Differentiation of immature enterocytes into enteroendocrine cells by Pdx1 overexpression

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Yamada, Syu, Hideto Kojima, Mineko Fujimiya, Takaaki Nakamura, Atsunori Kashiwagi, and Ryuichi Kikkawa. Differentiation of immature enterocytes into enteroendocrine cells by Pdx1 overexpression. Am J Physiol Gastrointest Liver Physiol 281: G229–G236, 2001.—The development of a variety of enteroendocrine cells of the gut is poorly understood. We tested whether immature intestinal stem cells were switched to multiple enteroendocrine hormone-producing cells by in vitro transfer of a homeobox gene. We transfected the pancreatic-duodenal homeobox 1 gene (Pdx1) into IEC-6 cells, an embryonic intestinal epithelial cell line derived from a normal rat, and selected the cells that overexpressed Pdx1 by 150-fold compared with control. The cells were examined for differentiation into enteroendocrine cells by immunocytochemical and electron microscopic analyses. Transfected cells cultured on micropore filters formed a trabecular network piled up on monolayer cells. These trabecular cells showed nuclear localization of Pdx1 protein and contained well-developed rough endoplasmic reticulum as well as many secretory granules of pleomorphic shape in the cytoplasm. Antibodies against chromogranin A, serotonin, cholecystokinin, gastrin, and somatostatin stained these secretory granules in the cytoplasm. Furthermore, immunofluorescence double staining analysis showed that different hormones were produced within a cell. These results provide the evidence that immature intestinal epithelial cells can differentiate into multiple hormone-producing enteroendocrine cells in response to overexpression of Pdx1.

Enteroendocrine cells of the digestive tract consist of many subtypes, which are stained with a variety of peptides as well as serotonin. Recent studies in vitro and in vivo suggest that enteroendocrine cells may share a common lineage with epithelial absorptive, goblet, and Paneth cells. All of these cells are derived from primitive intestinal stem cells located in the intestinal crypts (19, 27). However, the process by which the stem cells become enteroendocrine cells is not well known (6). Many transcription factors have been suggested to be involved in this process (1, 15, 29). In particular, BET2 is critical for the normal development of specialized cell types arising from the gut endoderm, because secretin- and CCK-producing enteroendocrine cells fail to develop in the absence of BET2 (15). Another homeobox gene, pancreatic/duodenal homeobox 1 (Pdx1) (10, 14, 17), is also expressed in duodenum, stomach, and pancreatic islets, although, compared with basic helix-loop-helix factor (bHLH) NeuroD/Beta2 (15) expression, it is suggested that Pdx1 has a relatively minor role in the normal differentiation of enteroendocrine cells (8, 16). However, it is also true that Pdx1 is required for organogenesis of the pancreas as well as enteroendocrine cells as revealed in Pdx1 knockout mice (5, 8). Thus the overexpression of Pdx1 may affect differentiation of immature intestinal cells into enteroendocrine cells.

To test this hypothesis, we selected an immature intestinal stem cell line, IEC-6, that is derived from normal rat small intestine (21). These cells have characteristics of immature intestinal crypt cells. They exhibit undifferentiated morphology and have limited expression of intestinal cell-specific genes. Interestingly, Suh and Traber (23) showed that overexpression of a caudal-related homeodomain protein, Cdx2, in IEC-6 cells enabled them to differentiate into two cell types, a goblet cell-like cell and an absorptive enterocyte-like cell. Recently, we showed (9) that exposure of IEC-6 cells to insulin-like growth factor-1 and insulin leads to differentiation of IEC-6 cells to some extent through stimulation of the autocrine/paracrine secretion of transforming growth factor-β1. These observations show that IEC-6 cells provide a suitable model for examining enteroendocrine cell formation.

Thus, in this study, we transfected and overexpressed Pdx1 in IEC-6 cells. Fifty positive clones were obtained, and two stable cell lines (IEC-6-YK14 and IEC-6-YK15) showing the highest levels of Pdx1 mRNA expression were examined for enteroendocrine characteristics by immunocytochemical and electron microscopic analyses.

