Changes in the Expression of Transcription Factors in Pancreatic AR42J Cells During Differentiation Into Insulin-Producing Cells

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Pancreatic AR42J cells possess both exocrine and neuroendocrine properties and convert to insulin-producing cells upon treatment with activin A and hepatocyte growth factor (HGF). We studied changes in the mRNA expression of various transcription factors during the course of differentiation. Among the transcription factors studied, expression levels of Pax4 and neurogenin3 changed significantly. These two factors were not detected in naive cells, whereas their mRNA levels were markedly increased after treatment with activin A and HGF. Thus, these two factors were induced by activin A. Transfection of Pax4 did not induce any changes in morphology or expression of pancreatic polypeptide (PP). Furthermore, introduction of antisense Pax4 did not affect the conversion into insulin-producing cells induced by activin A and HGF. In contrast, transfection of neurogenin3 induced morphological changes similar to those induced by activin A. In addition, transfection of neurogenin3 induced the expression of PP. Conversely, introduction of antisense neurogenin3 blocked the differentiation of AR42J cells induced by activin A and HGF. These results indicate that activin A regulates the expression of neurogenin3, which is critical for the differentiation of AR42J into endocrine cells. *Diabetes* 50 (Suppl. 1):S10–S14, 2001

Pancreatic endocrine and exocrine cells are thought to arise from common precursor cells located in the epithelium of pancreatic anlage during development (1–3). Recent genetic studies indicate that pancreatic development depends on an integrated network of distinct transcription factors operating at various levels. Several homeodomain and basic helix-loop-helix transcription factors have been postulated to play important roles in regulating differentiation of pancreatic endocrine cells. A mouse homeobox protein, insulin promoter factor-1 (IPF-1/PDX-1), is required for development of the murine pancreas (4,5). Islet-1 (Isl-1), a LIM homeodomain-containing protein, is necessary for the development of the dorsal pancreas and is required for generation of islet cells (6). In addition, inactivation of Beta-2/NeuroD causes a striking reduction in the number of insulin-producing cells, and mice lacking the functional Pax4 gene lack differentiated β- and δ-cells and fail to develop mature islets (7,8). In contrast, the number of all differentiated endocrine cell types, especially the number of α-cells, is markedly reduced in mice lacking the Pax6 gene (9). These transcription factors are thought to be involved in a complex regulatory network and cascade to exert their functions (10,11). However, because differentiation of pancreatic β-cells involves many steps, it is not clear how these steps are regulated by transcription factors.

Pancreatic AR42J cells are derived from a chemically induced pancreatic tumor and express both exocrine and neuroendocrine properties (12). Upon treatment with activin A, AR42J cells stop growing, and their morphology changes significantly by extending neurites (13). In addition, activin-treated cells express mRNA for GLUT2, ATP-sensitive potassium channel, and pancreatic polypeptide (PP). Thus, activin A converts AR42J cells into endocrine cells (13,14). Furthermore, in the presence of betacellulin (14) or hepatocyte growth factor (HGF) (15), activin-treated AR42J cells further convert to insulin-producing cells. Thus, AR42J cells provide a model system to study the molecular mechanism involved in β-cell differentiation. The present study was conducted to investigate how the expression of islet-associated transcription factors is changed during the differentiation of AR42J cells. We also assessed the role of such transcription factors in differentiation.

RESEARCH DESIGN AND METHODS

Materials. Recombinant human activin A was provided by Dr. Y. Eto (Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan). Recombinant HGF was purchased from R & D Systems (Minneapolis, MN). Anti-flag M2 mouse monoclonal antibody was purchased from Eastman Kodak Systems (New Haven, CT). Polyclonal antibodies against porcine insulin and Pax4 were provided by Dr. K. Wakabayashi (Gunma University, Maebashi, Japan) and Dr. Y. Oka (Yamaguchi University, Ube, Japan). Other commercially available antibodies were from sources described previously (16).

Cell culture and transfection. AR42J-B13 cells, a subclone of AR42J cells (15) that convert into insulin-producing cells after treatment with activin A and HGF, were cultured as described (15). Transfection for immunofluorescence and immunoblotting was performed as described (16). The transfection efficiency was 20–30%.

