Neurogenin3 Cooperates with Foxa2 to Autoactivate Its Own Expression*1,5

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The transcription factor Neurogenin3 functions as a master regulator of endocrine pancreas formation, and its deficiency leads to the development of diabetes in humans and mice. In the embryonic pancreas, Neurogenin3 is transiently expressed at high levels for a narrow time window to initiate endocrine differentiation in scattered progenitor cells. The mechanisms controlling these rapid and robust changes in Neurogenin3 expression are poorly understood. In this study, we characterize a Neurogenin3 positive autoregulatory loop whereby this factor may rapidly induce its own levels. We show that Neurogenin3 binds to a conserved upstream fragment of its own gene, inducing deposition of active chromatin marks and the activation of Neurog3 transcription. Additionally, we show that the broadly expressed endodermal forkhead factors Foxa1 and Foxa2 can cooperate synergistically to amplify Neurogenin3 autoregulation in vitro. However, only Foxa2 colocalizes with Neurogenin3 in pancreatic progenitors, thus indicating a primary role for this factor in regulating Neurogenin3 expression in vivo. Furthermore, in addition to decreasing Neurog3 autoregulation, inhibition of Foxa2 by RNA interference attenuates Neurogenin3-dependent activation of the endocrine developmental program in cultured duct mPAC cells. Hence, these data uncover the potential functional cooperation between the endocrine lineage-determining factor Neurogenin3 and the widespread endoderm progenitor factor Foxa2 in the implementation of the endocrine developmental program in the pancreas.

The pancreas consists of exocrine tissue (acinar and duct cells) and endocrine cells (insulin (β), glucagon (α), somatostatin (δ), pancreatic polypeptide (PP), and ghrelin (e) cells) that are organized in the islets of Langerhans. During embryonic development, differentiation of these distinct cell types is controlled by the ordered and coordinated activation and inactivation of many transcriptional regulators. Among them, the basic helix-loop-helix (bHLH)5 transcription factor Neurogenin3 (Neurog3) implements the endocrine differentiation program in multipotent pancreatic progenitor cells (MPCs). Mice deficient in Neurog3 are born with no pancreatic endocrine cells (1), and genetic lineage tracing studies have demonstrated that all islet endocrine cells derive from Neurog3 positive progenitors (2). The finding that deficiency in Neurog3 caused by mutations in the NEUROG3 gene underlies neonatal and childhood-onset diabetes confirms the importance of this transcription factor in islet cell development and function in humans (3–5). Moreover, Neurog3 is sufficient to drive endocrine differentiation in a variety of in vivo and in vitro cellular contexts (6–9), highlighting its potential as a tool to generate replacement β-cells from other cell types for treatment of diabetes.

Neurog3 is transiently expressed in scattered MPCs within the trunk region of the developing pancreas, it is progressively down-regulated as the endocrine program is initiated and remains expressed at low levels in some adult islet cells (6, 10). Despite its relevance for endocrine cell formation, the molecular mechanisms that control Neurog3 expression are poorly understood. The transcription factors HNF6/OneCut1, HNF1b/Tcf2, HNF3b/Foxa2, Sox9, Pdx1, and Glis3 have all been acknowledged to be upstream regulators of the Neurog3 gene (11–15). Conversely, in clear parallelism to neural development, the Notch signaling pathway negatively regulates Neurog3 expression through the transcriptional repressor Hes1 (16), implying that release from Hes-1-mediated repression is required for Neurog3 gene activation in MPCs. Consistent with this notion, loss of Hes1 in the developing foregut endoderm is sufficient to induce ectopic endocrine cell formation (17).

Recent studies have pointed out that attaining high Neurog3 levels is critical for endocrine cell commitment. Thus, low Neu-
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Neurog3-expressing MPCs can adopt alternative exocrine fates (18, 19) and Neurog3 haploinsufficiency results in decreased endocrine cell mass (18). Hence, activation mechanisms must operate to allow for rapid and substantial increases in Neurog3 expression in a narrow time window that is estimated to be <24 h (20, 19). One of the proposed mechanisms whereby Neurog3 may amplify its protein levels is through a positive feedback loop involving the Neurog3 target Myt1b, which activates Neurog3 gene transcription (21). Another mechanism used by transcription factors to control their protein levels is self-regulation. In this regard, exogenous Neurog3 has been shown to induce the endogenous mouse Neurog3 gene in pancreatic duct-like mPAC cells (8), thus revealing positive autoregulation as a potential mechanism that may contribute to the rapid accumulation of Neurog3 protein in endocrine progenitors. However, in apparent contradiction to results in mPAC cells, Neurog3 has also been shown to inhibit its own promoter in NIH3T3 fibroblasts (16). Given the transient nature of Neurog3 expression in endocrine progenitors, it is conceivable that positive and negative regulatory mechanisms function in a timely coordinated manner to ensure tight regulation of Neurog3 expression during pancreatic development.

Because of the essential role played by Neurog3 in the determination of endocrine cell fate in the pancreas, deciphering the molecular mechanisms that regulate its expression is important to fully understand how pancreatic endocrine cell differentiation is accomplished. Based on previously published studies indicating that Neurog3 is able to activate its own expression (8), here we sought to gain further insight into the mechanisms governing Neurog3 autoregulation. Using reporter luciferase and ChIP assays, we show that Neurog3 activates its own promoter by binding to a conserved upstream regulatory region. In addition, we demonstrate that the forkhead transcription factor Foxa2 synergizes with Neurog3 to autoactivate the Neurog3 gene. Importantly, we reveal that Foxa2 is not only instrumental for Neurog3 autoregulation but it is also required for the activation of other Neurog3 target genes, indicating that Neurog3 and Foxa2 functionally cooperate to switch on the endocrine differentiation program in the pancreas.

