375 down-regulated the expression of gut-endoderm/pancreatic progenitor markers Hnf1β and Sox9. Therefore, miRNAs are very likely to play important regulatory roles in the differentiation and maturation of pancreatic islet cells. Changing the levels of miRNAs during the differentiation may be an alternative strategy for promoting the maturation of hES cell-derived IPCs. To our knowledge, this is the first report to describe the dynamic expression of miRNAs during the hES cell differentiation towards IPCs.

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