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miRNA-302 facilitates reprogramming of human adult hepatocytes into pancreatic islets-like cells in combination with a chemical defined media

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ABSTRACT

The direct conversion of one cell type to another without an intermediate pluripotent stage is required for regenerative therapies. The ventral pancreas and liver share a common developmental origin. Recent studies have shown that hepatocytes could be induced to transdifferentiate into insulin-producing cells. In this paper, we showed a new strategy to achieve the direct conversion of human hepatocytes into surrogate β cells. Hepatocytes were transfected with microRNA-302 (miR-302) mimic and *Pdx1*, *Ngn3* and *MafA* expressed plasmids, followed by a chemical-defined culture system for maturation of insulin-secreting cells. Co-transfection of miR-302 mimic increased the transcription of pancreatic development-related genes (*Sox17*, *Foxa2*, and endogenous *Pdx1*). Furthermore, at the end of this treatment, hepatocytes became insulin expressed cells that released the hormone in response to a physiological glucose change *in vitro*. This work shows that miR-302 participation may facilitates the conversion of adult hepatocytes into pancreatic islets-like cells.

1. Introduction

Insulin insufficiency due to reduced pancreatic β cell number is the hallmark of diabetes [1,2], resulting in an intense focus on the development of β cell replacement therapy. Although whole-pancreas and islet transplantation have exerted beneficial effects, the availability of insulin-expressing cells is limited and immunosuppressive therapy is required [3]. One approach with overcoming the problem is to search for alternative sources to induce insulin-producing surrogate β cells, through the guided differentiation of stem cells or the transdifferentiation of mature cells [4,5]. Numerous studies have shown that insulin-producing cells (IPCs) can be generated from embryonic stem cells (ESCs) [6–8], mesenchymal stem cells (MSCs) [9,10], pancreatic exocrine cells [11,12], etc.

The advent of induced pluripotent stem cells (iPSCs) technology enabled the conversion of adult cells into any other cell type passing through a pluripotency state [13–15]. However, indefinite pluripotency is unphysiological and inherently labile. The direct conversion of mature cells of adult organisms without an intermediate pluripotent stage is required for regenerative therapies [16,17]. Liver is gaining much attention as both liver and ventral pancreas appear to arise from the same cell population located within the embryonic endoderm in the process of embryogenesis [18]. Several groups have demonstrated that ectopic expression of key transcription factor genes for pancreas development in hepatic cells can induce them to differentiate into IPCs [19–21]. Pdx1 is critical for development, regeneration and maintenance of β cell function [22]. Ectopic over expression of Pdx1 in liver stem cells and adult hepatocytes activated endogenous insulin expression and ameliorated hyperglycemia in diabetic mice [19,23]. Other studies showed that MafA, NeuroD/Beta2 and Ngn3 were potential inducers of islet cell differentiation [24–26]. Pdx1, MafA, and NeuroD/Beta2 induced expression of endogeneous insulin gene in liver [26]. Co-expression of Pdx1 and Ngn3 activated the transfected hepatocytes acquired the ability to synthesize and secrete insulin [24].

The miR-302∼367 cluster encodes several miR-302 family members, including miR-302a/b/c/d and miR-367. The genomic sequence encoding miR-302 is located in the 4q25 locus of human chromosome 4, a conserved region frequently associated with longevity [27]. MiR-302 is predominantly expressed in human ESCs and iPSCs, but expressed in other cell types at lower level. The highest expression of miR-302 is observed in undifferentiated cells and declines rapidly as they begin to differentiation [27,28]. Recent

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studies have shown that miR-302 can replace the defined factors (Oct4, Sox2, etc) to reprogram human and mouse somatic cells to iPSCs [29–31]. Mechanistically, miR-302 acts as a cytoplasmic gene silencer and represses the translation of multiple key epigenetic regulators to induce global DNA demethylation.