EXPERIMENTAL PROCEDURES

Mice—CD1 mice used in this study were maintained in a barrier facility according to protocols approved by the University of Barcelona Animal Welfare Committee. The morning of the appearance of a vaginal plug was considered as embryonic day (E) 0.5.

Luciferase and Expression Vectors—A 5-kb fragment of 5’-flanking sequence extending from −4864 nt to +88 nt (+1 is the transcription start site) of the mouse Neurog3 gene was amplified from mouse tail genomic DNA using primers A/B. The 5-kb fragment was then used as template to generate shorter promoter fragments by PCR using primers C/B (3kb, −3057 nt to +88 nt) and D/E (−3.2 kb cluster region, −3885 nt to −3217 nt), or by restriction digest with Nhel-XhoI (0.9 kb, −932 nt to +88 nt). Primer sequences are listed in supplemental Tables. The 5, 3, and 1 kb fragments were cloned upstream of the firefly luciferase gene in the KpnI-XhoI sites of the pGL3-promoter vector (Promega, Madison, WI). To generate mutations, we used the QuickChange II XL Site-directed mutagenesis kit (Stratagene, Santa Clara, CA) and the primers listed in supplemental Tables. The artificial E box-driven enhancer luciferase reporter constructs used to test E box preference of the Neurog3/E47 heterodimers were kindly provided by K. Kroll (Washington University). The Renilla luciferase reporter plasmid pRL-CMV (Promega) was used as transfection control in all luciferase assays.

The expression vectors for mouse Neurog3 (pCMV-TNT-Neurog3) and mouse E47 (pCMV-TNT-E47) were previously described (13, 22). Expression vectors encoding rat Foxa1 (pCMV-Bgal-Foxa1) and rat Foxa2 (pCMV-Bgal-Foxa2) were kindly provided by M. A. Navas (Universidad Complutense de Madrid, Spain). Expression vectors encoding mouse HNF6 (pCMV4-HNF6) and rat HNF1b (pRSV-HNF1b) were kindly provided by M. Gannon (Vanderbilt University, TN) and J. Ferrer (IDIBAPS, Barcelona, Spain) respectively.

Cell Culture and Adenoviral Infection—All cell lines (mPAC, L20, αTC1.6, BTC3, NIH3T3, MIN6, PANC-1) were grown in Dulbecco’s Modified Eagle’s Medium: 4.5 g/liter of glucose (Sigma-Aldrich) supplemented with 10% fetal bovine serum plus antibiotics. MIN6 cells were additionally supplemented with 2 mM L-glutamine and 50 μM β-mercaptoethanol.

For adenoviral infection experiments, cells were seeded onto 6-well plates or 10-cm plates 1 day earlier (or when reaching 70–80% confluence) with adenoviruses at a multiplicity of infection (moi) of 40 for 2–5 h at 37 °C in culture medium. Then, virus-containing media was replaced, and cells were cultured for an additional 44–48 h period. Generation and amplification of recombinant adenoviruses encoding human NEUROG3 and β-galactosidase was previously described (8). The adenovirus encoding the HA-tagged version of mouse Neurog3 used in ChIP and co-immunoprecipitation assays was kindly provided by G. Gradwohl (IGBMC, INSERM, University of Strasbourg, France).

Transient Transfections and Luciferase Assays—For luciferase assays, 1.2–1.5 × 10⁵ mPAC or NIH3T3 cells were plated onto 96-well culture tissue plates 1 day before transfection. Transient transfections were performed using Metafectene (mPAC) from Biontex Laboratories GmbH ( Martinsried, Germany) or Transfast (NIH3T3) from Promega according to the manufacturer’s instructions. The amount of DNA used per well was 125 ng of firefly luciferase reporter vector, 2.5 ng of pRL.CMV and 10 ng of any cotransfected transcription factor cDNA. Empty expression vector was added when necessary to keep the amount of DNA equal in all wells. Cells were harvested 48 h after transfection and luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega) and a Veritas microplate luminoimeter (Promega). Luciferase readings were normalized to activities of the internal control vector pRL.CMV.

For siRNA transfections, 2 × 10⁴ mPAC cells were seeded onto 12-well plates and simultaneously transfected with 100 pmols of SMART pool mouse Foxa2 siRNA or a siControl (Dharmacon, Lafayette, CO) using Metafectene® SI (Biontex) following the manufacturer’s instructions. The following day,
cells were infected with recombinant adenoviruses as detailed above.

**RNA Isolation and RT-PCR**—Total RNA was prepared from
cell lines and embryonic mouse pancreas using the RNeasy kit
(Qiagen, Hilden, Germany). RNA samples were treated with
DNase to remove contaminating genomic DNA. First-strand
cDNA was prepared using 1–2 μg of total RNA, Superscript III
enzyme (Invitrogen, Carlsbad, CA) and random hexamer prim-
ers (Invitrogen) in a total volume of 20 μl. Reverse transcription
reaction was carried for 90 min at 50 °C and an additional 10
min at 55 °C. 1/20 or 1/40 of the transcribed cDNA was used as
template for conventional RT-PCR or real time PCR, respec-
tively. Real time PCR was performed on an ABI Prism 7900
sequence detection system using SybrGreen reagents (Express
Greener, Invitrogen). PCR primer sequences are provided in
the supplemental Tables.

**Chromatin IP (ChIP) Assays**—mPAC cells or E15.5 pancreata
were fixed in 1% formaldehyde for 10–15 min and cross-linking
was quenched by addition of 0.125 mM glycine. Histone ChIPs
were performed as described elsewhere (22). ChIP against tran-
scription factors were performed using the EpiQuik kit (Epi-
gentek, Farmingdale, NY) following the manufacturer’s
instructions. Antibodies used were: rabbit anti-dimethylated
and, after 3 washes with coIP buffer, they were eluted by boiling
recovered using protein G-coupled magnetic beads (Millipore).