Analysis of mRNA by reverse transcription–polymerase chain reaction. Total RNA was extracted from AR42J cells cultured with or without activin A and/or HGF at different time points using TRIzol Reagent (Gibco-BRL, Grand Island, NY). Messenger RNA was extracted using the Quick Prep Kit (Pharmacia LKB Biotechnology, Piscataway, NJ). RNA was also obtained from rat pancreatic islets or INS-1 cells as the control. RNA or mRNA samples were pretreated with DNase to remove contamination of genomic DNA. First-strand cDNA was synthesized by using a Preamplication System for First Strand cDNA Synthesis Kit (Gibco BRL). To confirm no contamination of genomic DNA, samples without reverse transcription (RT) treatment were prepared. Oligonucleotide primers

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HGF, hepatocyte growth factor; PCR, polymerase chain reaction; PP, pancreatic polypeptide; RT, reverse transcription.

TABLE 1
Sequences of PCR primers used in the study

Gene	Sense primer	Antisense primer	Size of product (bp)	GenBank accession number
lmx1.1	GGGATGAGTTTGTCCTGAAGG	GTCAATGGGGTTTCCCTC	657	X81406(ha)
lmx1.2	GACTACGAGAAGGAGAAGGA	TGGTTCTGAAACCAGACCTGG	308	U77457(hu)
lim1(lmx2)	TGCGTCCAGTGCTGTGAATG	GCTGCTTGTTACACATCATGCA	212	Z27410(m)
islet-1	ACGTCTGATTTCCCTATGTGTTGG	TCGATGTGGTACACCTTAGAGCGG	276	S69329(r)
cdx2/3	AGCCAAGTGAAAACCAGGAC	CTGCTGCTGCTGTTGCTGCTGC	234	AF007884(hu)
cdx4	GCAGGTGACGGGGAAAACCAGG	CTCTCCTTGGCTCTGCGATTCTG	194	AF029877(hu)
Nkx2.2	CCGAGAAAGGTATGGAGGTGAC	CTGGGCGTTGTACTGCATGTGCTG	187	X81408(ha)
Nkx6.1	ATGGGAAGAGAAAACACACCAGAC	TAATCGTCGTCGTCCTCCTCGTTC	280	AF004431(r)
Pax4	TGGCTTTCTGTCCTTCTGTGAGG	TCCAAGACTCCTGTGCGGTAGTAG	214	AF053100(r)
Pax6	AAGAGTGGCGACTCCAGAAGTTG	ACCACACCTGTATCCTTGCTTCAGG	545	U69644(r)
PDX-1/IDX-1	ACATCTCCCCATACGAAGTGCC	AAGTTGAGCATCACTGCCAGCTCC	364	U04833(r)
HB9	CAGCACCCGGCGCTCTCCTA	GAACTGGTGCTCCAGCTCCAGCAGC	250	NM-005515(hu)
Hox1.11	GCGTCTGAGAACTGCTTACACCAAC	TTGTGTCCATTGGGAGCCTG	366	M91802(r)
neurogenin3	AAGAGCGAGTTGGCACTGCGCAG	GCTGTGGTCCGCTAGAGGAC	222	AJ133776(hu)
NeuroD/Beta-2	AGCCATGAATGCAGAGGAGGAC	GGACAAACCTTTGCAGAGGACGAG TGTCTG	400	AF107728(r)
GAPDH	CATGACCACAGTCCATGCCATC	CACCCTGTTGCTGTAGCCATATTC	451	M17701(r)

ha, hamster; hu, human; m, murine; r, rat.

used in this study are shown in Table 1. The reactions were conducted in a DNA Thermal Cycler (PerkinElmer, Norwalk, CT) under the following conditions: for insulin, PP, glucagon, and Pax4, denaturation at 94°C for 1 min, and annealing and extension at 65°C for 2 min; for Pdx-1, Pax6, Nkx2.2, Nkx6.1, Beta2, neurogenin3, and GAPDH, denaturation at 94°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s; and for lmx1.2, lmx2, cdx4, and hox1.11, denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 45 s. The number of cycles was 30, with the exception of 17 cycles for GAPDH.

