Brn-4 Transcription Factor Expression Targeted to the Early Developing Mouse Pancreas Induces Ectopic Glucagon Gene Expression in Insulin-producing β Cells*

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The endocrine pancreas is comprised of β and α cells producing the glucostatic hormones insulin and glucagon, respectively, and arises during development by the differentiation of stem/progenitor cells in the foregut programmed by the β cell lineage-specific homeodomain protein Idx-1. Brain-4 (Brn-4) is expressed in the pancreatic anlage of the mouse foregut at e10 in the α cells and transactivates glucagon gene expression. We expressed Brn-4 in pancreatic precursors or β cell lineage in transgenic mice by placing it under either Idx-1 or insulin promoter (rat insulin II promoter) control, respectively. Idx-1 expression occurs at developmental day e8.5, and insulin expression occurs at e9.5, respectively. Misexpression of Brn-4 by the Idx-1 promoter results in ectopic expression of the proglucagon gene in insulin-expressing pancreatic β cells, whereas misexpression by rat insulin II promoter did not. The early developmental expression of Brn-4 appears to be a dominant regulator of the glucagon expressing α cell lineage, even in the context of the β cell lineage.

The mammalian pancreas develops by the differentiation of endodermal stem/progenitor cells in the foregut of the developing embryo (Refs. 1–3 and references therein). These progenitor cells differentiate into the exocrine acinar cells that produce digestive enzymes and the endocrine islets of Langerhans consisting of the four types of cells specialized for the synthesis of the hormones glucagon (α cells), insulin (β cells), somatostatin (δ cells), and pancreatic polypeptide (PP cells). Islet cell-specific differentiation requires the actions of the basic loop helix transcription factors neurogenin-3, β2/NeuroD, and the homeodomain proteins Isl-1, Pax4, Pax6, Nkx2.2, Nkx6.6, and Idx-1 (see Refs. 1–4 for review). Idx-1 (Pdx-1/Stf-1/Ipf-1) is transiently expressed in all pancreatic cells as well as in the epithelial layer of the duodenal mucosa during early embryogenesis (5–8). During development, Idx-1 becomes progressively confined to the endocrine β cells where it is critical for the transcriptional regulation of the insulin gene (6, 9). Idx-1 null mice (7), as well as a child homozygous for an inactivating mutation of the Idx-1 (Ipf-1) gene (10), result in a failure of the pancreas to develop (pancreatic agenesis).

Brn-4 is a member of the class III family of pou-homeodomain proteins that are highly expressed in neural stem cells and regulate stem cell-specific genes, such as the intermediate filament protein nestin (11, 12). During early pancreas development, the expression of Brn-4 is specifically restricted to stem/progenitor cells that later differentiate into glucagon-producing α cells (13). Brn-4 has not been detected in either differentiating or mature β cells. Brn-4 also stimulates glucagon gene expression by interaction with the α cell-specific G1 promoter element within the glucagon gene (14).

Here we describe that the developmental misexpression of Brn-4 under the control of the mouse Idx-1 promoter (MIP)1 in mice results in the ectopic expression of the glucagon gene in pancreatic β cells that continue to express insulin. Furthermore, Brn-4 expression in the undifferentiated pancreatic ductal cell line AR42J, a pancreatic precursor cell line, results in the expression of the proglucagon gene. Misexpression of Brn-4 under the control of rat insulin II promoter (RIP) failed to yield an altered β cell phenotype in transgenic mice. Brn-4 expression in the differentiated mouse insulinoma cell line Min6 also failed to induce glucagon gene expression. Expression of Idx-1 occurs at mouse embryonic development days e8.0–e8.5, ~24 h earlier than the expression of insulin. Our findings suggest that the ectopic expression of Brn-4 at an early developmental stage under Idx-1 promoter control, both in transgenic mice and under cytomegalovirus promoter control in AR42J cells, directs glucagon gene expression in precursors of pancreatic β cells. Expression of Brn-4 later during mouse embryo development under RIP control and in differentiated clonal β cell Min6 cells fails to alter the phenotype of the pancreatic β cell. Our results directly demonstrate in vivo and in vitro the temporal importance of Brn-4 expression in the determination of the proglucagon-expressing α cell lineage. Our findings underscore the importance of temporal windows during development when the expression of critical transcription factors has the capability of determining the phenotype of specific cell lineages of hormone-producing cells of the endocrine pancreas.