Here, we describe a strategy to convert HL-7702 (a human hepatocytes cell line) cells into pancreatic islets-like cells. We showed that co-transfection of miR-302 mimic with Pdx1, Ngn3 and MafA activated the transcription of pancreas progenitor cells related genes including *Sox17*, *Foxa2*, and endogenous *Pdx1*. Furthermore, a chemical-defined culture system promotes differentiate cells to mature. The differentiated cells at the final stage secreted insulin and C-peptide in response to glucose stimulation.

2. Materials and methods

2.1. Plasmid construction

We generated two plasmids: pCAG-Pdx1-2A-Ngn3 (pCPAN) and pCAG-Pdx1-2A-MafA (pCPAM). Full-length human *Pdx1*, *Ngn3* and *MafA* cDNA were PCR-amplified from primary adult human islet cDNA. We placed *pdx1* and *ngn3*, or *pdx1* and *mafA* into a plasmid vector containing a CAG constitutively active promoter and a 2A peptide of Foot-and-mouth Disease Virus (FMDV).

2.2. Cell culture and transfection

HL-7702 cells were obtained from the Chinese Academy of Sciences Committee Typical Culture Collection Cell Bank in Shanghai and cultured in RPMI-1640 (Hyclone) containing 10% fetal bovine serum (Hyclone), 1× penicillin/streptomycin (Hyclone). All cells were cultured at 37 °C in a 5% CO_2 atmosphere. Transfection of HL-7702 was performed according to the manufacture protocols of Lipofectamine 2000 (Life technologies) and Fugene HD (Promega). We prepared reagent-DNA complexes at ratios of 2:1 and 3:1, respectively. Nucleofection of HL-7702 was performed according to the manufacturer's optimized protocol (Amaxa Biosystem). HL-7702 cells were mixed with 3 μg of plasmid DNA, and pulsed with the program T-028.

Table 1
List of primers used for quantitative RT-PCR analysis of human cells.

Gene	Forward sequence (5′3′)	Reverse sequence (5′3′)
Insulin	CTACCTAGTGTGCGGGGAAC	GCTGGTAGAGGGAGCAGATG
Glucagon	CATTCACAGGGCACATTCAC	ATGAATTCCTTGGCAGCTTG
Somatostatin	CCCAGACTCCGTCAGTTTCT	CCATAGCCGGGTTTGAGTTA
Foxa2	AGCCTCCGGTTTCCACTACT	TGGATTTCACCGTGTCAAGA
Sox17	CCTGGGTTTTTGTTGTTGCT	GAGGAAGCTGTTTTGGGACA
endo Pdx1	TGGAAAAAGGAGGAGGACAA	AGTGGTTGAAGCCCCTCAG
Glut2	TTGGTGTGATCAATGCACCT	CACAGTCTCTTCCTCAGCCC
Nkx6.1	ATTCGTTGGGGATGACAGAG	TCAACAGCTGCGTGATTTTC
Albumin	CTGCACAGAATCCTTGGTGA	CTCCTTATCGTCAGCCTTGC

2.3. Differentiation of HL-7702 into insulin producing cells

HL-7702 cells were transfected with pCPAN, pCPAM and synthetic miR-302a mimic or non-targeting controls (GenePhama, Shanghai) together on days 1, 3, 5, 7 and 9. After the first transfec-