Generation of a flag epitope–tagged form of Pax4 and neurogenin3 and their antisenses. To generate the NH_2-terminal flag epitope–tagged Pax4, an oligonucleotide containing the flag-tagged sequence GGGAATTCCCACCATG GACTACAAAGACGATGACGACAAG was used with another primer that was located around the initiation codon of rat Pax4 (ATGCAGCAGGACGGTCTC AGC) in high-fidelity polymerase chain reaction (PCR) (Pyrobest DNA polymerase; Takara Shuzo, Tokyo) as the sense primer. The antisense primer for Pax4 is 5′-GGGAATTCTTATGGCCAGTGTAAGTAATAGGTTGATG-3′. Neurogenin3 was cloned as for Pax4. The sense primer for neurogenin3 is 5′-GGGGATCCCCACCATGGACTACAAAGACGATGACGACAAGATGGCGCCTC ATCCCTTG-3′ and for antisense is 5′-GGGGATCCTCACAAGAAGTCTGAGA ACACCAG-3′. The PCR product was then confirmed by sequencing using an ABI Prism Dye Terminator Cycle Sequencing FS Ready Reaction Kit and Applied Biosystems DNA sequencer model 373S (ABI, Foster City, CA) and cloned into the PCDNA3 and/or pIRES-EGFP expression vectors inserted in both the sense and antisense orientation.

Immunofluorescence study. Cells were cultured on noncoated glass coverslips at a density of 2×10^5 cells/ml and transiently transfected with sense and antisense of Pax4 or neurogenin3. Cells were washed, and fresh medium was added. HGF or HGF plus activin A was added according to the experimental protocol. Cells were washed, fixed, and immunostained with specific anti-flag, anti-Pax4, or anti–green fluorescent protein (GFP) antibodies 48 hours later as described in detail elsewhere (16). Images were obtained using a Zeiss microscope equipped with fluorescein and rhodamine filter sets (Axiophoto; Carl Zeiss, Thornwood, NY).

RESULTS

Expression of islet-associated transcription factors during differentiation of AR42J cells. In the first set of experiments, we investigated the mRNAs for various transcription factors expressed in islets. We found that the expression for lmx1.2, lmx2, cdx4, Nkx2.2, Pdx-1, and Beta2 was detected in naive AR42J cells and had no significant changes during the differentiation into insulin-secreting cells. The mRNA for Pax6, Nkx6.1, and Isl-1 was undetectable before and after differentiation. The mRNA for lmx1.1, Pax4, Hox1.11, and neurogenin3 was upregulated during differentiation. Among them, the expression of Pax4 and neurogenin3 was markedly increased during differentiation (Fig. 1 and Table 2). We then measured the effect of HGF and activin A on the expression of Pax4 and neurogenin3, respectively. mRNA for Pax4 and neurogenin3 was induced by activin A but not by HGF. The expression of Pax4 was detected as early as 6 h after treatment with activin A, whereas the expression of neurogenin3 was detected 24 h after the stimulation (data not shown).

Effects of transfection of Pax4 and antisense Pax4 into AR42J cells. The above results indicate that activin A induces the expression of Pax4 and neurogenin3 during the differentiation of AR42J cells. We therefore expected that Pax4 and/or neurogenin3 may be involved in the differentiation into endocrine cells. To assess the significance of Pax4, we transfected a flag-tagged Pax4 into these cells. To identify effectively transfected cells, we measured the immunofluorescence using the anti-Pax4 antibody. Pax4-specific staining localized in the nuclei but transfection of Pax4 did not induce morphological changes in these cells. In addition, PP was not expressed in Pax4-expressing cells. We then examined whether Pax4-expressing cells were differentiated into insulin-secreting cells when treated with HGF. HGF did not induce the expression of insulin in Pax4-expressing cells.

To further determine the significance of Pax4 during the differentiation of AR42J cells, an antisense Pax4 was introduced to reduce its expression. Cells were transfected with antisense Pax4 and then treated with HGF and activin A for 48 h. Double staining was carried out to determine the expression of insulin with anti-insulin antibody and the efficiency of transfection with anti-GFP antibody. Even in cells effectively transfected with antisense Pax4, immunoreactive insulin was detected. We analyzed the efficacy of the antisense method by measuring the expression of transfected Pax4 because endogenous Pax4 was not detected by anti-Pax4 antibody. AR42J cells were transfected with a pIRES-EGFP expression vector containing the DNA sequence of Pax4 in