MATERIALS AND METHODS

Transgenic Mice—Two plasmid constructs carrying ~4.6 kb of the MIP and the ~0.64 kb of RIP were used for inserting mouse Brn-4 and Pax6 cDNAs that were amplified by PCR. These constructs carry a rabbit β-globin exon 2 splice signal between the transcriptional start site of the respective promoters and the translational start codon of the

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† The abbreviations used are: MIP, mouse Idx-1 promoter; RIP, rat insulin II promoter; TG, transgenic; WT, wild type; RT, reverse transcription; CREB, cAMP-response element-binding protein.
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A rabbit β-globin poly(A) signal was ligated 3' in the respective cDNAs (see Fig. 2B). Linearized vectors excluding plasmid-derived sequences were used as transgenes for microinjection. Generation of transgenic animals was performed by the Beth-Israel Transgenic Facility in Boston. The time of implantation of the transgene-injected eggs was taken as e0.5 (15). Pregnant foster mothers were sacrificed, and the embryos were removed at e8.5, e10, e15, and e18. Embryos (e8.5 and e10) or pancreata (e15 and e18) were extracted for protein, DNA, and total RNA with TRIZOL (Invitrogen). Protein extracts were analyzed by Western immunoblot, and nucleic acid extracts were analyzed by RT-PCR. Mice carrying the transgene were identified by PCR amplification of DNA derived from tail-snip tissue (15). Upstream primers corresponded to 3' of the transcriptional start site (Idx-1, 5'-AGCTCTATTGG-GAGCGGTTTTG-3'; rat insulin II, 5'-ACAGCTATCAGTGGAACGT-GTGA-3'); the downstream primer was within the cDNA sequence of the transgene construct 5'-TTGGAGGACCTGTGGCCATGTCG-3' (see Fig. 2B).

Histological Analysis—Tissues were fixed in 4% paraformaldehyde. After dehydration, tissues were embedded in paraffin, and 5 μm serial sections were dewaxed in xylene and rehydrated in ethanol. Nonspecific serum binding was blocked with 10% donkey nonimmune serum, and sections were stained with primary antibodies: rabbit anti-Idx-1 HM253, 1:500 (8), rabbit anti-Brn-4 4R2, 1:500 (16), guinea pig anti-insulin C peptide 1:1000 (Linco), rabbit anti-glucagon 1:500 (Linco). Biotinylated secondary antibodies were detected with the ABC Elite System (Vector Systems).

Western Immunoblot—Proteins were isolated from transgene-negative (wild-type, WT) and transgene-positive (TG) mice were separately pooled, and 100 μg of protein was fractionated via 10% SDS-polyacrylamide gel electrophoresis and transferred to a nylon membrane for protein immunodetection. Antibodies and their dilutions used were anti-Brn-4 4R2, 1:500; anti-Pax6, 1:1000 (Developmental Studies Hybridoma Bank, University of Iowa); anti-Nkx 6.1, 1:1000 (provided by J. Jensen and O. Madsen, Hagedorn Research Institute, Copenhagen, DK); and anti-CREB, 1:500 (17).

Radioimmunoassay—Protein was extracted for insulin and glucagon assays with commercially available assay kits (Linco) from portions of e15 and e18 embryonic pancreas (see Fig. 2B). Target sequences were amplified using Taq polymerase as a template. Biocompatible secondary antibodies were detected with the ABC Elite System (Vector Systems).

RT-PCR of Embryos and Pancreas Tissue—To analyze for transgene expression, RNA was extracted from embryonic mouse tissue (e8.5 and e18 embryonic pancreas (as pancreata) as described (18). The radiolabeled assay detects glucagon specifically with little cross-reactivity with glucagon-like peptide-1 and -2.