**Immunofluorescence and Immunocytochemistry**—Mouse
embryos were dissected in phosphate-buffered saline (PBS) and
their intestinal tracts, including stomach, pancreas, and spleen,
were fixed in 4% paraformaldehyde (PFA) for 3 to 6 h. After
three washes in PBS, tissues were subsequently dehydrated,
embedded in paraffin wax, and sectioned at 3 μm. For immu-
nofluorescence, a standard immunodetection protocol was fol-
lowed. Briefly, tissues were rehydrated and subjected to heat-
mediated antigen retrieval in citrate buffer in a pressure cooker
for 10 min. After a blocking step in 3% donkey serum/0.2%
Triton X-100 tissue sections were incubated with the following
primary antibodies: goat anti-Foxa2/HNF3β 1:100, rabbit anti-
Sox9 1:200, rabbit anti-mucin-1 1:100 (Santa Cruz Biotechnol-
ogy), mouse anti-Neurogenin3 1:500, mouse anti-Nkx6.1 1:500
(Developmental Studies Hybridoma Bank, Iowa City, IA),
guinea pig anti-Pdx1 1:500 (Abcam, Cambridge, UK), guinea
pig anti-insulin 1:500 (DAKO, Glostrup, Denmark), rabbit anti-
chromogranin A 1:200 (Thermo Scientific). The secondary
antibodies used were donkey anti-goat Cy2, donkey anti-mouse
Cy3, donkey anti-rabbit Cy2 Cy3, or Alexa643, and donkey
anti-guinea pig Cy3 (Jackson ImmunoResearch, Suffolk, UK).

For immunocytochemistry, mPAC and MIN6 cells were
seeded onto coverslips and transduced with the β-galactosidase
and NEUROG3 adenoviruses as described above. Cells were
fixed 44 – 48 h after infection in 4% PFA for 20 min and permea-
bilized in PBS with 0.2–0.5% Triton X-100. After a blocking
step of 1 h in 3% normal donkey serum, cells were incubated
overnight with an antibody against mouse Neurog3 at 1:500
dilution. The secondary antibody used was donkey anti-mouse
Cy3. Before mounting onto slides, nuclei were stained for 3 min
in a 1:500 dilution of Hoechst 33258 (Sigma). Fluorescent
images were captured using a Leica DMi 6000B widefield
microscope or a Leica TCS SPE confocal microscope.

For quantification of Neurog3 and Foxa2 levels, single con-
focal scans were analyzed using image J software. Briefly, Foxa2
and Neurog3 fluorescence intensities of >100 Neurog3 positive
cells were quantified and normalized for each channel to the
maximum intensity (100%) for each of the images. To assign
cells a qualitative fluorescence intensity value (HIGH or LOW),
cells displaying fluorescence levels over 60% were arbitrarily
considered HIGH and cells a qualitative fluorescence intensity value (HIGH or LOW),
cells displaying fluorescence levels over 60% were arbitrarily
considered HIGH and vice versa.

**Statistical Analysis**—Data are presented as means ± S.E. of
the mean (S.E.). Statistical significance was tested using
Student’s t test.

**RESULTS**

**Neurog3 Activates Its Own Promoter**—As a first step to locate
cis-regulatory sequences potentially involved in Neurog3 auto-
regulation, we searched a 20 kb genomic region surrounding
the mouse Neurog3 gene for evolutionarily conserved regions.
We identified two areas that displayed extensive conservation
throughout vertebrates: a ~2 kb region located between −3 kb
and −5 kb and a 0.3 kb region lying immediately upstream of
the Neurog3 transcription initiation site (Fig. 1A).
To define sequences potentially involved in Neurog3 auto-regulation, we focused on the 5-kb region located upstream of the Neurog3 TSS and cloned 5’ deletion fragments of a 5 kb mouse Neurog3 genomic construct, depicted in Fig. 1A, in front of the luciferase gene. We used the pancreatic cell line mPAC and NIH3T3 fibroblasts, where positive and negative Neurog3 self-regulatory loops have been previously described (8, 16), to test whether Neurog3 alone, or in conjunction with its dimeric partner E47, increased the transcriptional activity of these reporter vectors. We found that, in both cell types,
Neurog3+E47 activated the 5kb Neurog3 promoter but were unable to do so when using the shorter promoter constructs containing 3 kb and 0.9 kb promoter fragments (Fig. 1, B and C). Altogether, our results demonstrate that Neurog3 can autoregulate its own expression and point to the distal conserved area (between −3 and −5 kb) as a required element in this autoregulatory loop. In agreement with these data, it is worth noting that sequences upstream from −2.6 kb have been shown to be necessary for high expression levels of a human gene. Moreover, we located 8 E boxes as putative Neurog3 binding sites within this highly similar fragment, all of which were conserved between both species. To determine whether this region alone could mediate Neurog3 autoregulation, we cloned a 670-bp fragment (−3885 nt to −3217 nt) containing all 8 E boxes in front of the SV40 promoter and co-transfected it along with Neurog3 and/or E47 in mPAC cells. As depicted in Fig. 1D, Neurog3+E47 significantly stimulated the activity of the Neurog3 (−3885 to −3217)-SV40 heterologous promoter by 3.5-fold, thus confirming that this region alone (hereafter referred to as −3.2 kb cluster) confers Neurog3 responsiveness to a heterologous promoter.

