Nestin Action During Insulin-secreting Cell Differentiation

So-Yoon Kim, Song Lee, Seok-Woo Hong, Bon-Hong Min, Ki-Up Lee, Moise Bendayan, and In-Sun Park

Department of Anatomy and BK21 Center for Advanced Medical Education, College of Medicine, Inha University, Incheon, Korea (S-YK, SL, S-WH, I-SP); Department of Pharmacology and BK21 Program for Medical Sciences, College of Medicine, Korea University, Seoul, Korea (B-HM); Department of Internal Medicine, College of Medicine, University of Ulsan, Seoul, Korea (K-UL); and Department of Pathology and Cell Biology, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada (MB)

SUMMARY Nestin, which was initially identified as a marker of neural stem cells, has been reported in regenerating pancreas as well as in early embryonic stem (ES) cell derivatives. However, little is known about its specific roles in stem cells as a functional regulator. We investigated the source of the action of nestin in ES and adult pancreatic ductal stem (PDS) cells in regard to the neogenesis of insulin-secreting β-cells. In ES cells, suppression of nestin by gene silencing led to an increased expression of the pluripotency-associated genes, including Oct 4, Nanog, and SSEA-1, before embryoid body (EB) formation, whereas it reduced endodermal and pancreatic transcription factors in EBs. Inhibition of nestin expression in adult PDS cells caused a low expression of pancreatic transcription factors and islet hormones, leading to poor β-cell development and insulin secretion. These data may indicate not only that nestin is a simple stem cell marker, but also that it constitutes a functional factor at the time of stem cell differentiation. We suggest that nestin plays pivotal roles as an intermediate regulator governing both stemness and differentiation of stem cells in the process of their differentiation into insulin-secreting cells.

KEY WORDS nestin pancreatic stem cell β-cell stemness differentiation

Pancreatic β-cells differentiating from embryonic stem (ES) cells and from adult pancreatic stem cells have emerged as a new and promising source of β-cell replacement therapy for diabetes (Kroon et al. 2008; Xu et al. 2008). However, understanding the differentiation mechanisms of β-cells remains elusive, and understanding them will contribute to enhancing our knowledge of both the quality and the quantity of these newly formed β-cells.

Nestin is an intermediate filament protein transiently expressed during early development in neuronal cells as well as in non-neuronal embryonic and adult cells (i.e., cardiac, skeletal, hepatic, and pancreatic cells) and during tissue regeneration (Lendahl et al. 1990; Dahlstrand et al. 1992; Morshead et al. 1994; Matsuoka et al. 2002; Shibuya et al. 2002; Wiese et al. 2004). In pancreas, nestin expression is considered a marker for pancreatic stem cells and for islet progenitor cells (Kim et al. 2004). Previous studies have shown that insulin-secreting cells are generated from mouse and human (Soria et al. 2000; Assady et al. 2001) ES cells by the selection of progenitor cells expressing nestin (Lumelsky et al. 2001; Blyszczuk et al. 2003). In adult rat pancreas, nestin-positive cells were identified in the islet as a non-endocrine subpopulation that can differentiate ex vivo into cells having pancreatic endocrine, exocrine, and hepatic phenotypes (Zulewski et al. 2001). In addition, we previously observed that nestin is highly expressed at the earlier stages of ductule morphogenesis. Culture of these nestin-positive duct stem cells led to their full

Correspondence to: I.S. Park, Department of Anatomy and BK21 Center for Advanced Medical Education, College of Medicine, Inha University, Shinheung-Dong, Jung-Gu, Incheon, 400-103, Korea. E-mail: sunpark@inha.ac.kr

Received for publication December 15, 2009; accepted February 12, 2010 [DOI: 10.1369/jhc.2010.955682].

© 2010 Kim et al. This article is distributed under the terms of a License to Publish Agreement (http://www.jhc.org/misc/lopub.shtml). JHC deposits all of its published articles into the U.S. National Institutes of Health (http://www.nih.gov/) and PubMed Central (http://www.pubmedcentral.nih.gov/) repositories for public release twelve months after publication.