MATERIALS AND METHODS

Cell lines and culture conditions. We purchased IEC-6 cells, a nontransformed, immature intestinal cell line derived...
from rat small intestine, from the American Type Culture Collection (Rockville, MD) at passage 11. The IEC-6 stock cells were maintained in T-150 flasks in DMEM supplemented with 5% dialyzed fetal bovine serum (FBS), 1 g/l d-glucose, 3.7 g/l NaHCO₃, 0.1 g/l streptomycin, and 10⁵ U/l penicillin G. The flasks were incubated at 37°C under a humidified atmosphere of 95% air-5% CO₂. We subcultured IEC-6 cells with or without Pdx1 overexpression were seeded onto a six-well 0.5-μm micropore filter (Falcon cell culture insert, Becton Dickinson) and cultured for 4 days in DMEM containing 5% FBS before studies. The cells were then washed twice with PBS and used for histological and molecular biological analyses.

**Stable transfectants.** The complete coding sequence of the mouse Pdx1 cDNA was inserted into pcDNA3 plasmid to yield pcDNA3-PDX1 (28). Ten micrograms of pcDNA3-PDX1 or an equal amount of the empty vector, pcDNA3, were transfected into IEC-6 cells by electroporation at 250 V and 975 μF with a Gene Pulser (Bio-Rad, Hercules, CA). Clones resistant to selection medium containing 0.6 mg/ml of G418 (Calbiochem-Novabiochem, La Jolla, CA) were isolated and screened for Pdx1 expression by Northern blot analysis. The Pdx1 signal was compared with that from RNA extracted from neonatal rat duodenal mucosa. We obtained 50 positive clones and examined the two stable cell lines (IEC-6-YK14 and IEC-6-YK15) showing the highest levels of Pdx1 mRNA. In this report, we show the results of IEC-6-YK14 cells with the highest level of Pdx1 mRNA expression at passages 3–10, which was obtained similar results in IEC-6-YK15 cells. There was no difference in growth curve between IEC-6-YK14 cells and IEC-6 empty cells under these culture conditions.

**RNA extraction and Northern blot analysis.** Neonatal Sprague-Dawley rats were anesthetized and killed by intraperitoneal injection of pentobarbital sodium. The duodenal mucosa and pancreas were dissected, washed with PBS, snap frozen, and then stored at −70°C. Cultured cells were washed three times with PBS, harvested by digestion with 10% trypsin before addition of TRIZol (GIBCO BRL, Rockville, MD), and then stored at −70°C for completion of RNA extraction at a later date. Total RNA was extracted by using acid guanidinium isothiocyanate-phenol-chloroform as described previously (13). Ten-microgram aliquots of the RNAs were used for Northern blot analysis. The Northern blots were hybridized with a fragment of the mouse Pdx1 cDNA that was released by digestion with SmaI subcloned into the pcDNA-PDX1 plasmid for amplification. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was used for normalization to evaluate differences between different samples.

**Immunocytochemical analysis.** The cells were fixed for 2 h with 4% paraformaldehyde, 0.2% picric acid, and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C and then incubated for an additional 12 h with 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB at 4°C. After the cells were washed for 24 h with PBS at 4°C, they were incubated for 48 h with the following antibodies diluted 1:5,000 in PBS containing 0.3% Triton X-100 (PBST) at 4°C. Rabbit antiserum to a synthetic peptide of Pdx1 (28), rat chromogranin A, CCK, gastrin, or gastric inhibitory polypeptide (GIP) (Yanaihara Shizuoka), mouse monoclonal antibodies to somatostatin (3) and serotonin (5-hydroxytryptamine; Ref. 26), and rabbit antiserum to apolipoprotein A-1 (25) were used in this study. After being washed with PBST, the cells were incubated for 2 h with species-specific biotinylated IgG (Vector Labs, Burlingame, CA) diluted to 1:1,000 in PBST at room temperature and then reacted for 1.5 h with avidin-biotin peroxidase complex (Vector Labs) diluted to 1:1,000 in PBST at room temperature. The immunoreaction was then visualized by developing with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3’-diaminobenzidine, 1% ammonium nickel sulfate, and 0.0005% H₂O₂ for 30 min at room temperature. The microprobe filters with stained cells were mounted on gelatin-coated glass slides, dehydrated by graded ethanol, coverslipped with Entellan (Merek, Darmstadt, Germany), and observed by using light microscopy. For negative-stained cells, counterstaining with 0.1% neutral red solution was performed.