RT-PCR of Embryos and Pancreas Tissue—To analyze for transgene expression, RNA was extracted from embryonic mouse tissue (e8.5 and e18 embryonic pancreas (as pancreata) as described (18). The radiolabeled assay detects glucagon specifically with little cross-reactivity with glucagon-like peptide-1 and -2.
gene construct carried a rabbit β-globin splice signal, correct expression of the transgene could be identified by a smaller PCR product lacking the inserted β-globin splice sequence as compared with the PCR from genomic DNA. Correct euakaryotic processing of the transgenes was verified by transfecting (Lipofectamine, Invitrogen) the rat immortalized β-cell line INS-1 with the MIP-Brn-4 and RIP-Brn-4 expression plasmids.

**Cell Culture and Transfection—**AR42J cells were obtained from the American Type Tissue Culture Collection, and INS-1 (rat insulinoma), Min6 (mouse insulinoma), and βTC-1 (mouse insulinoma) cells were generously provided by C. Wollheim (University of Geneva, Switzerland), J. Miyazaki (Kumamoto University), and S. Efrat (University of Tel Aviv, Israel), respectively. RN1056A cells were generated in our laboratory (19). AR42J and Min6 cells were transfected using Lipofectamine 2000 with a pcDNA3.1 vector (Invitrogen) carrying the full-length rat Brn-4 cDNA (provided by R.G. Rosenfeld, University of San Diego, CA) under control of the human cytomegalovirus promoter. RNA from cultured cells was extracted with Trizol (Invitrogen). Reverse transcription was performed as above using primers and cycling conditions used for glucagon and for rat and mouse adenosyl-phosphoribosyltransferase described previously (20).

**RESULTS AND DISCUSSION**

Immunohistochemical analyses of pancreata of e18 of WT and MIP-Brn-4 TG mice were done with antisera to insulin and glucagon (Fig. 1A). The pancreata of WT mice show the typical intense insulin staining of almost all of the cells of the islets because the β cells that produce insulin comprise 80–90% of all islet cells (Fig. 1A, left top panel). Also typical of WT pancreata, the α cells that express glucagon are sparse and are distributed around the periphery of the islets (Fig. 1A, left right top panel). In contrast to the pancreata of control WT mice, the pancreata of TG mice show a striking difference in the distribution of glucagon-expressing cells (Fig. 1A, right middle and lower panels). In the pancreata of TG mice, glucagon and insulin staining is seen in essentially all of the cells of the islets (Fig. 1A, left middle and lower panels). Therefore, we conclude that insulin and glucagon are co-expressed in the same cells of TG mice.

The coexpression of glucagon and insulin in the same cells is evidenced by the strong similarities in the patterns of cellular immunostaining for insulin and glucagon (Fig. 1A, left and right panels, respectively). Notably, the immunostaining of e18 pancreata of TG mice expressing Brn-4 directed by either the RIP (Fig. 1B) or the expression of Pax6 directed by MIP (data not shown) gave a pattern of glucagon and insulin expression indistinguishable from that of WT mice. Therefore, neither the RIP promoter driving Brn-4 expression nor the MIP promoter driving Pax6 expression is capable of inducing the ectopic expression of glucagon in β cells.

The MIP promoter effectively expresses Brn-4 ectopically in the β cells of e18 in WT mice, as shown by the appearance of Brn-4-positive nuclei throughout the cells of the islets (Fig. 1C, left lower panel) as compared with the typical restricted distribution of Brn-4 expression to the α cells located at the periphery of the islets of WT mice (Fig. 1C, left upper panel). Of importance is the finding that the distribution of endogenous Idx-1 expression in the WT and TG mice is indistinguishable, i.e. the ectopic expression in β cells of Brn-4 driven by MIP does not alter the expression of Idx-1 (Fig. 1C, right upper and lower panels). This finding in vitro is consistent with the findings of Brn-4 and Idx-1 expression interactions in INS-1 cells in vitro (see Ref. 21 and see below).