We next analyzed which E boxes in the −3.2 kb cluster were required for Neurog3 autoregulation. Given the high number of E boxes present in this region and the fact that bHLH factors exhibit preference for specific E box core sequences, we first determined which E box types are specifically activated by Neurog3. To this aim, we used ten different artificial enhancer luciferase reporter constructs containing three copies in tandem of each possible E box type, CANNTG (23), and tested their activation by Neurog3/E47. These assays revealed activation of the CATATG, CAGATG, and CAGCTG boxes by the Neurog3/E47 heterodimer, albeit the latter was more induced by E47 alone, indicating preference of the E47 homodimer for this site (supplemental Fig. S1). Based on these results, we then searched for potential target E boxes within the −3.2 kb cluster region and identified the closely located E boxes E2 and E3 (5ˈ to 3ˈ end) as putative Neurog3 binding sites. Thus, we disrupted E2 and E3, individually or in combination, and tested activation of the 5 kb Neurog3 promoter construct by Neurog3+E47. We found that mutation of E2 significantly impaired transcriptional activation of this construct, whereas mutation of E3 resulted in a reduced but still significant response. The effect of mutating both E2 and E3 was undistinguishable from that of E2 alone. Thus, these data suggest that E box E2 likely mediates Neurog3 autoregulation (Fig. 1E). Remarkably, the sequence of E box E2 (CAGATG) coincides with the Neurog3 responsive E boxes identified in the Pax4, NeuroD, and Atoh8 genes (22, 24–25).

**Neurog3 Binds and Induces Chromatin Modifications at the Distal −3.2 kb Cluster Region of the Endogenous Neurog3 Gene**—We next assessed whether Neurog3 was directly involved in Neurog3 gene autoregulation using ChIP assays. We determined Neurog3 binding to genomic sequences of the endogenous Neurog3 gene in mPAC cells transduced with a HA-tagged Neurog3 version. Our studies confirmed Neurog3 binding to the −3.2 kb cluster region but not to the proximal promoter (Fig. 2A). In addition, we confirmed that Neurog3 could bind to the −2 kb region of the Pax4 gene, as previously demonstrated by EMSA (24), whereas it did not bind the MyoD promoter, chosen as a negative control, as it is not induced by Neurog3 in
mPAC cells (Fig. 2A). Altogether, these data demonstrate that Neurog3 binds and activates its own promoter in vitro.

Functional activation of developmentally regulated enhancers has been associated with the deposition of active histone H3 lysine 4 methylation marks (H3K4me2/3) within these enhancer regions (26, 27). Consistent with this notion, we found that exogenous NeuroG3 expression resulted in increased H3K4me2 levels at the upstream −3.2 kb cluster region as well as at the Pax4 enhancer in mPAC cells (Fig. 2B). Also correlating with transcriptional induction of the endogenous Neurog3 gene, NeuroG3 increased deposition of H3K4me2 marks at the Neurog3 proximal promoter while it had no effect on the MyoD or Actb proximal promoters, neither of which is activated by Neurog3 (Fig. 2B). On the other hand, NeuroG3 had no statistically significant effects on H3K4me3 levels at any of the genes studied, even though it tended to increase this mark at the Neurog3 proximal promoter and Pax4 upstream enhancer (Fig. 2B). We next assessed whether the −3.2 kb cluster region was enriched in active histone marks in vivo by performing ChIP assays using chromatin prepared from whole pancreases from embryonic day (E) 15.5 mouse embryos. As shown in Fig. 2C, the −3.2 kb cluster region of the Neurog3 gene as well as the Pax4 enhancer exhibited similar and significantly higher H3K4me2 levels than the MyoD promoter (Fig. 2C). Despite that Neurog3 expression peaks at E15.5 (6), it should be noted that endocrine progenitor cells represent a minor fraction of total pancreatic cells, thus explaining the modest enrichment levels observed in endocrine-specific genes relative to Actb. In summary, these data demonstrate deposition of active chromatin marks at the −3.2 kb cluster region in response to exogenous Neurog3 in vitro and confirm the presence of these marks in this region in vivo, supporting the notion that the −3.2 kb cluster becomes activated by this transcription factor and could thus function as a developmentally regulated enhancer.

mPAC Cells Express a Set of Transcription Factors Present in Embryonic MPCs—To further understand the molecular cues underlying Neurog3 autoactivation, we assessed whether the autoregulatory loop was dependent upon cell context. We found that, among the tested cell lines, only mouse ductal mPAC and human ductal PANC-1 cells exhibited positive Neurog3 autoregulation (Fig. 3A). Indeed, despite correct Neurog3 transgene expression, neither the endocrine cell lines αTC1.6 (glucagon-expressing) and MIN6 (insulin-expressing) nor NIH3T3 fibroblasts induced the endogenous mouse Neurog3 gene (Fig. 3A). Furthermore, Neurog3 positive nuclei were detected by immunocytochemistry using an antibody against mouse Neurog3 in mPAC cells treated with the adenovirus encoding human NeuroG3 but not in control cells (Fig. 3B), confirming endogenous mouse NeuroG3 protein accumulation in addition to mRNA induction upon NeuroG3 transgene introduction.

Pancreatic ductal cells are known to express several of the transcription factors found in MPCs in the embryonic pancreas, namely HNF6, HNF1b, Sox9, Pdx1, and Foxa2. Hence, we postulated that the responsiveness of mPAC cells to Neurog3 autoregulation might be related to the presence of these pancreatic progenitor transcription factors. We compared expression of these and other known Neurog3 upstream regulators among different cell lines, and found that mPAC cells expressed the complete set of mRNAs encoding these proteins (Foxa2, Glis3, HNF6, HNF1b, Pdx1, and Sox9), whereas the endocrine and non-pancreatic tested cell lines expressed some but not all of these regulators (Fig. 3C). Therefore, it is plausible that because of this particular expression profile, mPAC cells are uniquely equipped to allow Neurog3-dependent autoactivation. Moreover, it may be argued that the similarities in pancreatic progenitor factor expression between mPAC cells and MPCs may also underlie the reported ability of these cells and of primary ductal cells to recapitulate the endocrine differentiation program upon Neurog3 expression (7, 8).