For the double immunofluorescence staining, the fixed cells were incubated for 48 h in either a mixture of the specific antibodies against chromogranin A and serotonin or antibodies against serotonin and somatostatin, which were diluted to 1:5,000 in PBST at 4°C. After being washed with PBST, the cells were incubated for 2 h at room temperature with either a mixture of FITC-labeled anti-mouse IgG (Vector Labs) and Texas red-labeled anti-rabbit IgG (Vector Labs) for chromogranin A-serotonin or serotonin-somatostatin double staining. After rinsing cells with PBST, we mounted microprobe filters with attached cells on the glass slides, and cells were dried, coverslipped with liquid paraffin, and observed under a confocal laser scanning image system (MRC-600; Bio-Rad). We also performed immunofluorescence staining for Pdx1, CCK, gastrin, GIP, and apolipoprotein A-1, in which Texas red-labeled anti-rabbit IgG (Vector) was used as a second antibody and was observed by using the confocal laser scanning imaging system.

The specificity of the positive staining was examined by an immunocytochemical absorption study. The primary antibodies were replaced with an antigen-antibody mixture in which we used recombinant rat chromogranin A, serotonin, synthetic somatostatin 14, and recombinant Pdx1 peptide at a concentration of 10 mM each.

**Electron microscopy.** The cultured cells were fixed as described in Immunocytochemical analysis and incubated for 1 h with 1% OsO₄ in 0.1 M PB at 4°C. The samples were then dehydrated with a graded series of ethanol and propylene oxide and embedded in epoxy resin. Ultrathin sections were made in an ultramicrotome (Ultracut E; Reichert-Jung, Vienna, Austria) vertically or horizontally along the filter and mounted on 200-mesh copper grids. Cells were stained for 20 min with 2% uranyl acetate and for an additional 5 min with Reynolds’ solution and then observed under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

For the immunoelectron microscopic study, fixed cells were incubated overnight with LR Gold resin (London Resin, Basingstoke, UK) at −20°C and incubated for an additional 1 h with LR Gold resin containing 0.1% benzil (Pelco; Ted Pella, Redding, CA) at −20°C (18). The embedded specimens were allowed to polymerize for 4 h in an Ultraviolette Cryo Chamber (Pelco; Ted Pella) at −20°C. The embedded specimens (60 nm) were cut in an ultramicrotome and picked up on nickel grids. The nickel grids were incubated with 3% normal goat serum (DAKO Japan, Tokyo, Japan) dissolved in a reaction buffer (0.1 M PBS containing 0.2% bovine serum albumin, 0.2% saponin, and 0.05% NH₄Cl, pH 7.4) for 30 min at room temperature. The sections were incubated with antibody against chromogranin A (1:300) diluted with the reaction buffer. The sections were washed with 0.1 M PBS and incubated for 1.5 h with immunogold-conjugated goat anti-rabbit IgG (15 nm gold; British BioCell International, Cardiff, UK) diluted 1:40 with the reaction buffer, followed by washing with 0.1 M PBS and then with distilled water. The sections
were stained with 2% uranyl and Reynolds’ solution and then examined by electron microscope (H-7100; Hitachi).