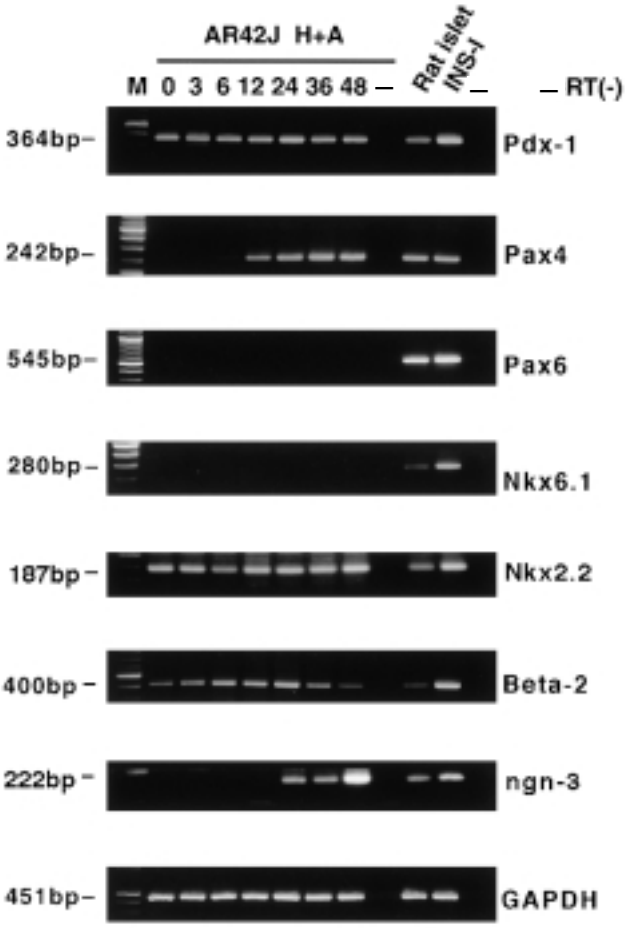

FIG. 1. Changes in the mRNA expression of transcription factors in AR42J cells before and after treatment with activin A and HGF. AR42J cells were incubated for various periods with 100 pmol/l HGF and 2 nmol/l activin A (H+A). mRNA expression of Pdx-1, Pax4, Pax6, Nkx6.1, Nkx2.2, Beta-2, neurogenin3 (ngn-3), and GAPDH was measured by RT-PCR. mRNA from rat islets and Ins-1 cells was also used as a positive control. (–), RNA without RT.

antisense or sense orientations. Expression of Pax4 was detected with anti-Pax4 antibody. The expression of exogenous Pax4 was blocked by cotransfection with antisense Pax4, whereas cotransfection of pIRES-EGFP had no effect on the expression of Pax4 (data not shown).

Effect of transfection of neurogenin3 and antisense neurogenin3. To assess the role of neurogenin3, we first transfected AR42J cells with flag-tagged neurogenin3. As shown in Fig. 2, transfection of neurogenin3, detected by the fluorescence of flag, induced morphological changes, which included extention of long neurite-like processes. Furthermore, transfection of neurogenin3 induced the expression of mRNA for PP (Fig. 3). However, HGF did not convert neurogenin3-transfected cells into insulin-producing cells. We then examined whether transfection of antisense neurogenin3 blocked the differentiation of AR42J cells by activin A and HGF. As shown in Fig. 4, cells converted into insulin-producing cells by activin A and HGF. Introduction of antisense neurogenin3, detected by the fluorescence of GFP, blocked the conversion into insulin-producing cells (Fig. 4 and Table 3).

DISCUSSION

The pancreas comprises endocrine, exocrine, and ductal cell types. Each of these distinct pancreatic cell types is derived from common precursor cells. We used AR42J cells as a model system to investigate the role of transcription factors involved in the differentiation of endocrine cells. Among the transcription factors investigated, we found that the expression of Pax4 and neurogenin3 is markedly upregulated during differentiation. Pax4 has been shown to be essential for the differentiation of β- and δ-cells (8), and a recent study showed that neurogenin3 acts as a proendocrine gene in the development of the pancreas (17,18).

In AR42J cells, activin A markedly increased the expression of Pax4. However, transfection of Pax4 did not reproduce the effect of activin A—namely morphological changes and the expression of PP. It was shown recently that Pax4 functions as a transcription repressor (19,20). It binds to the potential

TABLE 2
Changes in the mRNA expression of transcription factors

Transcription factor	Length (bp)	Native AR42J	Activin + HGF–treated	Ins-1	Islet
lmx1.1	657	–	+	+	+
lmx1.2	308	+	+	+	+
lmx2 (lim1)	212	+	+	+	+
islet-1	276	–	–	+	+
cdx 2/3	234	–	–	+	+
cdx4	194	+	+	+	+
Nkx2.2	187	+	+	+	+
Nkx6.1	280	–	–	+	+
alx3	170	–	–	–	+
Pax4	242	–	++	+	+
Pax6	545	–	–	+	+
Pdx-1	364	+	+	+	+
HB9	250	–	–	+	+
Hox1.11	366	–	+	–	+
neurogenin3	222	–	++	–	+
Beta-2	400	+	+	+	+

AR42J cells were incubated for 72 h with 2 nmol/l activin A and 100 pmol/l HGF. mRNA was obtained from cells before and after differentiation. Expression of mRNA for various transcription factors expressed in islets was measured by RT-PCR. Semiquantified results are presented.