Neurog3 Synergizes with Foxa2 to Autoactivate Its Own Gene—In addition to E boxes, the −3.2 kb cluster region contains potential binding sites for several of the pancreatic transcription factors previously identified as Neurog3 upstream regulators, including the HNF factors HNF6, HNF1b, and Foxa2 (Fig. 4A). HNF6 is required for Neurog3 expression in the embryonic pancreas (11), while HNF1b and Foxa2 have been connected to Neurog3 expression through co-immunolocalization (28) and in vitro DNA binding assays (12), respectively. Furthermore, as shown in Fig. 3C, these three factors are endogenously expressed in mPAC cells. To investigate their possible involvement in Neurog3 autoregulation, we co-transfected expression vectors for HNF6, HNF1b, and Foxa2 in the presence or absence of Neurog3+E47 along with the 5 kb Neurog3 promoter reporter construct into mPAC cells. We found that both HNF6 and Foxa2 individually transactivated the 5kb Neurog3 promoter, albeit with different potencies (8-fold by
HNF6 and 2-fold by Foxa2), whereas HNF1b did not (Fig. 4B). Conversely, co-transfection of Neurog3 and its partner E47 with each of these factors revealed that only Foxa2 functioned in a synergistic manner with the bHLH heterodimer to trans-activate the Neurog3 promoter (Fig. 4B). The synergism between Foxa2 and the bHLH heterodimer was corroborated in

**FIGURE 4.** A, schematic representation of the −3.2 kb cluster region of the mouse Neurog3 distal promoter showing potential binding sites for several pancreatic progenitor transcription factors. B, luciferase reporter vector containing the −4.9 kb Neurog3 promoter fragment was transiently cotransfected with a CMV promoter-driven Renilla luciferase reporter plasmid and expression vectors encoding the indicated transcription factors or an empty vector (no cDNA) into mPAC and NIH3T3 cells. Reporter gene activity is expressed relative to the activity of the promoter-less parental vector pFOXLuc (backbone) and the empty expression vector, set at 1. All data are presented as the mean ± S.E. from 4–10 independent transfection experiments. *, p < 0.05, **, p < 0.01, ***, p < 0.001 versus no cDNA (empty bar) and #, p < 0.05, ##, p < 0.01 versus Neurog3 + E47. C, luciferase reporter vector carrying the −3.2 kb cluster region of the Neurog3 promoter ligated in front of the SV40 promoter (same as used in Fig. 1D) was transiently cotransfected with a CMV promoter-driven Renilla luciferase reporter plasmid and expression vectors encoding the indicated transcription factors or an empty vector into mPAC and NIH3T3 cells. Data shown represent the fold-activation relative to the activity of the parental reporter vector and the empty expression vector (set at 1) and are expressed as mean ± S.E. from 6–8 independent transfection experiments. *, p < 0.05, **, p < 0.01 versus no cDNA (empty bar), and #, p < 0.05, ##, p < 0.01 versus Neurog3 + E47. D, chromatin was prepared from mPAC cells and immunoprecipitation was carried out using an anti-Foxa2 antibody. Associated DNA was analyzed by real-time PCR using primers that amplified the distal conserved region and proximal promoter of the Neurog3 gene and the MyoD proximal promoter, which was used as negative control. Percentage enrichment of input was calculated as described under “Experimental Procedures.” Data represent mean ± S.E. from five independent experiments. P values relative to MyoD are shown on top of each bar. E, mPAC cells were transfected with a siRNA directed against Foxa2 (siFoxa2) or with a control siRNA (siCont). Foxa2 protein levels were analyzed by immunoblot analysis 48 h after transfection. Representative immunoblot and densitometric quantification (normalized by Actb). Data are mean ± S.E. of three independent experiments. F, mPAC cells were transfected with siFoxa2 or siCont and infected with an adenovirus encoding NEUROG3. 48 h later, total RNA was extracted and expression of the NEUROG3 transgene and the endogenous Neurog3 gene were assayed by real-time PCR. Results were normalized to the Tbp gene and expressed relative to levels in mPAC cells transfected with siCont (set at 1). Data are mean ± S.E. for five independent experiments. ***, p < 0.001 versus siCont.
**Neurog3 Positive Autoregulation**

![FIGURE 5. Co-expression of Foxa2 and Neurog3 in the mouse embryonic pancreas by immunofluorescence.](image_url)

NIH3T3 fibroblasts (Fig. 4B). As expected, the synergistic action of Neurog3 and Foxa2 was specific for the 5 kb construct and was not detected on the 3 kb promoter luciferase construct in either mPAC or NIH3T3 cells (data not shown). To determine if the distal Neurog3 autoregulatory region was sufficient for synergistic activation by Foxa2, we used the Neurog3 (−3885 to −3217)-SV40 heterologous promoter in transient transfection assays as in Fig. 1D. We found that Foxa2 exerted a more moderate effect on Neurog3 promoter autoactivation from this reporter vector than from the 5 kb Neurog3 promoter construct in mPAC and NIH3T3 cells (Fig. 4C), which may indicate that additional sequences are needed for full response of the Neurog3 promoter to Foxa2. In fact, the mouse Neurog3 gene harbors a Foxa2 site at the proximal promoter, conserved in the human gene, which has been shown to compete equally for Foxa2 binding as the distal site by shift assays (12). Further corroborating these evidences, we found that Foxa2 bound both the upstream and proximal Neurog3 promoter regions in mPAC cells using ChIP assays (Fig. 4D). However, mutation of the Foxa2 proximal site had no significant effect on the induction by Foxa2 of the Neurog3 promoter construct in luciferase assays (supplemental Fig. S2), precluding a major involvement of this site in the Foxa2 synergistic effect.