**RT-PCR analysis.** Using 1 μg of the total RNA as a template and a set of antisense oligonucleotides complementary to each mRNA, we reverse transcribed the first cDNA strands in 30 μl of a reaction mixture containing reagents (Takara, Kyoto, Japan). For the subsequent PCR, 1 μl of the reaction mixture was used each time. The specific oligonucleotide primers (5’ and 3’) were used for the amplification of each transcription factor. The thermal cycle profile was as follows. A single 1-min denaturing step at 94°C was followed by 30 cycles of 30 s at 94°C, 45 s at 54°C, and 1 min at 72°C. The primer pairs were as follows (forward and reverse): NeuroD/Beta2, 5’-GCAAAAGTTTGTGCCAGC-3’ and 5’-ACGT-GGAAGAGCTGGGAG-3’; hepatocyte nuclear factor (HNF)-1α, 5’-ATGAGCCCTGCTCCTCC-3’ and 5’-GTTGGATGCAGCAGTG-3’; HNF-3α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-3β, 5’-GGCTCCTTCCGACCCTCCTG-3’ and 5’-ACCTGGCTTGCTGCTCTG-3’; HNF-4α, 5’-TACATCAACGACCGGCAG-3’ and 5’-GTGGAGTACGTGAC-3’; glucose-6-phosphate dehydrogenase, 5’-GCAAAGGTTTGTCCCAGC-3’ and 5’-ATGAGCCCTGCTCCTCC-3’; HNF-1α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-3α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-4α, 5’-TACATCAACGACCGGCAG-3’ and 5’-GTGGAGTACGTGAC-3’; hepatocyte nuclear factor (HNF)-1α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-3α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-4α, 5’-TACATCAACGACCGGCAG-3’ and 5’-GTGGAGTACGTGAC-3’; glucose-6-phosphate dehydrogenase, 5’-GCAAAGGTTTGTCCCAGC-3’ and 5’-ATGAGCCCTGCTCCTCC-3’; HNF-1α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-3α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-4α, 5’-TACATCAACGACCGGCAG-3’ and 5’-GTGGAGTACGTGAC-3’; glucose-6-phosphate dehydrogenase, 5’-GCAAAGGTTTGTCCCAGC-3’ and 5’-ATGAGCCCTGCTCCTCC-3’; HNF-1α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-3α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-4α, 5’-TACATCAACGACCGGCAG-3’ and 5’-GTGGAGTACGTGAC-3’; glucose-6-phosphate dehydrogenase, 5’-GCAAAGGTTTGTCCCAGC-3’ and 5’-ATGAGCCCTGCTCCTCC-3’; HNF-1α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-3α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-4α, 5’-TACATCAACGACCGGCAG-3’ and 5’-GTGGAGTACGTGAC-3’; glucose-6-phosphate dehydrogenase, 5’-GCAAAGGTTTGTCCCAGC-3’ and 5’-ATGAGCCCTGCTCCTCC-3’; HNF-1α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-3α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-4α, 5’-TACATCAACGACCGGCAG-3’ and 5’-GTGGAGTACGTGAC-3’; glucose-6-phosphate dehydrogenase, 5’-GCAAAGGTTTGTCCCAGC-3’ and 5’-ATGAGCCCTGCTCCTCC-3’; HNF-1α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-3α, 5’-AACAATGAACTCCGGCCTGGG-3’ and 5’-AGCTGCTAGCTTTCCTCCTG-3’; HNF-4α, 5’-TACATCAACGACCGGCAG-3’ and 5’-GTGGAGTACGTGAC-3’; glucose-6-phosphate dehydrogenase.

**RESULTS**

**Morphological changes in IEC-6 cells that overexpress Pdx1.** We obtained 50 G418-resistant clones after transfection of IEC-6 cells with pcDNA3-Pdx1 and then used two stable clones, IEC-6-YK14 and IEC-6-YK15, exhibiting the highest level of Pdx1 mRNA expression in the following experiments (Fig. 1). The level of Pdx1 mRNA in IEC-6-YK14 cells was 150-fold higher than that in IEC-6 cells containing empty vector. This increase was specific for Pdx1, because the level of Cdx2 mRNA in the IEC-6-YK14 cells was not increased (data not shown).