0022-1554/10/$3.30

The Journal of Histochemistry & Cytochemistry

Downloaded from jhc.sagepub.com by guest on May 2, 2016
differentiation into endocrine cells, mostly β-cells, having the phenotype of glucose-stimulated insulin secretion (Kim et al. 2004). However, it remains unclear whether nestin in ES and adult stem cells supports intermediate regulators for the self-renewal of ES cells and for the differentiation into β-cells. To address this, we have tested two different cells, ES cells and pancreatic ductal stem (PDS) cells in which nestin was depleted using the gene silencing approach.

Materials and Methods

Culture of ES Cells

Mouse D3 ES cells obtained from the American Type Culture Collection (ATCC; Rockville, MD) were maintained undifferentiated on mitomycin C–treated STO cells as feeder layers in DMEM-high glucose (GIBCO/BRL; Grand Island, NY) supplemented with 15% defined fetal bovine serum (FBS; Hyclone, Logan, UT), 10^7 U/ml ESGRO (Chemicon; Charders Ford, Hampshire, UK), 1% non-essential amino acids (GIBCO/BRL), 2 mM l-glutamine (GIBCO/BRL), 0.1 mM β-mercaptoethanol (Sigma; St. Louis, MO), and 100 U penicillin/100 μg streptomycin/ml (GIBCO/BRL) at 37°C in 5% CO₂. ES cells were routinely seeded onto gelatin-coated 12-well plates at 2 × 10⁵ per well without feeder layers for 2 days.

In Vitro Differentiation of ES Cells

According to a previously described protocol (Kahan et al. 2003), with some modifications, we promoted the differentiation of mouse ES cells into insulin-secreting β-cells. After transfection, to establish embryoid bodies (EBs), these cells were plated onto bacteriological culture dishes in DMEM containing 15% FBS in suspension for 4 days (early EB). After 4 days of suspension culture, intact EBs were plated onto gelatin-coated surfaces in 24-well or 60 × 15-mm culture plates (BD Biosciences; Franklin Lakes, NJ). EBs were allowed to differentiate further for 10 days (mid-EB) and 24 days (late EB) with the same medium. Each stage of EBs was characterized by their developmental maturity as follows: early EB as the initial stage at which the EB starts to form, mid-EB as the stage at which differentiation into ecto-, meso-, and endodermal lineages and outgrowth occurs, and late EB as the terminal stage of differentiation that commits progenitors.

Isolation and Culture of PDS Cells

Subtotal pancreatectomy (Px) was performed on 5-week-old male Sprague-Dawley rats (100–120 g; Daehan Experimental Animal, Seoul, Korea) to induce pancreatic regeneration. All animals were treated according to the guidelines of the Laboratory Animal Care Committee of the School of Medicine at Inha University. We isolated the neogenic ductules from the pancreatic remnants 3 days after Px to harvest PDS cells according to protocols described previously (Kim et al. 2004). The isolated ductules were plated onto coverslips in 12-well plates. The tissue fragments were cultured with RPMI medium (GIBCO/BRL) supplemented with 10% heat-inactivated FBS (Hyclone), 100 U penicillin/100 μg streptomycin/ml (GIBCO/BRL), and 2.5 μg/ml of fungizone (GIBCO/BRL) for 4 days until the cell explants developed from the primary ductal tissues. No growth factor or glucose was added to the culture medium, to exclude their effects on cell proliferation and differentiation during the culture (Soria 2001).

Synthesis of Small Interfering RNAs (siRNAs)

The siRNAs corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (as a control template in a standard silencer siRNA cocktail kit reaction) and nestin gene were synthesized using the Silencer siRNA cocktail kit (Ambion; Austin, TX). To generate a transcriptional template for both strands, single-stranded RNA (ssRNA) and long ssRNA were synthesized from the gene-specific PCR products amplified with T7 promoter containing PCR primers (sense and antisense) using separate PCRs. The GAPDH control template is a linear GAPDH double-stranded DNA (dsDNA) fragment with opposing T7 promoters that yields a 440-bp double-stranded RNA (dsRNA) product. Nestin template was used in separate PCR procedures amplified with the following sequences. Producing nestin siRNAs requires four primers containing the T7 promoter: sense 5′-GGGATACGACTCACTATAGGGAGCTTCCCCTGAGCGTGGA-3′ and antisense 5′-GAGAATTCACAGGCTTGAGTTCG-3′; sense 5′-GGGATACGACTCACTATAGGGAGCTTCCCCTGAGCGTGGA-3′ and antisense 5′-GGGATACGACTCACTATAGGGAGCTTCCCCTGAGCGTGGA-3′ (primers giving a 1.1-kb product). After the transcription reaction, both strands of RNA were annealed to form nestin dsRNA. Then 15 μg of dsRNA for each gene was digested with RNase III (Ambion) and the siRNAs were purified according to the manufacturer’s specifications. Here, RNase III–generated siRNA cocktails are a good alternative to standard methods of siRNA production. The siRNA population generated by this method does not exhibit high toxicity or nor does it have nonspecific effects on either nestin or GAPDH gene expression.