We next investigated whether Foxa2 is necessary for autoregulation of the endogenous Neurog3 gene by using siRNA-based transient knockdown of Foxa2 expression in mPAC cells. Transfection of the Foxa2-specific siRNA resulted in a significant reduction of Foxa2 protein levels in mPAC cells (Fig. 4E) and a concomitant marked reduction (~60%) in the activation of the Neurog3 gene upon exogenous NEUROG3 expression in these cells (Fig. 4F). Therefore, these data show that Foxa2 is required to induce the expression of the endogenous Neurog3 gene. All together, these findings point to a possible role of Foxa2 in endocrine fate specification through the modulation of Neurog3 expression levels in pancreatic progenitors.

**Foxa2 Protein Localization during the Secondary Transition**—While Foxa2 protein expression has been well characterized in early stages of pancreas development and in adult islets (29), its expression pattern during the secondary transition, the major endocrine differentiation wave that begins at approximately E14 in the mouse, has not been investigated in detail. To further define the expression pattern of Foxa2 during this period and determine whether Neurog3 and Foxa2 co-exist in endocrine progenitors, we performed a series of immunofluorescence staining using antibodies specific to Foxa2 in E13.5, E14.5, and E15.5 pancreata. We found that Foxa2 is expressed and readily detectable throughout the pancreatic epithelium at the three stages analyzed, but with distinct expression patterns between them (Fig. 5A and supplemental Fig. S3). Thus, at E13.5, Foxa2 was homogeneously expressed throughout the branching pancreatic epithelium, with the exception of a few cells displaying higher levels of this transcription factor (Foxa2^HIGH^) (supplemental Fig. S3). These cells appeared both grouped in clusters (corresponding to glucagon-expressing cells, data not shown) or as single cells within or in close proximity to the pancreatic epithelial chords (supplemental Fig. S3). In contrast, at E14.5-E15.5, Foxa2 was expressed at lower levels in the cells lining the lumen of the epithelial chords, while Foxa2^HIGH^ cells were detected within or adjacent to the epithelial chords (Fig. 5A and supplemental Fig. S3). Simultaneous staining for Foxa2 and

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Neurog3 revealed that Neurog3 positive cells were also positive for Foxa2 from E13.5 to E15.5 (Fig. 5B and supplemental Fig. S3). Remarkably, cells expressing high levels of Neurog3 (Neurog3HIGH, arrowheads in Fig. 5B) largely localized with Foxa2HIGH cells. To confirm a possible correlation between Neurog3 and Foxa2 expression levels, we quantified their respective fluorescence intensities in a series of confocal images from E15.5 pancreata. We found that indeed, there is a statistically significant correlation between Neurog3 and Foxa2 expression levels (Fig. 5D). In addition, cell quantification revealed that the ratio of Foxa2HIGH/Foxa2LOW cells is dramatically different in Neurog3LOW and Neurog3HIGH endocrine progenitor populations (Fig. 5E), indicating that Neurog3HIGH endocrine progenitors express Foxa2 at higher levels. Altogether, these studies show that Foxa2 and Neurog3 co-exist in vivo in endocrine progenitor cells and support a role for Foxa2 in enhancing the Ngn3 auto-regulatory loop during endocrine differentiation in vivo.

The fact that Foxa2 is present throughout the pancreatic epithelium at E14.5 and E15.5, prompted us to investigate its expression in the different pancreatic cell lineages. Thus, we performed a series of simultaneous immunofluorescence staining to evaluate the presence of low and/or high levels of Foxa2 together with well-known pancreatic markers. Co-staining of Foxa2 and Nkx6.1, which at these stages is excluded from tip cells, revealed that Foxa2 is expressed in this domain, which mainly contributes to acinar fates by E14.5 (30) (supplemental Fig. S4). Notably, some of the scattered double positive cells for Nkx6.1 and Foxa2HIGH were detected adjacent to the epithelial chords, suggestive of cells in an intermediate state of β-cell differentiation (supplemental Fig. S4). Given that Foxa2 levels appeared to be more variable in the cells located in the trunk of the developing pancreas, we performed a more extensive analysis on endocrine (Pdx-1, insulin, ChromograninA) and ductal (Sox9, mucin-1) markers, the cell lineages distinguishable in the trunk domain at E14.5. Staining against Foxa2 and Pdx1 in E14.5 pancreata revealed an almost complete overlap between these two factors. At this stage, Pdx1 is found in all pancreatic epithelial cells, albeit at much higher levels in β-cells. Most of the Pdx1HIGH cells did not display the highest levels of Foxa2, indicating that Foxa2HIGH cells did not correspond to β-cells (supplemental Fig. S4). Accordingly, immunodetection of insulin showed that most of the Foxa2HIGH cells are not β-cells (supplemental Fig. S4). However, detection of the pan-endocrine marker Chromogranin A (Chga), which is temporarily expressed after Neurog3, revealed an almost complete overlap with Foxa2HIGH cells (supplemental Fig. S4), showing their likely specification toward an endocrine fate.

