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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.

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 mRNAs 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

Abbreviations: EB, embryoid body; hES cell, human embryonic stem cell; IPCs, insulin-producing cells; miRNA, microRNA; PP, pancreatic polypeptide.

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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% CO_2 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 2 μM retinoic acid (RA, Sigma), 20 ng/ml fibroblast growth factor 7 (FGF7, Peprotech), 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 RNeasy 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

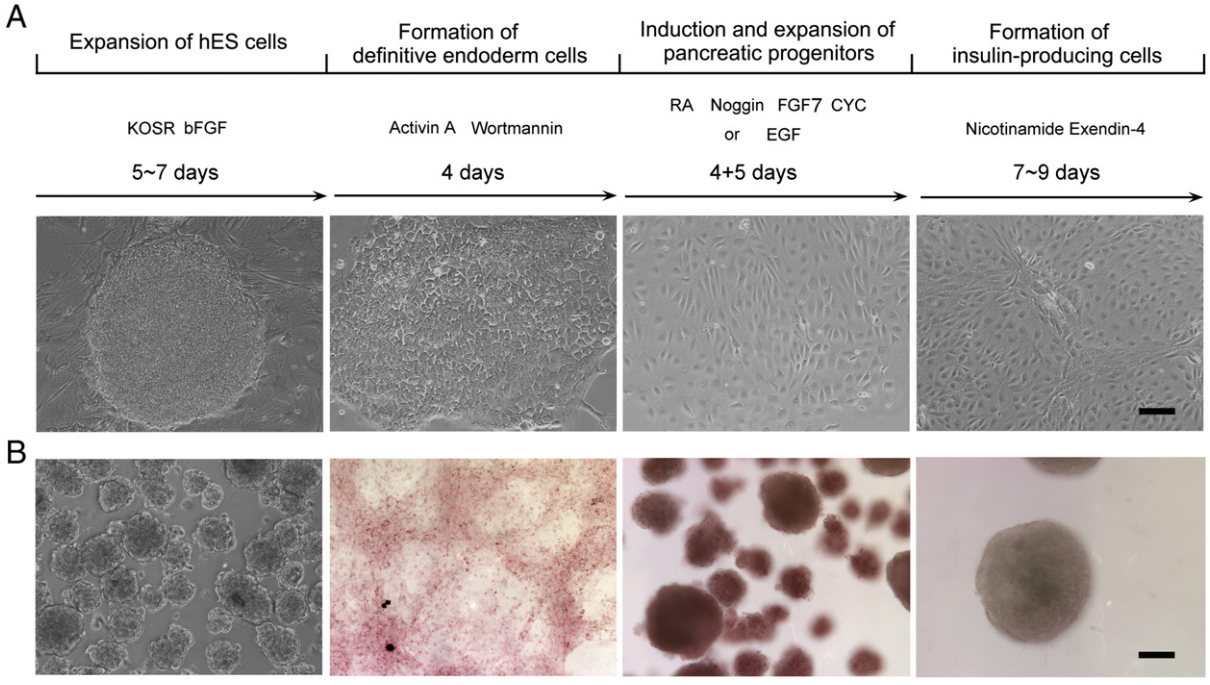

Fig. 1. Differentiation of insulin-producing cells (IPCs) from hES cells. A: The upper panel displaying the differentiation scheme for generating IPCs. The lower panel showing the morphologies of an undifferentiated hES cell colony, definitive endoderm cells, pancreatic progenitors and IPCs in monolayer respectively from left to right. B: The pictures of left to right were the IPCs in suspension culture, the dithizone (DTZ) staining of IPCs in monolayer and in suspension, and an embryoid body (EB) served as negative control of DTZ staining. Scale bars: 50 μm. KOSR: KnockOut™ serum replacement; bFGF: basic fibroblast growth factor; RA: retinoic acid; FGF7: fibroblast growth factor 7; CYC: KAAD-cyclopamine; EGF: endothelial growth factor.