Within 3–4 days after seeding onto micropore filters, IEC-6-YK14 cells grew in monolayers and then formed trabecula-shaped cell networks (Fig. 2), although no such cell networks appeared in cultures of IEC-6 cells containing empty vector (Fig. 2a). Staining of IEC-6-YK14 cells with a specific antibody against Pdx1 revealed that trabecular cells grown on micropore filters showed strong positive staining for Pdx1 in the nucleus (Fig. 3a, b and b’). In contrast, trabecular cells grown on micropore filters showed very little staining for Pdx1 in the cytoplasm without staining in the nucleus (Fig. 3a).

**Immunocytochemical analyses of production of multiple hormones in IEC-6-YK14 cells.** To characterize the trabecular cells, we stained them with an antibody against chromogranin A, an acidic protein present in the secretory granules of a wide variety of endocrine cells, and stained them with an antibody against apolipoprotein A-1 as a marker of absorptive enterocytes. IEC-6 cells containing empty vector cultured on micropore filters showed no staining for chromogranin A (Fig. 4a) but positive staining with apolipoprotein A-1 (Fig. 4c). In contrast, IEC-6-YK14 cells showed positive staining for chromogranin A only in trabecular network-forming cells (Fig. 4b) but negative staining with apolipoprotein A-1 (Fig. 4d). Furthermore, those trabecular cells also positively stained with antisera against serotonin (Fig. 5a, c, and g) and gastrin (Fig. 5k), showing positive staining for chromogranin A (Fig. 4a) and nontrabeculated cells were only stained in cytoplasm. However, IEC-6 cells containing empty vector cultured on micropore filters showed very little staining for Pdx1 in the cytoplasm without staining in the nucleus (Fig. 3a). YK14 cells with a specific antibody against Pdx1 revealed that trabecular cells grown on micropore filters showed strong positive staining for Pdx1 in the nucleus (Fig. 3a, b and b’). In contrast, IEC-6-YK14 cells showed positive staining for chromogranin A only in trabecular network-forming cells (Fig. 4b) but negative staining with apolipoprotein A-1 (Fig. 4d). Furthermore, those trabecular cells also positively stained with antisera against serotonin (Fig. 5a, c, and g) and gastrin (Fig. 5k), showing positive staining for chromogranin A (Fig. 4a) and nontrabeculated cells were only stained in cytoplasm. However, IEC-6 cells containing empty vector cultured on micropore filters showed very little staining for Pdx1 in the cytoplasm without staining in the nucleus (Fig. 3a).

**Fig. 1.** Expression of Pdx1 mRNA in IEC-6 cells with or without Pdx1 overexpression by Northern blot analysis. Total RNA from neonatal rat duodenum (lane 1), IEC-6 empty cells cultured on micropore filters (lane 2), IEC-6-YK14 cells cultured on micropore filters (lane 3), or IEC-6-YK15 cells cultured on micropore filters (lane 4) were subjected to gene electrophoresis, blotted, and hybridized with radiolabeled probes as described in MATERIALS AND METHODS. Top: result for Pdx1 mRNA. Bottom: result for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The blot shown is representative of 3 independent experiments.

**Fig. 2.** Photomicrographs of IEC-6 empty (a) and IEC-6-YK14 (b) cells cultured for 4 days on micropore filters. Trabecular formation was seen in cultured IEC-6-YK14 cells but not in cultured IEC-6 empty cells. Bar = 50 μm. The picture was taken by a phase-contrast light microscope.
Fig. 3. 3,3’-Diaminobenzidine-nickel (a, b) and fluorescence (b’) immunocytochemical staining of Pdx1. a: IEC-6 empty cells cultured on micropore filters. Pdx-1 staining was not found above the background level. Counterstaining with 0.1% neutral red solution was performed. b and b’: IEC-6-YK14 cells cultured on micropore filters. Pdx1 was strongly stained in trabecular network-forming cells compared with nontrabeculated cells (b). At higher magnification, the trabecular network-forming cells (surrounded by small arrows) were strongly stained in the nucleus (large arrows) (b’). In contrast, nontrabecular cells were stained by Pdx1 antibody only in cytoplasm. Bars = 50 μm (a, b) and 25 μm (b’).