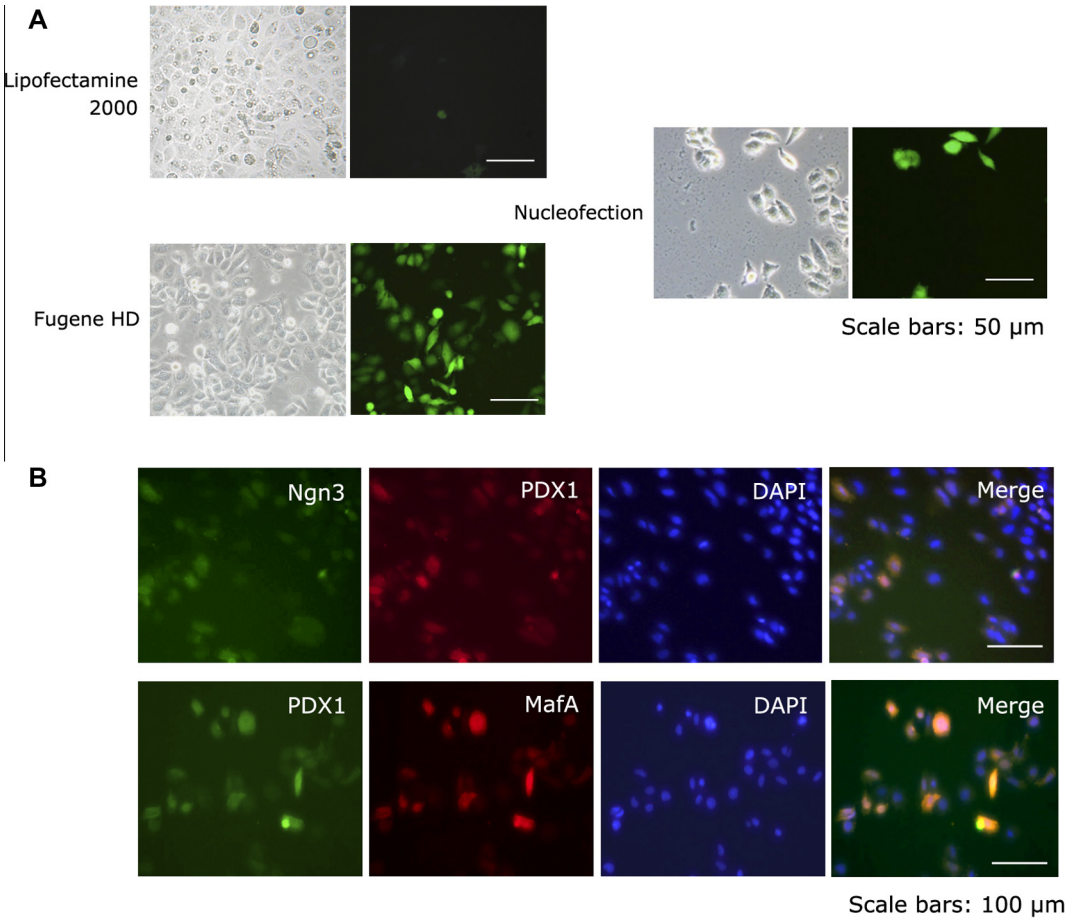

Fig. 1. Gene expression in transfected HL-7702. (A) Live images of pCAG-EGFP transfected HL-7702. (Scale bars, 50 μm). (B) Immunofluorescence staining of transfected HL-7702. pCPAN transfected HL-7702 expressed both PDX1 and Ngn3. pCPAM transfected HL-7702 expressed both PDX1 and MafA (Scale bars, 100 μm).