Western immunoblot analyses of levels of expression of transcription factors in total pancreatic protein extracts of e18 embryos showed increased expression of Brn-4 in both the MIP-Brn-4 and RIP-Brn-4 transgenic pancreata, as compared with wild-type pancreata (Fig. 1D). This observation makes it unlikely that a dose effect of Brn-4 expression is a cause for the differences in the phenotypes of MIP-Brn-4 and RIP-Brn-4 transgenic mice. Idx-1 immunoreactivity was unchanged in the transgenic animals, suggesting that Brn-4 does not influence the expression of Idx-1, i.e. Brn-4 does not suppress Idx-1 in β cells (Fig. 1, C and D).

The paired-homeodomain transcription factor Pax6 is implicated in α cell development shown by a reduction in glucagon-producing cells in Pax6 null mice (18, 22), and Pax6 binds to a proximal element in the glucagon promoter and up-regulates glucagon gene transcription (23, 24). An increase in Pax6 may increase glucagon gene expression. The effect of Brn-4 overexpression in pancreatic β cells does not alter Pax6 levels as determined by Western immunoblot (Fig. 1D). Nkx6.1 is a homeodomain transcription factor suggested to be β cell-specific (13, 25, 26). Nkx6.1 levels in total pancreas extract are not altered by Brn-4 misexpression (Fig. 1D). Altered expression of Idx-1, Nkx6.1, or Pax6 does not appear to account for the phenotype of co-expression of Brn-4, Idx-1, insulin, and glucagon found in the islets (β cells) of mice carrying the transgene consisting of the MIP driving misexpression of Brn-4.

We measured the contents of glucagon and insulin in protein extracts prepared from pancreata of MIP-Brn-4 transgenic and non-transgenic littermates (control) at e15 and e18. The animals carrying the transgene had increased pancreatic glucagon content (TG mice, 600 ± 110 and 650 ± 120 as compared with control mice, 330 ± 70 and 430 ± 90 ng/mg protein for e15 and e18, respectively). Insulin levels did not appear to be affected by the presence or absence of a transgene (TG mice, 10 ± 0.9 and 7.6 ± 0.3 as compared with control mice, 9.2 ± 1.2 and 7.4 ± 0.8 μg/mg protein for e15 and e18, respectively). The radioimmunoassay used is capable of distinguishing glucagon from other glucagon gene products (i.e. glucagon-like peptide-1 and glucagon-like peptide-2), suggesting that the increased glucagon immunoreactivity was properly detecting processed glucagon derived from the precursor protein proglucagon.

Analysis of the expression of the transgenes in mouse embryonic tissues was performed with the identical PCR primers that were used for transgene detection. Total RNA from e8 and e12 embryos and pancreata from e15 and e18 were extracted, and RT-PCR was performed to detect transgene expression (Fig. 2A). Correct processing of the transgene-derived RNAs was tested in the rat β cell line INS-1 transfected with the transgene expression DNA plasmids (Fig. 2, B and C). Proper expression of the transgene-derived mRNA yielded a shorter PCR product as compared with the genomic DNA template due to the removal of the rabbit β-globin splice site (see “Materials and Methods”). Expression of the MIP-Brn-4 transgene was detectable in e8 embryos (Fig. 2C, leftmost panel), whereas the RIP-Brn-4 transgene, although present, was not expressed at the RNA level. At a later developmental stage (e10), both the MIP-Brn-4 (not shown) and RIP-Brn-4 transgenes were expressed (Fig. 2C, rightmost panel). This agrees with previous observations that Idx-1 is expressed earlier in development (i.e. e8.0–e8.5) than is insulin, which is detectable around e9.0–e9.5. Thus, a different time of transgenic misexpression of Brn-4 may account for the different islet phenotype generated using the two different promoters, MIP and RIP. However, the issue of timing of expression is further complicated by the evidence that MIP is activated in pancreatic precursor cells, and the RIP is activated in cells presumably already committed to become β cells. Thus, both temporal and spatial compartmental expression of the Idx-1 and insulin genes must be considered.