Transfection of siRNAs

To determine whether inhibition of nestin affects stemness of ES cells at the early stage, transfection into feeder-free ES cells was carried out with nestin siRNAs at a starting confluence between 40% and 60% at day 1 of culture using the siPORT lipid transfection agent (Ambion). Transfection with reagent alone served as a negative control. Briefly, 100 nM siRNA was incubated with 3 μl siPORT Lipid in 100 μl OPTI-MEM
(GIBCO/BRL) for 35 min at room temperature. The mixture was added to the cells in a final volume of 0.5 ml. PDS cells were also transfected at day 3 of culture as above. The cells were provided with fresh medium 4 hr after transfection and were cultured for 24 hr. The cells were harvested by trypsinization to assess modification of mRNA transcripts of genes and proteins. In addition, the pellets of harvested cells were fixed with Bouin’s solution or 4% paraformaldehyde for analysis of protein expression by immunocytochemistry.

Quantitative PCR (qPCR) Analysis
RNA was isolated with TRIzol (Invitrogen; Carlsbad, CA). Using the ImProm-II Reverse Transcriptase (Promega; Madison, WI), 1 μg of total RNA was then reverse transcribed into cDNA. qPCR analysis was performed on an iCycler iQ multicolor detection system (Bio-Rad, Foster, CA; 94°C for 30 sec, 56°C or 58°C for 30 sec, 72°C for 30 sec) with several PCR primer sets, as listed in Tables 1 and 2. All transcript levels were normalized against GAPDH. The cycle number for the nestin siRNA–transfected cells was calculated relative to mock control. For each evaluated marker, the amplification threshold was defined as a fold value of 1.

Western Blot
ES and PDS cells were harvested 24 hr after transfection. Cells were extracted with 1% NP-40, 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris (pH 7.4), and 1 mM PMSF. Cellular debris was removed by centrifugation at 13,000 rpm for 15 min at 4°C. Supernatants were collected, and proteins were separated by electrophoresis through 10% Tris-HCl polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) and incubated with mouse anti-nestin [clone rat 401, 1:500; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA] and rabbit anti-Oct 4 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hr at room temperature. Finally, membranes were revealed using the Enhanced Chemiluminescence Kit (Roche; Mannheim, Germany).

Immunocytochemistry
For ES cells in culture, coverslips were rinsed three times with PBS and fixed with 4% paraformaldehyde gels. Proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) and incubated with mouse anti-nestin [clone rat 401, 1:500; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA] and rabbit anti-Oct 4 (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hr at room temperature. Finally, membranes were revealed using the Enhanced Chemiluminescence Kit (Roche; Mannheim, Germany).