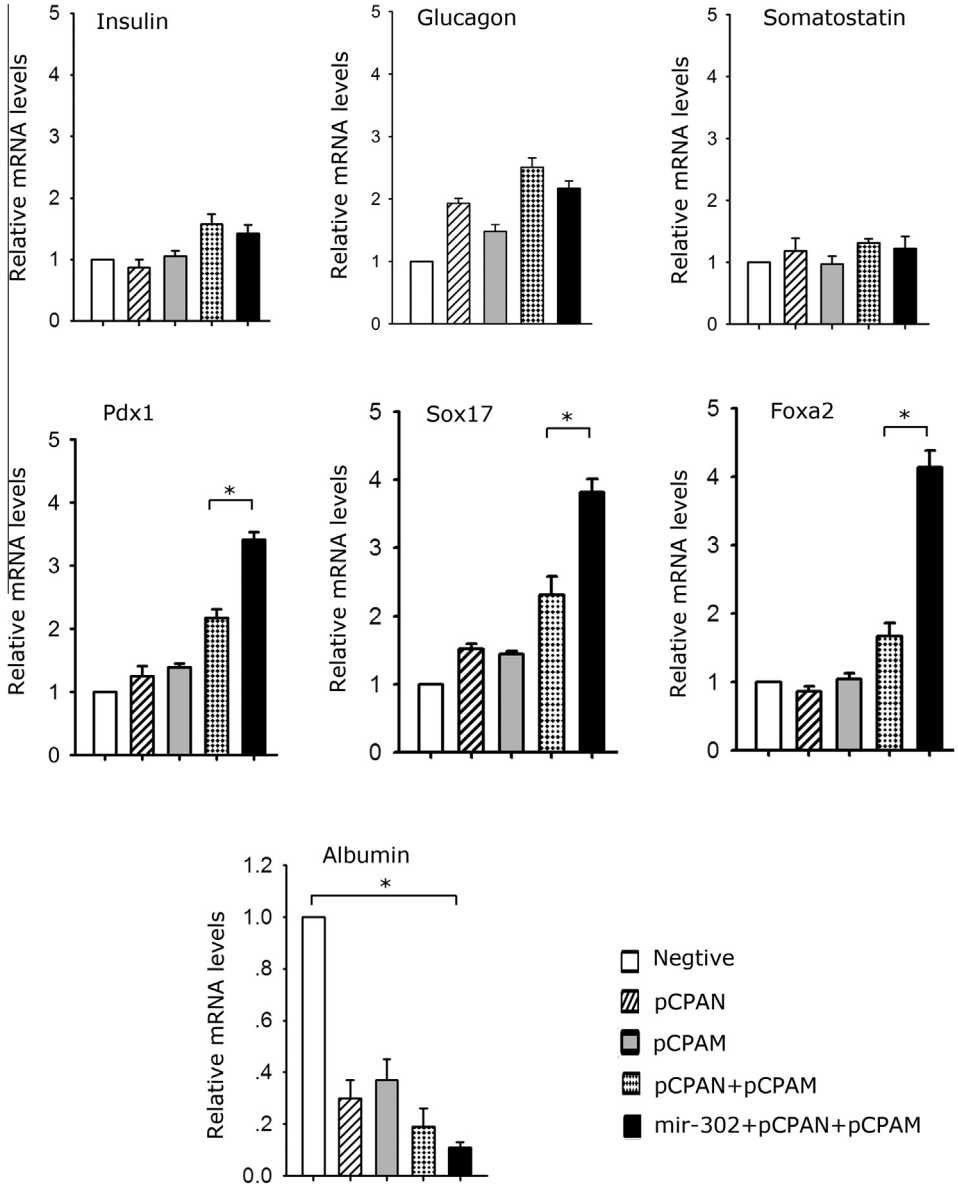

Fig. 2. Expression pattern of markers of pancreatic cells (*Insulin, Glucagon, Somatostatin, Pdx1, Sox17, Foxa2, Albumin*), in untreated HL-7702 cells (negative), in HL-7702 cells transfected with pCPAN, pCPAM, both plasmids together with non-target control or synthetic miR-302 mimic.

tion, medium was refreshed and replaced with the basal medium (Dulbecco's Modified Eagle Medium/Ham's F12 supplemented with 0.5% BSA, 1% B27, 1% N2, 1% non-essential amino acids, 1% Glutamax, and 0.1 mM β-mercaptoethanol). For further transdifferentiation, on day 11, cells were cultured in basal medium with 10 μM RA (Sigma). Two days later, medium was replaced with basal medium containing 1% insulin-transferrin-selenium (ITS), 20 ng/ml bFGF, 50 ng/ml Exendin4 , and 1 mM nicotinamide (Sigma) for another 28 days to encourage differentiation. Medium was refreshed daily. All media were from Hyclone, supplements were from Gibco, and growth factors were from Peprotech.

2.4. Quantitative RT-PCR analysis of gene expression

Quantitative RT-PCR was performed as described previously [32]. PCR analysis was performed on ABI PRISM 7900 sequence detection system using SYBR Green PCR Master Mix (Roche). For each sample, three independent experiments were performed. The primer sequences are shown in Table 1.