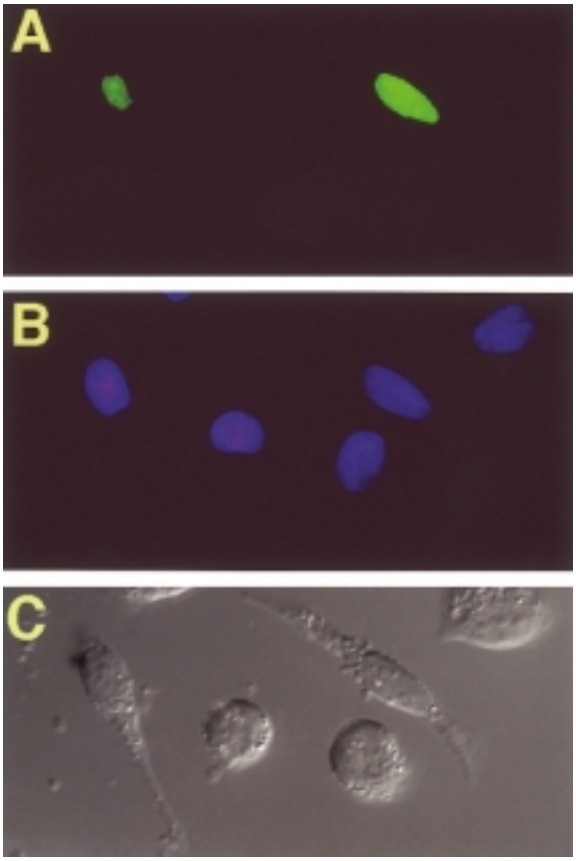

FIG. 2. Effect of transfection of neurogenin3 on the morphology of AR42J cells. AR42J cells were transfected with flag-tagged neurogenin3 and incubated for 48 h. Cells were then stained with anti-flag antibody (green, *A*), and nuclei were stained with 4′,6-diamidino-2-phenylindole (*B*). Effectively transfected cells are identified by green color. *C* shows the morphology of the cells.

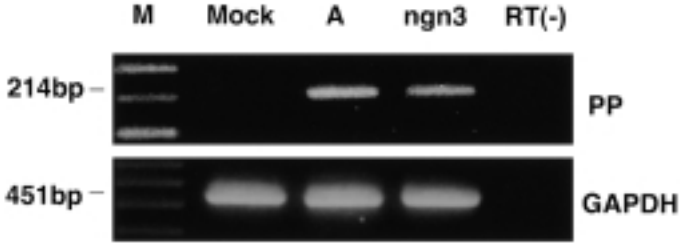

FIG. 3. Effect of transfection of neurogenin3 on PP expression. AR42J cells were transfected with neurogenin3 and incubated for 24 h. RNA was obtained from cells before and after transfection. RNA from activin-treated cells was also obtained as a positive control. mRNA for PP was measured by RT-PCR.