During the secondary transition, Sox9 and mucin-1 serve as markers of a bipotential endocrine/ductal progenitor population, and are absent in both young and mature endocrine cells. Simultaneous antibody staining against Sox9 and Foxa2 revealed that Sox9+ bipotential ductal/endocrine progenitors contained Foxa2 at low levels. Conversely, Foxa2HIGH cells did not express Sox9 (Fig. 5C and supplemental Fig. S4). Triple immunofluorescence staining against Foxa2, Sox9, and Neurog3 further confirmed that most endocrine progenitors do not localize in the Foxa2LOW/Sox9+ ductal domain (Fig. 5C). Similarly, Foxa2LOW cells lining the epithelial chords contained mucin-1 on its apical side, thus confirming the low Foxa2 expression levels in ductal/endocrine progenitors (supplemental Fig. S4). In summary, we found that Foxa2 is expressed in low abundance in the ductal/endocrine progenitors lining the epithelial chords, but its expression level seems to peak in the Neurog3HIGH endocrine precursors supporting a role for Foxa2, together with Neurog3, in the specification of endocrine cells.

**Foxa2 and Foxa1 Display Similar Abilities for Neurog3 Autoactivation**—The closely related transcription factor Foxa1 shares gene targets with Foxa2 in multiple tissues including the pancreas. Both factors are thought to exert both common and distinct functions during pancreatic development as well as in the maintenance of adult islet functionality (31, 32). Hence, we postulated that Foxa1 and Foxa2 might also share the ability to enhance Neurog3 gene autoactivation. Consistent with this notion, we found that Foxa1 synergistically cooperated with Neurog3+E47 in activating the Neurog3 promoter in transient transfection assays in mPAC and NIH3T3 cells (Fig. 6, A and B), thus demonstrating that Foxa1, like Foxa2, can regulate Neurog3 promoter activity in vitro.

The Foxa1-expressing cells in the fetal pancreas have not been precisely defined and hence, we performed immunofluorescence staining against Foxa1 in E14.5 pancreata. In agreement with previous reports (29, 33), we failed to detect Foxa1 protein in endocrine progenitors at this stage (data not shown), thus ruling out a relevant role of this protein in the regulation of Neurog3 expression levels in vivo. Interestingly, Foxa1 mRNA is faintly expressed in mPAC cells (Fig. 6C), but it is significantly induced upon Neurog3 introduction in these cells (Fig. 6D), uncovering Foxa1 as a potential Neurog3 downstream target during endocrine cell differentiation. In this regard, it should be noted that Foxa1 expression is enriched in islets but negligible in acinar cells in the adult pancreas (29). Thus, our results indicate that although Foxa2 and Foxa1 are both equally capable of potentiating the autoactivation of Neurog3 in in vitro luciferase assays, in vivo, Foxa1 probably functions at later stages of endocrine differentiation.

**Foxa2 Knockdown Impairs Neurog3-induced Activation of the Endocrine Differentiation Program**—To determine the molecular basis of the synergy between Neurog3 and Foxa2, we investigated whether Foxa2 physically interacts with Neurog3. We used an antibody against Foxa2 to immunoprecipitate Foxa2-interacting partners from cell extracts of mPAC cells transduced with an adenovirus expressing a HA-tagged version of Neurog3. We observed that Foxa2 physically interacts with Neurog3 in mPAC cells transduced with an adenovirus expressing a HA-tagged version of Neurog3 or cells left untreated. The anti-HA antibody detected Neurog3 in the Foxa2 immunoprecipitate, whereas it failed to detect it in the IgG immunoprecipitate (Fig. 7A). These results indicate that Foxa2 interacts with Neurog3 both physically and functionally to regulate Neurog3 gene transcription.

Disruption of Foxa2 prevents full differentiation of fetal α- and β-cells (34, 35). These evidence together with our observation that Neurog3 and Foxa2 co-immunoprecipitate in the same complexes prompted us to investigate whether Foxa2 is broadly involved in Neurog3-dependent activation of the endocrine differentiation program. To this aim, we compared activation of several Neurog3 gene targets in mPAC cells transduced with
AdCMV-NEUROG3 in the presence of the siRNA against Foxa2 or a siRNA control as established previously (see Fig. 4). We found that Foxa2 knockdown resulted in significantly reduced induction of genes encoding markers of differentiating (NeuroD1, Pax4, Ins1) as well as fully differentiated (IAPP, Gck) endocrine cells (Fig. 7B). However, not all Neurog3 targets were blocked by siFoxa2 (i.e. Atoh8, Sst), disclosing gene-specific effects of Foxa2 knockdown. Interestingly, siFoxa2 did reduce Foxa1 activation but to a lesser extent (16%) than the other studied genes, suggesting that Foxa1 inhibition is unlikely to be primarily responsible for the impairment in endocrine gene activation in response to siFoxa2. Taken together, these results uncover the possible direct contribution of Foxa2 to the establishment of the Neurog3-dependent endocrine transcriptional program (Fig. 7C).

**DISCUSSION**

Neurog3 is both essential and sufficient for endocrine cell differentiation in the pancreas, and understanding how Neurog3 is regulated will be valuable not only for attaining mechanistic insights into pancreatic endocrine cell genesis during development, but also for devising better in vitro differentiation protocols for β-cell replacement therapies in diabetes. In this study, we define a positive direct autoregulatory mechanism for Neurog3, which involves binding of Neurog3 to a highly conserved 0.7 kb region located at −3.2 to −3.9 kb of the Neurog3 promoter. In addition, we show that the forkhead-containing transcription factor Foxa2, which is expressed in MPCs, synergizes with Neurog3 to enhance this autoactivation loop, revealing its potential contribution to regulation of Neurog3 expression in endocrine progenitors. Furthermore, our study shows that the functional interaction between Neurog3 and Foxa2 extends to other Neurog3 target genes in vitro, thus uncovering a possible cooperation of these transcription factors in the implementation of the endocrine differentiation program in the pancreas.