2.5. Immunofluorescence assay

The sample cells were fixed in 4% paraformaldehyde and blocked with 5% serum and 0.5% triton X-100 in PBS, and then incubated with the primary antibody: Ngn3 antibody (mouse IgG, 1:50, Santa Cruz), Pdx1 antibody (rabbit IgG, 1:50, Santa Cruz; goat IgG, 1:200, Cell Signaling Technology), MafA antibody (rabbit IgG, 1:100, Abcam), FoxA2 antibody (mouse IgG, 1:100, Abcam), Sox17 antibody (mouse IgG, 1:100, Abcam), C-peptide antibody (rabbit IgG, 1:200, Cell Signaling Technology), overnight at 4 °C and further incubated with respective secondary antibody: Dylight488-conjugated anti-mouse IgG (1:100, Thermo), FITC-conjugated anti-goat IgG (1:100, Jackson Lab.), TRITC-conjugated anti-rabbit IgG (1:100, Jackson Lab.), TRITC-conjugated anti-mouse IgG (1:100, Jackson Lab.).

2.6. Measurement of insulin and C-peptide in the medium

The insulin and C-peptide levels in culture supernatants were measured by Human insulin or C-peptide ELISA Kit (Millipore).

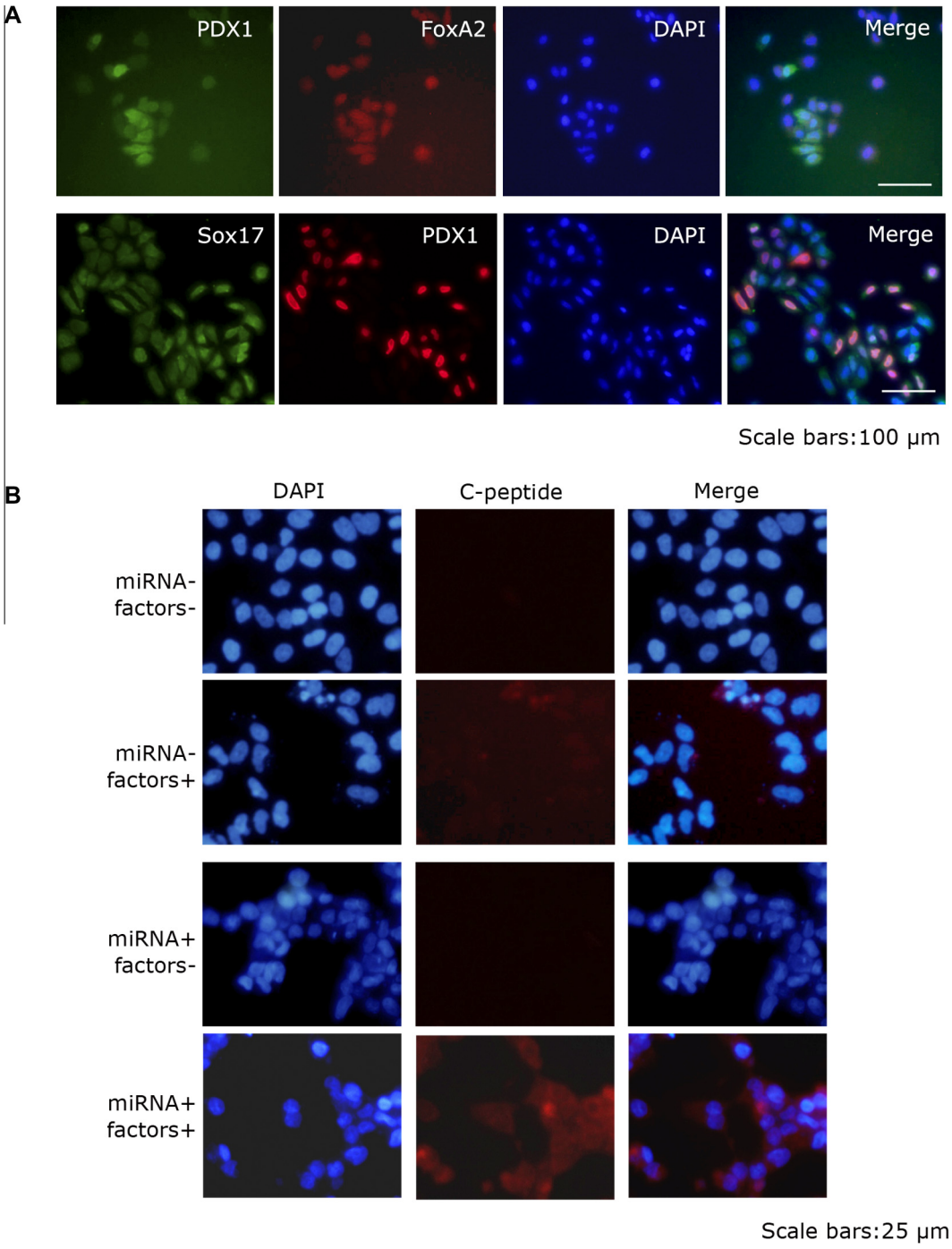

Fig. 3. Immunocytochemical localization. (A) Immunocytochemical staining of Pdx1, FoxA2 and Sox17 in co-transfected HL-7702 cells. (Scale bars, 100 μm). (B) The induced HL-7702 cells subjected to pancreatic induction for another 30 days. Immunoassay showing the C-peptide-labeled cells in before or after treatment group. (Scale bars, 25 μm).

After pre-incubation with Krebs-Ringer buffer (KRBB) for 90 min, the differentiated cells were incubated with KRBB containing 5 mM glucose or 25 mM glucose for 1 h. Then the respective conditioned supernatant was collected and analyzed. The total protein content was measured with a BCA Protein Assay Kit (PIERCE).