To examine the property of Brn-4 to activate the expression of the proglucagon gene, we performed Brn-4 transfection-expression studies in cell lines in vitro. The rat pancreatic duct precursor cell line AR42J expresses Idx-1 but not endocrine
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FIG. 2. Onset of Idx-1 and insulin II gene transcription as detected with RT-PCR of -4.6-kb MIP and -0.64-kb RIP constructs matches the expression pattern of the endogenous Idx-1 and insulin genes in transgenic mice during development. Expression of endogenous Idx-1 (A) as well as Idx-1 promoter-driven transgene (B) is detected from e8.5 onwards (C), and expression of insulin (A) as well as insulin II promoter-driven transgene (C) is detected from e10 onwards. Detection of the correct transgene expression was confirmed by RT-PCR and by accurate removal of β-globin splice signal sequence site (C) during the generation of transgenic mRNA.

Our results suggest that the expression of Brn-4 is important in the determination of whether or not an early developing pancreatic endocrine cell lineage will express glucagon at later stages of development, and the suppression of hormones (27)2 and can be differentiated in vitro into a pancreatic endocrine phenotype (27–29). AR42J cells can be considered a pancreatic precursor cell line. In contrast, mouse Min6 cells exhibit a phenotype of differentiated pancreatic endocrine β cells that express most if not all transcription factors specific for β cells, maintain a glucose-induced insulin secretory response, and express insulin but not glucagon (Fig. 3) or somatostatin (not shown).

Transfection of AR42J cells with Brn-4 cDNA results in the expression of the proglucagon gene, whereas the Min6 cells transfected with Brn-4 cDNA do not express proglucagon (Fig. 3). The experimental expression of Brn-4 induces proglucagon gene expression in undifferentiated islet precursor cells (AR42J), but experimental expression of Brn-4 in a differentiated β cell line (i.e. Min6) fails to induce proglucagon gene expression. The results of the studies of misdirected expression of Brn-4 in transgenic mice in vivo and transfection expression studies in AR42J cells in vitro indicate that Brn-4 expressed at an early developmental stage, either in vivo or in vitro, can induce proglucagon gene expression. However, when Brn-4 is expressed in cells that are already committed to differentiate into β cells (i.e. RIP-Brn-4 transgenic mice, or Min6 cells), Brn-4 fails to alter the phenotypic determination of those respective cells.

Our results suggest that the expression of Brn-4 is an important step in the determination of whether or not an early developing pancreatic endocrine cell lineage will express glucagon at later stages of development, and the suppression of Brn-4 expression may be a key event in the phenotypic determination of a cell programmed to become an insulin-producing β cell. Our results also suggest that an ectopic gain of function of Brn-4 leads to a change in the pattern of glucagon expression when under the control of a promoter (Idx-1) that is active early during pancreatic development. An altered phenotype is not detected if Brn-4 is later expressed under the control of the insulin promoter (RIP) that is active 24 h later during development (1) and when the endocrine cell lineage is committed irreversibly to a β cell lineage. Thus, there appears to be a temporal window (24 h between e8.5–9.0 and e9.5–10.0) during early pancreas development that allows for a change in the phenotypic programming of cells destined to become endocrine cells and, at a later developmental stage, the program cannot be changed. However, these temporal considerations must take into account the spatial constraints of gene expression likely to be at play; Idx-1 appears to be expressed in early pancreatic precursor cells, whereas insulin is presumably expressed in cells already committed to become β cells.