Table 1 The primers used for qPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct 4</td>
<td>CTCACCCCTGGGCTTCCCCTT</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>GAGAACACCATCCTCTTAG</td>
<td></td>
</tr>
<tr>
<td>Nanog</td>
<td>CAGCCCTGTATCTTCTTACAGG</td>
<td>384</td>
</tr>
<tr>
<td></td>
<td>GATGGGTCACACAGAATGCC</td>
<td></td>
</tr>
<tr>
<td>Hnf3β</td>
<td>ACCGTAGGCCCCGAGTACC</td>
<td>346</td>
</tr>
<tr>
<td></td>
<td>GGGACCTTGAAGAAGGACGTC</td>
<td></td>
</tr>
<tr>
<td>GATA4</td>
<td>GCGCGCCCTGCTGCCCTCC</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>TTGGCTCTCCGTTTTCTGTGGTTGA</td>
<td></td>
</tr>
<tr>
<td>Pdx-1</td>
<td>CTCCGTTGGAACCTGGTAAACA</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>GCTTTGTTGAAATTCATCCAGGG</td>
<td></td>
</tr>
<tr>
<td>Pax4</td>
<td>AATTGGCCAGAGAAGAGAA</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>ATGGGAGGAGGAGCCAGAGAGA</td>
<td></td>
</tr>
<tr>
<td>Nkx2.2</td>
<td>CAGGGGATGTTGATGGTCG</td>
<td>188</td>
</tr>
<tr>
<td>Insulin</td>
<td>TCTTCGCCCTGGCTGCCCTGC</td>
<td>312</td>
</tr>
<tr>
<td>Gluc-2</td>
<td>CGGTGCAAGCATGTGTCGTGG</td>
<td>416</td>
</tr>
<tr>
<td></td>
<td>AGTTCGATAGTCTTCCAG</td>
<td></td>
</tr>
<tr>
<td>Glucagon (mouse)</td>
<td>ACTCACAGGCCCCGACATCCAGCCAGG</td>
<td>353</td>
</tr>
<tr>
<td>Glucagon (rat)</td>
<td>ATCCTTCAGCTGCCAGATCCAGG</td>
<td>197</td>
</tr>
<tr>
<td>Nestin (mouse)</td>
<td>GAGAAGAGAAGGCTGAGAACGAGAAGA</td>
<td>430</td>
</tr>
<tr>
<td>Nestin (rat)</td>
<td>GGGGTACCTGCTGACTACCTG</td>
<td>1092</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, quantitative PCR.

Table 2 The primers used for conventional and qPCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ngn3 (mouse)</td>
<td>TGGCACTCAGCAGAAGCAGGA</td>
<td>444</td>
</tr>
<tr>
<td></td>
<td>ACCCAGGAGCGAGAGGGCTT</td>
<td></td>
</tr>
<tr>
<td>Ngn3 (rat)</td>
<td>GGATGGGAGGAGACATGGGGATG</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>CTTGACAAGGAATGCTGGAGAACACC</td>
<td></td>
</tr>
<tr>
<td>Isl-1 (mouse)</td>
<td>AGATAGGGAGACATGGGGCATG</td>
<td>327</td>
</tr>
<tr>
<td></td>
<td>ACACAGGGAAAAACACTGGATG</td>
<td></td>
</tr>
<tr>
<td>Isl-1 (rat)</td>
<td>CACTTCTGACGTACGGCACC</td>
<td>255</td>
</tr>
<tr>
<td></td>
<td>AAAATCAGTTACACTCCGCCG</td>
<td></td>
</tr>
<tr>
<td>Glucagon (mouse)</td>
<td>ACTCACAGGCCCCGACATCCAGGCAGG</td>
<td>353</td>
</tr>
<tr>
<td>Glucagon (rat)</td>
<td>ATCCTTCAGCTGCCAGATCCAGG</td>
<td>197</td>
</tr>
<tr>
<td>Nestin (mouse)</td>
<td>GAGAAGAGAAGGCTGAGAACGAGAAGA</td>
<td>430</td>
</tr>
<tr>
<td>Nestin (rat)</td>
<td>GGGGTACCTGCTGACTACCTG</td>
<td>1092</td>
</tr>
</tbody>
</table>

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qPCR, quantitative PCR.
anti-nestin (1:250; Abcam, Cambridge, UK), mouse anti-insulin monoclonal antibody (1:1000; Biogenex, San Ramon, CA), rabbit anti-insulin (1:1500; Santa Cruz Biotechnology), rabbit anti-Oct 4 (1:500; Santa Cruz Biotechnology), stage-specific embryonic antigen (SSEA-1) (clone MC480, 1:100; Chemicon), anti-glucagon (1:1000; Dako), anti-somatostatin (1:1000; Dako, Glostrup, Denmark), and goat anti-rat C-peptide (1:200; LINCO Research, Inc., St. Charles, MO). Secondary antibodies were biotinylated rabbit anti-goat IgG, goat anti-mouse IgG, or goat anti-rabbit IgG (1:250; Vector Labs) and the Alexa Fluor 488 goat anti-mouse IgG (1:2000; Molecular Probes, Eugene, OR). Stained ES and PDS cells were examined with a light and a confocal microscope (Bio-Rad).

Measurement of Insulin Secretion
Culture media were collected for measurement of insulin secreted by ES and PDS cells after transfection with mock or nestin siRNAs. Immunoassay for insulin concentrations was performed using an ultrasensitive insulin ELISA kit displaying standard ranges between 0.02 and 5.5 ng/ml (Alpco Diagnostics; Windham, NH).