Fig. 4. Immunocytochemistry for chromogranin A (a and b) and apolipoprotein A-1 (c and d) in IEC-6 empty cells (a and c) and in IEC-6-YK14 cells (b and d). Counterstaining with 0.1% neutral red solution is shown in a. Chromogranin A was strongly stained in the cytoplasm of trabecular-forming cells in b (arrows) in contrast to no positive staining in cells in a, where the reaction was of background level. Apolipoprotein A-1 was positively stained in IEC-6 empty cells (c) but was not stained in IEC-6-YK14 cells (d). Bars = 50 μm.
Fig. 5. Double staining for serotonin-chromogranin A (a–f) and serotonin-somatostatin (g–i) in IEC-6-YK14 cells cultured on micropore filters, in lower (a–c)- and higher (d–i)-magnification views, and single staining for CCK (j) and gastrin (k). Serotonin (a, d) and chromogranin A (b, e) immunoreactivities seen in the cytoplasm completely overlapped (c, f) in the trabecular network-forming cells. Serotonin (g) and somatostatin (h) immunoreactivities also overlapped (i) in a trabecular network-forming cell. CCK (j) and gastrin (k) were also stained in the cytoplasm of trabecular network-forming cells. Arrows represent the presence of each hormone in the cells. Bars = 50 μm.
Finally, we examined whether each cell expressed one or more hormones by using confocal laser scanning microscopy (Fig. 5). Under low magnification, the staining of chromogranin A appeared in the trabecular network-forming cells as red staining (Fig. 5b) and the staining of serotonin in the same field showed a similar pattern but in green (Fig. 5a). Colocalization analysis of these two hormones revealed an identical pattern outlined in yellow (Fig. 5c). We observed identical results at a higher magnification (Fig. 5, d–f). Similar findings were also observed for somatostatin (Fig. 5b) and serotonin (Fig. 5g), with overlapping staining (Fig. 5i).

Electron microscopic features of IEC-6-YK14 cells. IEC-6-YK14 cells cultured on micropore filters for 4 days were examined by electron microscopy. Two different types of cells were seen, i.e., a monolayer of thin-body cells that were in contact with the micropore filter and thick-body cells piled up on the monolayer cells (Fig. 6a).

These findings and those obtained by light microscopy indicated that the trabecular network-forming cells were thick-body cells. In these cells, the rough endoplasmic reticulum was well developed, and, most characteristically, the cells had many cytoplasmic granules of pleomorphic shapes (Fig. 6b). Moreover, immunoelectron microscopic analysis for localization of chromogranin A showed that the cores of some granules were colocalized with immunogold particles, but other granules were not (Fig. 6c). In IEC-6 empty cells, on the other hand, the endoplasmic reticulum was poorly developed and granules were not observed in the cytoplasm (Fig. 6d).

mRNA expression of transcription factors in IEC-6-YK14 cells. We examined the expression of some important nuclear transcription factors in IEC-6 cells; expressions in IEC-6-YK14 cells were compared with those of either IEC-6 empty cells or neonatal rat small intestine by RT-PCR (Fig. 7). IEC-6 cells, IEC-6 empty cells, and IEC-6-YK14 cells were expressed with NeuroD/Beta2, HNF-1α, HNF-3α, HNF-3β, and HNF-4α, which were similar to those of neonatal rat small intestine.