2.7. Statistical analysis

Values are expressed as the mean ± standard deviation (SD). Statistical differences between the groups were analyzed with the paired t test, and the significant level was defined as $P < 0.05$. The data were analyzed by SPSS statistical software (SPSS, Chicago. IL).

3. Results

3.1. Fugene HD allows high-efficiency transfection of hepatocytes

Since hepatocytes are difficult to transfection, we compared the transfection efficient of Lipofectamine 2000, Fugene HD and Nucleofection using pCAG-EGFP plasmid, which encodes enhanced green fluorescent protein (EGFP). The efficiency was determined as the

proportion of EGFP-positive cells to the total number of cells at 24 h by counting 10 fields at 200× magnification in three independent experiments. Quantitative assessment revealed that transfection using Lipofectamine 2000, Fugene HD and Nucleofection yielded 2.8 ± 0.9%, 64.3 ± 2.4% and 26.5 ± 1.7% EGFP positive cells, respectively (Fig. 1A). This result indicates that Fugene HD is a suitable non-viral transfection approach for gene transfer in HL-7702.

3.2. Co-expression of Pdx1-Ngn3 and Pdx1-MafA in HL-7702

We constructed two 2A-dependent bicistronic expression plasmids (pCPAN: pCAG-Pdx1-2A-Ngn3 and pCPAM: pCAG-Pdx1-2A-MafA). HL-7702 cells were transduced with pCPAN or pCPAM. Immunofluorescence staining demonstrated that the expression of Pdx1-Ngn3 or Pdx1-MafA was mostly visualized in the same nucleus at 24 h after transfection (Fig. 1B). Therefore, we got a strategy, co-expression both of Pdx1 and Ngn3, or Pdx1 and MafA.