Morphometry
For morphometric analysis, we counted insulin-positive cells as well as non-immunoreactive duct epithelial cells in explants immunocytochemically stained on coverslips (18 x 18 mm). Data on differentiation are presented as number of insulin-positive cells per 1000 cells of the explants at 24 hr after mock or nestin siRNA transfection.

Statistical Analyses
All values are expressed as means ± SEM. Comparisons were performed by two-tailed unpaired Student’s t-test. Statistical significance was defined as p<0.05 or p<0.001.

Results
Nestin Expression in ES and PDS Cells
We previously reported that neogenic ductules isolated from the regenerating pancreas following partial Px show eccentric expansion with an increase in nestin-positive cells (Kim et al. 2004). Consistent with those results, nestin expression in PDS cells as found in the present study increased and peaked at day 3, but it subsequently decreased to levels lower than those of day 1 (Figure 1A). For ES cells, nestin mRNA expression was significantly increased in EBs at early stages compared with ES cells (Figure 1B), but decreased afterward in mid- and late EBs. Nestin expression in mid- and late EBs was significantly less than that in ES cells.

siRNA-mediated Inhibition of Nestin Expression in ES and PDS Cells
We suppressed nestin expression in stem cells by siRNA to reveal some of the roles that nestin plays in differentiating stem cells. Almost complete absence of nestin expression was reached in both embryonic and adult pancreatic stem cells at the mRNA level as well as protein upon transfection of the cells with nestin siRNA (Figure 2).

Modifications of Stage-specific Genes in ES Cells by Nestin Suppression
We examined the changes of stage-specific genes of stem cells, including Oct 4, Nanog, and SSEA-1, following suppression of nestin expression. These genes are known as specific markers of stem cells (Lumelsky et al. 2001; Zulewski et al. 2001; Blyszczuk et al. 2003). Expression of Oct 4 and Nanog was monitored by qPCR and Western blot analysis 24 hr after transfection (Figures 3A and 3B). Expression of Oct 4 and Nanog was significantly increased 2.4-fold and 6.8-fold respectively, in nestin siRNA–transfected cells (Figure 3A). Consistent with this increase in mRNA transcriptions, Oct 4 protein was also enhanced by suppressing nestin (Figure 3B). SSEA-1 is expressed at the surface of early mouse embryos and mouse cells, and its expression is downregulated along with differentiation of the cells (Solter and Knowles 1978). By suppressing nestin in stem cells, immunofluorescence signal for SSEA-1 showed increased intensities (Figure 3C), which reflects enhanced protein content and suggests maintenance of pluripotency.

Nestin siRNA–mediated Suppression of Transcription and Differentiation Factors
To highlight some of the roles of nestin during insulin cell differentiation, we assessed the expression of

Figure 1 Nestin expression in embryonic stem (ES) cells and pancreatic ductal stem (PDS) cells. Changes of nestin mRNA transcripts were determined by quantitative PCR (qPCR) in PDS cells (A) and ES cells (ESCs), including various stages of embryoid bodies (EBs) (B). The x-fold induction is presented as relative to values of day 1 of culture for PDS cells and ESCs. PDS, pancreatic ductal stem cell. Mean values ± SEM. *p<0.05, **p<0.001 vs control.
specialized transcription factors, islet hormones, and insulin cell–specific genes in nestin-suppressed cells (Figures 4 and 5). qPCR analyses were performed in mid- and late-EB and PDS cells. Most of the factors determined by qPCR analysis showed a decreased mRNA expression after transfection with nestin siRNAs in both ES and PDS cells, when compared with non-transfected control cells. The x-fold induction is represented as a relative value to controls in B. Expression of nestin in ES and PDS cells was also determined by Western blot analysis (C), showing absence or marked reduction in both cell types after transfection with nestin siRNAs. Nestin expression was also revealed by immunocytochemical staining (D). ES cells (upper panel) and PDS cells (lower panel) transfected with nestin siRNAs (right column) and non-transfected (left column) were stained for nestin (green fluorescence). Nuclei were counterstained with propidium iodide (PI; red fluorescence). Staining was carried out 24 hr after transfection. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Mean values ± SEM. **p<0.001 vs control. Bar = 50 μm.