DISCUSSION

In this report, we show that an immature intestinal crypt cell line undergoes differentiation into multiple
hormone-producing enteroendocrine cells after being overexpressed with Pdx1. Compared with wild-type IEC-6 cells cultured on micropore, IEC-6-YK14 cells overexpressing Pdx1 consisted of two cell types: one cell type formed a trabecular cell network, and the other formed a round cell mass surrounded by trabecular cells. The trabecular cells showed strong Pdx1-positive immunoreactivity in the nucleus compared with nontrabeculated cells. Only trabecular cells acquired enteroendocrine cell-like characteristics, leading to the synthesis of chromogranin A and hormones including serotonin, somatostatin, CCK, and gastrin. However, we could not show synthesis of insulin, glucagon, GLP-1, or GIP in those cells under these culture conditions. Consistently, an expression of apolipoprotein A-1, a typical enterocyte marker (30), was lost in IEC-6-YK14 cells, although IEC-6 cells expressed the marker. These data suggest that overexpression of Pdx1 induces the differentiation into endocrine cells specific for the upper small intestine but not the pancreas. Immunofluorescence double staining for these products showed that multiple hormones were colocalized in the same cells. These data are intriguing in view of the evidence that colocalization of multiple hormones in one cell is a characteristic feature of an early stage of endocrine cell differentiation (22).

Our results directly indicate that overexpression of Pdx1 can play some role in the differentiation of enteroendocrine cells from immature epithelial cells. Although Pdx1 seems to be important for enteroendocrine cell formation, the differentiation of intestinal stem cells into nonendocrine cells requires the factor Cdx2 (12, 23). This factor was not normally expressed in IEC-6 cells in the presence or absence of Pdx1. Wild-type IEC-6 cells form a simple monolayer of flat epithelial cells in culture. Overexpression of Cdx2 in IEC-6 cells enables them to differentiate into absorptive enterocytes or goblet cells. These cells grown in culture tend to form clusters of round cells and a surrounding trabecular cell network (latticelike structure) (23). Electron microscopic analysis also reveals that the cells overexpressing Cdx2 grow in piles. The piles consist of a lower layer of flat cells and an upper layer of trabecular cells, which are differentiated cells. These morphological characteristics were also seen in the cells overexpressing Pdx1 in the present study. We found that the upper cells had strong immunostaining for various hormones but the lower monolayer of nontrabeculated flat cells did not. On the basis of these observations, it appears that a divergent mature intestinal cell lineage can arise from IEC-6 cells in response to overexpression of either Pdx1 or Cdx2. Cdx2 expression stimulates immature cells to the formation of more differentiated absorptive enterocytes and goblet cells, and Pdx1 overexpression leads to the formation of enteroendocrine cells.

How do our results provide a better understanding of intestinal cell differentiation into enteroendocrine-like cells? According to published studies, enteroendocrine cell differentiation requires other more important transcription factors including bHLH NeuroD/Beta2 (15), neurogenin-3 (4), and members of the HNF family (2, 11, 20, 22, 24). Furthermore, it has also been reported that Hes1, which is activated by Notch signaling, is shown to inhibit neurogenin 3 and NeuroD cascade, resulting in impairment of terminal differentiation of endodermal endocrine cells (7). Mice homozygous for a null mutation in some of these transcription factors showed marked morphological changes, with defects in endocrine function not only in the small intestine but also in the pancreas. For example, secretin- and CCK-producing enteroendocrine cells failed to develop in the absence of NeuroD/Beta2 (15). Furthermore, foregut morphogenesis is severely affected and tube formation is defective in HNF-3β−/− embryos (1, 29). In the present study, we found that HNF-1α, HNF-3α, HNF-3β, HNF-4α, and NeuroD/Beta2 were expressed in IEC-6 cells at the basal culture conditions (Fig. 7). However, analysis of complex interactions among many transcription factors for the control of the differentiation of intestinal cells into enteroendocrine cells awaits future study, because the levels of these transcription factors in IEC-6 cells with or without Pdx1 overexpression were too low to analyze further. Thus the endocrine differentiation seen by Pdx1 overexpression may indicate that the relatively immature IEC-6 cells have retained some of the multipotent properties of the intestinal stem cell that permit forced differentiation into enteroendocrine cells.

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