3.3. Co-transfection of miR-302a mimic facilitates liver to pancreas reprogramming

To activate the reprogramming of hepatocytes into pancreatic islet-like cells, HL-7702 cells were transfected with pCPAN, pCPAM and miR302a mimic or non-targeting controls together on days 1, 3, 5, 7 and 9. After we performed the transfection for 5 times, the mRNA expression levels of *Insulin*, *Glucagon*, *Somatostatin*, *Sox17, Foxa2*, endogenous *Pdx1*, and *Albumin* of different groups were measured by Q-RT-PCR. Our data showed an increase in the mRNA levels of *Sox17, Foxa2*, and endogenous *Pdx1*, the expression of liver gene (*Albumin*) was detected in control HL-7702, but was dramatically decreased in differentiated cells, whereas there is no increase in insulin mRNA after transduction (Fig. 2). Furthermore, immunofluorescence staining showed that most co-transfected cells expressed Pdx1 and FoxA2 or Sox17 together (Fig. 3A). These data indicate that miR-302a may allow the conversion of human hepatocytes into pancreatic progenitor cells.

3.4. A cocktail of factors induce maturation

We further attempted to promote induced-HL7702 into insulin-producing cells (IPCs). We used a differentiation strategy by a cocktail of factors. The same strategy was used to promote differentiated endoderm cells which induced from the human ES or iPS cell line into IPCs [8,33]. After approximately 28 days of further induction, we examined C-peptide by immunoassay at the final stage. C-peptide-positive cells were observed in further induced miR302 mimic co-transfection group, and few in the control group or without in non-further induced groups (Fig. 3B). To characterize the generated IPCs, we examined their gene mRNA levels, quantitative RT-PCR revealed an increase in the expression of *Insulin*, *Glucagon*, *Somatostatin*, endogenous *Pdx1*, *Glut2*, and *Nkx 6.1*, but decrease in *Sox17* and *Foxa2* (Fig. 4A, $P < 0.05$, respectively). Therefore, the specific expression pattern of C-peptide and transcription factors indicates that mature β-like cells had been obtained *in vitro* at the final stage.

3.5. Insulin and C-peptide release in response to glucose

Based on the above findings, we further analyzed the potential of glucose-stimulated insulin and C-peptide release. Our data showed that the insulin and C-peptide release were increased by high glucose treatment over basal glucose stimulation (Fig. 4B and C, $P < 0.05$). These results suggest that human hepatocytes can be differentiated into insulin-producing cells by our induction protocol.