Figure 2  Inhibition of nestin expression by RNA interference in ES and PDS cells. Conventional (A) and qPCR analysis (B) showed a definite reduction of nestin expression after transfection with nestin siRNAs in both ES and PDS cells, when compared with non-transfected control cells. The x-fold induction is represented as a relative value to controls in B. Expression of nestin in ES and PDS cells was also determined by Western blot analysis (C), showing absence or marked reduction in both cell types after transfection with nestin siRNAs. Nestin expression was also revealed by immunocytochemical staining (D). ES cells (upper panel) and PDS cells (lower panel) transfected with nestin siRNAs (right column) and non-transfected (left column) were stained for nestin (green fluorescence). Nuclei were counterstained with propidium iodide (PI; red fluorescence). Staining was carried out 24 hr after transfection. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Mean values ± SEM. **p<0.001 vs control. Bar = 50 μm.

Insulin Cell Differentiation in ES and PDS Cells

Expression of C-peptide and insulin was assessed by immunocytochemistry in differentiated ES and PDS cells after nestin siRNA transfection and mock-control transfection (Figure 6). In late EB, C-peptide–positive cells were numerous in the control, whereas only a few of these cells were observed among the nestin
siRNA–transfected cells (Figure 6A). We also found a remarkable decrease of insulin immunoreactivity among the PDS cells after transfection of nestin siRNA (Figure 6A). The number of differentiated cells among the PDS cells was estimated by the number of insulin-positive cells ($1 \times 10^3$). The insulin-positive cells among nestin siRNA–transfected cells decreased by 2.1-fold ($4.5 \pm 1.14$ cells/$10^3$ duct cells) 24 hr after transfection compared with the control ($9.6 \pm 1.46$ cells/$10^3$ duct cells) (Figure 6B). To detect transitional cells undergoing transformation from nestin-positive cells to insulin-positive cells, we performed double labeling at day 7 of the culture. We found some cells that simultaneously expressed both insulin and nestin. Figure 7 illustrates a particular cell that displays nestin and insulin in two different poles, which indicates cellular transformation from nestin-positive stem stage into differentiated insulin cells.

**Figure 3** Activation of the pluripotent cell state–associated genes by suppression of nestin in ES cells. Oct 4 and Nanog mRNA transcripts assessed by qPCR were significantly increased in ES cells after transfection with nestin siRNAs, compared with non-transfected cells. The x-fold induction is represented as relative to values of mock control (A). Western blot analysis showed increased Oct 4 protein levels in cells transfected with nestin siRNAs (B). The immunostaining for SSEA-1 (green fluorescence in C) is more intense in nestin-deleted cells as compared with the mock control. SSEA-1 expression is evident at the surface of cells (green staining in upper and lower panels in C). PI stains nuclei (red in middle and lower panels in C). The lower panel is a merged image of upper (SSEA-1) and middle panels (PI). *p<0.05. Bar = 50 μm.

**Figure 4** Changes in differentiation factors in EBs. (A) The expression of all genes including Hnf3β, GATA4, Pdx-1, Ngn3, Pax4, Isl-1, insulin, glucagon, somatostatin, Glut-2, and PC2 was decreased by downregulation of nestin in mid-EB (A) and late EB (B) relative to non-transfected cells. The x-fold induction is presented as relative values of the control. Mean values ± SEM. *p<0.05, **p<0.001 vs control.
Insulin Secretion

Insulin concentration in the medium was found to be significantly lower in nestin siRNA–transfected ES cells at late EB (4.19 ± 0.11 pmol/l, n=6, p<0.001) than in the mock-control condition (5.22 ± 0.10 pmol/l, n=6) (Figure 8A). Insulin secretion from the PDS cells transfected with nestin siRNA was also significantly lower than that of the control cells (1.00 ± 0.03 µg/ml vs 1.34 ± 0.03 µg/ml, **p<0.001) (Figure 8B).