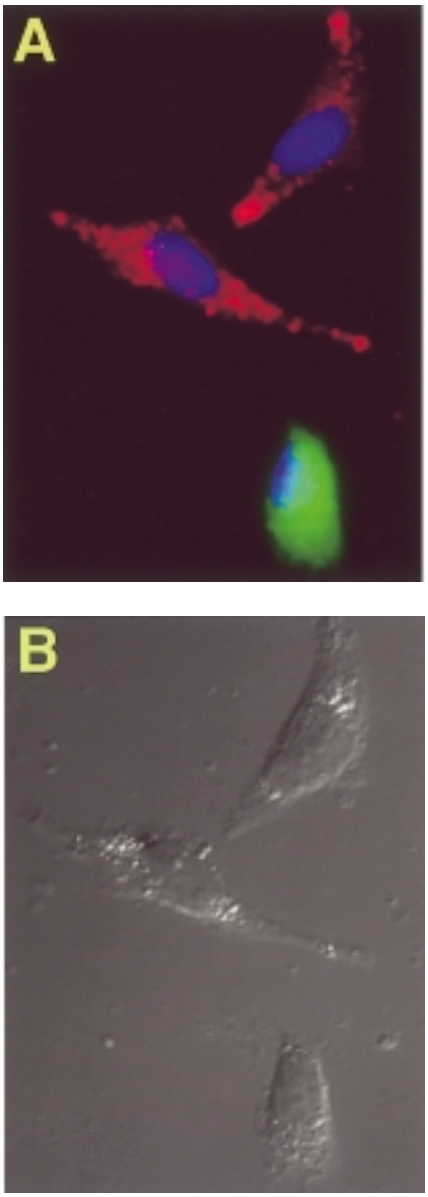

FIG. 4. Effect of transfection of neurogenin3 on the differentiation of AR42J cells to insulin-producing cells. AR42J cells were transfected with antisense neurogenin3 and incubated for 72 h with 2 nmol/l activin A and 100 pmol/l HGF. Cells were then stained with anti-insulin antibody (red) and anti-GFP antibody (green). Nuclei were stained with 4′,6-diamidino-2-phenylindole. The effectiveness of transfection was identified by the expression of GFP.

binding sites for Pax6 and blocks the Pax6-induced activity. As shown in Fig. 1, Pax6 is not expressed in AR42J cells either before or after differentiation. It is thus likely that Pax4 does not play a significant role in the differentiation of AR42J cells. Consistent with this notion, introduction of antisense Pax4 did not affect the differentiation of AR42J cells into insulin-producing cells. These results suggest that, although induced by activin A, Pax4 does not play a significant role in these cells. In mice with null mutation of the Pax4 gene, the number of β-cells is greatly reduced. However, even in these mice, insulin-positive cells were detected in pancreatic anlage. This implies that Pax4 is not absolutely necessary for the formation of insulin-producing cells. Rather, Pax4 may be critical for the subsequent formation and expansion of β-cells.

Activin A increased the expression of neurogenin3. The role of neurogenin3 was confirmed by two lines of observations. First, introduction of neurogenin3 induced morphological changes suggestive of differentiation to neuroendocrine cells. Neurogenin3 also induced the expression of PP. Second, decrease in the expression of neurogenin3 by the antisense method abolished the conversion to insulin-producing cells. The differentiation-inducing activity of activin A in these cells was reproduced at least partly by the expression of neurogenin3. It should be mentioned that expression of neurogenin3 did not completely reproduce the effect of activin A: treatment of neurogenin3-expressing cells with

TABLE 3
Effect of transfection of antisense neurogenin3 on the differentiation of AR42J into insulin-producing cells

Transfection	Number of cells	Insulin-positive cells (%)	Insulin-negative cells (%)
Antisense neurogenin3	326	53 (16)	273 (84)
Vector alone	353	299 (85)	54 (15)

AR42J cells were transfected with antisense neurogenin3 or GFP vector and incubated for 72 h with 100 pmol/l HGF and 2 nmol/l activin A. Cells were stained with anti-insulin and anti-GFP antibodies. The number of insulin-positive cells among effectively transfected cells was counted. Results are accumulated data from four separate experiments. Note that 80–90% of the untransfected cells became insulin-positive by activin A and HGF.

HGF did not convert them to insulin-producing cells. Hence, besides induction of neurogenin3, activin A may have other effects in these cells. In any event, the present results are consistent with the idea that neurogenin3 acts as a key transcription factor for the formation of pancreatic endocrine cells (18). Apelqvist et al. (17) reported that neurogenin3 is under the control of the Notch signaling system. They further showed that expression of neurogenin3 under the control of the IPF-1/ Pdx-1 promoter augmented the formation of pancreatic endocrine cells in transgenic mice. They postulated that formation of endocrine cells from their progenitors is regulated by "lateral inhibition" involving the Notch signaling system. Our results indicate that the expression of neurogenin3 is also regulated by activin A. Because activin A does not alter the expression of Hes-1 (Y.-Q.Z., I.K., unpublished data), a downstream target of the Notch signaling system, the expression of neurogenin3 may be regulated by the Notch and activin signaling systems. We showed previously that progenitor cells in pancreatic anlage express activin A (21). Miralles et al. (22). showed that conversion of progenitor cells to endocrine or exocrine cells is modulated by mesenchyme-derived follistatin, an inhibitor of activin A (23). Given that activin A is involved in the differentiation of pancreatic endocrine cells (24,25), these results imply that differentiation of progenitor cells is regulated by the activin-follistatin system via an autocrine/paracrine mechanism. Taken together, the present results suggest that differentiation of pancreatic progenitors is controlled by two distinct regulatory systems: the Notch signaling system and the activin-follistatin system.

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