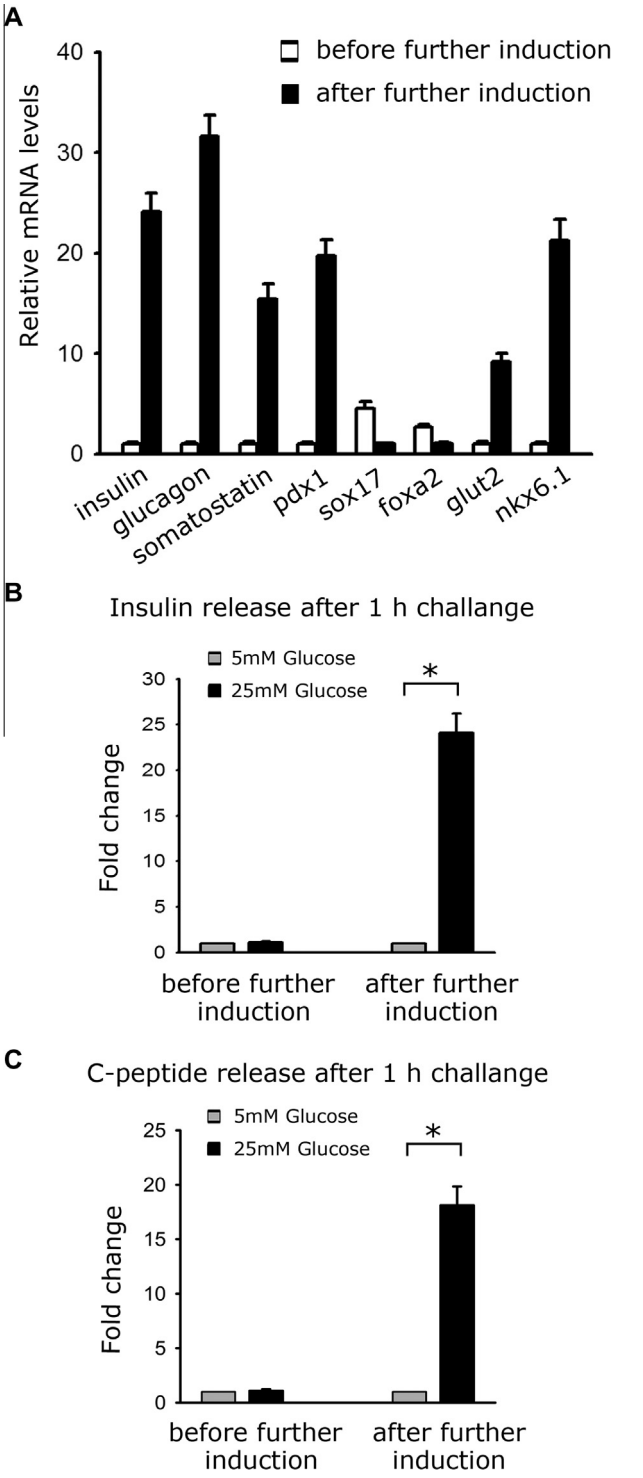

Fig. 4. Morphologic characterization of IPCs. (A) Gene expression changes in miR-302 mimic co-transfected group before or after further incubation in the chemical-defined culture system. Quantification of insulin release (B) and C-peptide (C) in the culture medium in response to 5 mM glucose or 25 mM glucose for 1 h in matured co-transfected cells.

4. Discussion

We have established a novel approach for inducing human hepatocytes to differentiate into insulin-producing cells. HL-7702 can be reprogrammed to pancreatic progenitor fate by transient co-expression of synthetic miR-302a mimic, Pdx1, Ngn3 and MafA.

Because the shortcomings of viral vehicles-mediated gene transfer strategies, we used bicistronic expression plasmids to deliver transcriptional factors. Furthermore, a cocktail of factors could promote differentiation and induce the functional secretion of insulin.

MiR-302 belongs to non-coding RNAs known as microRNAs (miRNA). Many studies have shown that miRNAs have key roles in diverse regulatory pathways, including control of development timing, cell proliferation and organ development [34–36]. The majority of miR-302-targeted genes are transcripts of development signals. In human, miR-302 is highly expressed in hESCs and iPSCs, and plays a critical role in regulating cell stemness and pluripotency [27]. In our study, we showed that co-transfection of miR-302a mimic activated the transcription of pancreas progenitor cells related genes, including *Sox17, Foxa2* and endogenous *Pdx1*.

In our system, we also identified a population of pancreatic progenitors by Sox17, Foxa2 and Pdx1 co-expression, which sufficiently mature into insulin-producing cells in a chemical-defined culture system *in vitro*. Numerous studies have developed induction protocols to generate pancreatic lineage cells from human embryonic stem cells (hESCs) by stepwisely mimicking the major events of the pancreas development [6–8,33]. For each differentiation step, a cocktail of factors was using to increase the differentiation efficiency. In this paper, we also used the culture system containing B27/N2/ITS/bFGF/Exendin4 to further promote pancreatic lineage differentiate to insulin-producing cells. Further research should be conducted to test the functional efficiency of pancreatic conversion *in vivo* by transplanting IPCs to SCID mice whose β-cells have been destroyed. In addition, as hepatocytes isolation leading to tissue damage and the hepatocytes primary cultures are hard to maintain, human fibroblasts will be used for this induction.

In summary, this newly established strategy would provide a method to induce liver-to-pancreas reprogramming with microRNA participation *in vitro*.

Author disclosure statement

The authors declare that no conflicting financial interests exist. J.L., H.Y.D and L.J.L. performed the experiments. J.L., H.L.H. and Q.H.W. evaluated the data. J.L. and J.M.T. wrote the manuscript.

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