Positive autoregulation is a transcriptional control mechanism used by many cell fate-determining bHLH factors in development (36–39), which is thought to sustain expression of these proteins throughout specific time windows so that they can reliably perform their developmental functions. In addition, because autoactivation can release these factors from control by external stimuli, it may also ensure cell fate commitment (40). Another suggested function of positive autoregulatory loops is to raise the expression levels of cell fate-determining factors over a threshold necessary for initiation of their respective developmental programs (41). The observation that low Neurog3 expression in MPCs results in their allocation to exocrine fates (18) underlines the potential importance of this
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et al. were published that support the concept that Neurog3 enforces its own expression in the embryonic pancreas (43). In their study, the authors found decreased Neurog3 promoter activity in Neurog3-deficient embryos, consistent with the existence of Neurog3 autoactivation in vivo. However, whether this occurs through direct positive autoactivation, is mediated by additional Neurog3-dependent factors, or both, is difficult to discriminate using in vivo models. Our present findings demonstrate that Neurog3 can activate its own promoter in vitro, thus validating at a molecular level the existence of a direct autoregulatory mechanism. Even so, we cannot exclude the possibility that additional positive feedback loops control Neurog3 expression in the embryonic pancreas (21). In any case, promoter occupancy data using chromatin from purified endocrine progenitors will be needed to provide definite confirmation of the existence of direct autoregulation.

In many instances, autoregulatory loops involving cell-fate determining factors rely on extrinsic cues that promote cell fate decisions. For example, in Drosophila's sensory organ development, proneural bHLH autoregulation is contingent on the activation of the EGFR pathway (39). Conversely, Notch inhibits its sensory organ precursor recruitment by directly antagonizing proneural bHLH autoactivation (44). Based on present and previously available data, we propose a model where inhibition of the Notch pathway would activate Neurog3 expression in progenitor cells (17, 12, 43, 45), while Neurog3 autoactivation would act to boost Neurog3 levels after transcription has been initiated. This last process would be independent of Notch activity as recently suggested (43). Nonetheless, we cannot rule out that, in addition to Neurog3 itself, other signals are required to trigger Neurog3 autoactivation in vivo. In other words, Neurog3 autoregulation may be restricted to some cells by additional signals, leading to differences in the amount of Neurog3 and, in turn, in different cell fate outcomes. In this regard, it is noteworthy that Neurog3 mRNA is more broadly expressed than Neurog3 protein in the pancreatic epithelium (46), implying that not all cells that activate the Neurog3 gene end up producing detectable amounts of this factor. Both the Notch and the GDF11/TGFβ signaling pathways, which are known to regulate the number of Neurog3+ cells in the pancreas (17, 47), could potentially interact with Neurog3 self-regulation.

In addition to extrinsic signals, intrinsic signals can also influence autoregulatory loops. Accordingly, our present data indicate that Neurog3 self-regulation depends on the cellular context. We show that Neurog3 autoactivation occurs in pancreatic ductal cell lines. Likewise, Neurog3 autoactivation has recently been reported in primary cultures of adult human pancreatic duct cells (48). We postulate that the presence of key transcription factors normally found in MPCs (and implicated as Neurog3 gene activators) makes duct cells competent to respond to Neurog3 autoregulation. It is possible that binding of these factors to cis-acting elements of the Neurog3 gene directs recruitment of Neurog3 to its own gene (note the clustering of progenitor factor binding sites within the −3.2 kb region), and/or warrants a chromatin structure permissive of transcription. Particularly, we show that Foxa2 affects Neurog3 autoactivation both in reporter luciferase assays and on activation of the endogenous Neurog3 gene in mPAC cells. Remark-
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ably, one of the key functions of the Foxa family of forkhead-containing transcriptional regulators is to serve as pioneer factors that initiate regulatory control of transcription through direct opening of chromatin (49). In this regard, we have found that the Neurog3 proximal promoter, which harbors a conserved Foxa binding site (12), exhibits moderate enrichment in the active histone mark H3K4me2 in control mPAC cells, indicating that despite being silent, the Neurog3 gene is transcriptionally competent in these cells. However, the sole presence of Foxa2 is not sufficient to confer competency for Neurog3 autoactivation as Foxa2 is expressed in all pancreatic cell lines studied, but not all cells exhibit Neurog3 self-regulation. Therefore, Neurog3 autoregulation likely requires several components, both transcriptional and epigenetic, to operate in some cells and not in others.

Cooperative interactions between transcription factors are instrumental to the regulation of gene expression. Here we show that Foxa2 synergizes with the Neurog3/E47 bHLH heterodimer to regulate not only Neurog3 autoactivation but also the induction of other Neurog3 target genes, uncovering a specific role of Foxa2 in the induction of endocrine cell differentiation. Foxa2 deletion in the early pancreatic epithelium results in normal endocrine differentiation, but α-cells fail to completely mature (34). However, single knockouts may not evidence the full complement of functions exerted by individual Foxa factors due to functional compensation by remaining Foxa proteins (50). Thus, compound Foxa2 and Foxa1 mutants exhibit severe pancreatic hypoplasia and impaired acinar and endocrine cell development (31). Nevertheless, whether the endocrine differentiation defects in this mouse model are due to the specific absence of Foxa2 in the prospective endocrine precursor or secondary to impaired expansion of the MPC population is unclear at present. Conditional deletion of Foxa genes in endocrine progenitors will be necessary to elucidate the role of these factors in the activation of the endocrine developmental program.

It has been argued that autoregulation positively correlates with the developmental and/or physiological importance of a given transcription factor (41). Because of the crucial role of Neurog3 in endocrine cell differentiation, it is expected that multiple direct and indirect positive and negative feedback loops will cooperate to control Neurog3 gene expression. In this study, we have characterized one of these loops that involve direct transcriptional autoactivation by Neurog3 of its own promoter. Further work aimed at extending our knowledge on the autoregulatory and crossregulatory circuits that regulate Neurog3 levels is needed to better comprehend how endocrine cell fate is established during pancreatic development.

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