How can the phenotype of the coexpression of Idx-1, Brn-4, insulin, and glucagon in the transient transgenic mice in this study be explained? Our results rule out a dose effect of Brn-4 expression in pancreatic β cells. Brn-4 protein levels were equally elevated (Fig. 1D, Western immunoblot) in mice transgenic for MIP-Brn-4 that expressed high levels of glucagon and for RIP-Brn-4 that did not have any detectable glucagon in pancreatic β cells. Protein levels of Idx-1 in pancreata (β cells) were no different in transgenic mice expressing either the MIP-Brn-4 or the RIP-Brn-4 transgenes (Fig. 1D). It is worth noting the important report of Wang et al. (21) in which the introduction (transfection) of either Brn-4 or dominant negative Idx-1 expression vectors into the INS-1 clonal β cell line resulted in the ectopic expression of the proglucagon gene. The conclusions drawn from these studies were that Idx-1 suppresses and Brn-4 activates the ectopic expression of the proglucagon gene (21). These intriguing findings support the findings that interactions exist among Idx-1 and Brn-4 in determining the β cell versus α cell phenotype are also consistent with our findings, which found no effects of ectopic Brn-4 expression on levels of Idx-1 (and of Nkx6.1, Pax6, and CREB) in the pancreas of RIP-Brn-4 transgenic mice as compared with control mice (Fig. 1D). Although we observed that ectopic expression of Brn-4 in the undifferentiated AR42J islet progenitor cell line induced the expression of glucagon, it failed to do so in the differentiated MIN6 clonal β cell line (Fig. 3). The ectopic expression of Idx-1 in stably transfected glucagonoma cell lines induced the expression of the insulin gene yet did not extinguish the expression of proglucagon (30). These intriguing disparate findings of the effects of the ectopic expression of Brn-4

2 M. A. Hussain, C. P. Miller, and J. F. Habener, unpublished observations.
on the induction of proglucagon expression in insulin-producing β cells remain unexplained but may be attributable to the different experimental conditions and/or the different β cell lines used.

Mice lacking the Pax6 gene have diminished numbers of α cells and β cells (18, 22). However, Pax6 has been shown to be important for the expression of the glucagon, insulin, and somatostatin genes, so it appears that Pax6 may be critical for the development of the endocrine pancreas phenotype development in general. A relevant report addresses the phenotypic consequences of the misexpression in transgenic mice of Pax6, a transcription factor important for pancreatic endocrine cell development and differentiation (31). Pax6 was expressed under the control of the insulin and Idx-1 (Pdx-1) promoters. Transgenic mice generated with both promoters displayed disturbed β cell development with increased β cell apoptosis and diabetes. Pax6 overexpression under the control of the insulin promoter specifically affected β cells, whereas overexpression under the control of the MIP resulted in hypertrophy of the exocrine pancreas, increased proliferation of both ductal epithelia and islet cells, and the development of cystic malformations of the pancreas. The differences between our observations and those of Yamaoka et al. (31) with Pax6 overexpression under apparent similar circumstances may be due to the different strains of mice used in our study and by Yamaoka et al. (31) and the fact that the phenotype they describe is present during postnatal life and not prenatal life (e18), as in our study. The transcription factor Nkx6.1 has been suggested to repress glucagon expression in β cells (1, 13). Our present findings of the expression of Brn-4 in pancreatic β cells along with unaltered Nkx6.1 expression suggest that the presence of Nkx6.1 alone is not sufficient to suppress glucagon gene expression in differentiating pancreatic endocrine cells, particularly if Brn-4 is expressed early in mouse pancreas development, i.e. under the control of the Idx-1 promoter.

Our results are supported by a recent report that the earliest sign of α cell development appears to be Brn-4 expression, which apparently precedes Isl-1 expression (13, 25). The results of our studies indicate that if Brn-4 is expressed early in the differentiation of pancreatic endocrine cells in conjunction with other transcription factors important for the epigenetic control of glucagon gene expression, it is capable of inducing permanent glucagon gene expression in the early cell lineage that becomes β cells. Whether this is due to a direct interaction of Brn-4 with the proximal G1 promoter element of the glucagon gene or via indirect mechanisms remains unanswered. One known target gene induced by Brn-4 is that encoding nestin, an intermediate filament protein expressed in neural stem cells (11, 12). Identification of the target genes of Brn-4 actions in the early stages of the development of pancreatic islet cells may lead to an understanding of how the differentiation of a common precursor endocrine cell into α cell versus β cell lineages is regulated.

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