Discussion

Stem cells can undergo either proliferation or multilineage differentiation, with the outcomes of either self-renewal or differentiation. Differentiation occurs under the influence of both intrinsic factors and extrinsic signals (Fujikura et al. 2002; Chambers et al. 2003; Boyer et al. 2005). Nestin is a class VI intermediate filament protein that is expressed transiently in early developmental stages and during tissue regeneration in response to injury in a large variety of cell types (Lendahl et al. 1990; Lin et al. 1995; Vaittinen et al. 2001; Shibuya et al. 2002). Recently, in vitro, intestinal epithelial cells derived from nestin-positive cells showed a high but definite proliferative capacity and were able to differentiate into cells expressing neural, pancreatic, or hepatic transcripts and proteins (Wiese et al. 2006). In addition, a distinct subpopulation of nestin-positive mesenchymal stem cells primed toward neural differentiation was described (Lamoury et al. 2006), indicating that nestin expression is a required step for the transformation of stem cells into differentiated functional cells. In pancreatic cells, nestin expression has been suggested as an essential process for stem cell differentiation into insulin-secreting cells (Lumelsky et al. 2001; Zulewski et al. 2001; Kim et al. 2004; Maria-Engler et al. 2004). Although it is still unclear whether nestin expression is required to trigger the differentiation of stem cells into functional cells, there is increasing evidence that intermediate filament proteins are involved in major biological processes, including regulation of cell differentiation, growth potential, and cytoprotection (Franke et al. 1982; Tolstonog et al. 2001,2002). Self-renewal and differentiation are inherent and programmed processes of stem cells, but are distinctive cellular events that are regulated individually by different
intrinsic factors (Niwa et al. 1998; Takeda et al. 2006).

In our previous study on pancreatic cells, we suggested that nestin expression is an intermediate cellular event in the process of differentiation (Kim et al. 2004). In the present study, we attempted to further elucidate the roles of nestin as an intermediate factor in the balance between self-renewal of stem cells and differentiation. We observed that spontaneous expression of nestin is markedly enhanced in early EB and at day 3 in PDS cell cultures. This implies that maximal expression of nestin is concurrent with a shifting stage in stem cells, from cell proliferation dominance to cell differentiation dominance. Several transcription factors used as stem cell markers are known to instigate self-renewal, suppressing differentiation of ES cells. Expression of Oct4, a class V transcription factor of POU (Pit-Oct-Unc) factors, is crucial for the maintenance of ES cell self-renewal (Niwa et al. 2000).

Nanog encodes a divergent homeodomain protein that directs propagation of undifferentiated ES cells (Chambers et al. 2003; Mitsui et al. 2003). This suggests that continuous expression of Oct4 and Nanog is concomitant with the maintenance of pluripotency and stem cell identity as well as prevention of differentiation. We found that deletion of nestin results in an enhanced expression of Oct4 and Nanog in ES cells at an early stage, leading to prevention of differentiation to endoderm and pancreatic lineages. Ectopic expression of Oct4 causes dysplasia by inhibiting cellular differentiation (Hochdelinger et al. 2005). Nanog also acts as a gatekeeper of pluripotency in human embryonic stem and carcinoma cells by preventing differentiation to extraembryonic endoderm and trophectoderm lineages (Hyslop et al. 2005). This suggests that nestin expression may play a role in the self-renewal identity of ES cells.

Inhibition of nestin expression not only downregulated the genes associated with insulin cell development, but also reduced the differentiation of insulin cells from stem cells. As shown in Figures 4 and 5, there was significant downregulation of Hnf3b, Pdx-1, Ngn3, Pax4, Nkx2.2, and Is1-1, factors known to be essential for the determination of the cell fate toward pancreatic development and particularly toward the insulin-secreting cells (Ahlgren et al. 1997; Sosa-Pineda et al. 1997; Dufort et al. 1998; Kaczorowski et al. 2002; Wilson et al. 2003). In addition, we also found that decreases in their expression correlated well with a significant reduction of differentiation of insulin cells and release of insulin (Figures 6 and 8). Nestin thus appears to be an intermediate regulator for differentiation influencing expression of Oct4 or Nanog. In the case of PDS cells, we should be concerned about a possible ambiguity in the cell differentiation process, owing to the possible presence of fibroblasts and other stromal cells with their own interactions during culture. We, however, found similar regulatory actions of nestin on stem cells from different sources. This
may indicate that commitment of stromal cells does not affect the characteristics of the ductal stem cells. On the basis of these data, we suggest an intermediate role for nestin, not only in cell fate determination but also in the differentiation of pancreatic stem cells. We also suggest that inducing nestin expression in stem cells could be a useful strategy to trigger an insulin cell differentiation surrogate for cell replacement therapy in diabetes.

Acknowledgments

This work was supported by the Korean Science and Engineering Foundation (2006-2005402 and 2009-0091914).

Literature Cited


