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OBJECTIVE—Neurogenin 3 plays a pivotal role in pancreatic endocrine differentiation. Whereas mouse models expressing reporters such as eGFP or LacZ under the control of the Neurog3 gene enable us to label cells in the pancreatic endocrine lineage, the long half-life of most reporter proteins makes it difficult to distinguish cells actively expressing neurogenin 3 from differentiated cells that have stopped transcribing the gene.

RESEARCH DESIGN AND METHODS—In order to separate the transient neurogenin 3-expressing endocrine progenitor cells from the differentiating endocrine cells, we developed a mouse model (Ngn3-Timer) in which DsRed-E5, a fluorescent protein that shifts its emission spectrum from green to red over time, was expressed transgenically from the NEUROG3 locus.

RESULTS—In the Ngn3-Timer embryos, green-dominant cells could be readily detected by microscopy or flow cytometry and distinguished from green/red double-positive cells. When fluorescent cells were sorted into three different populations by a fluorescence-activated cell sorter, placed in culture, and then reanalyzed by flow cytometry, green-dominant cells converted to green/red double-positive cells within 6 h. The sorted cell populations were then used to determine the temporal patterns of expression for 145 transcriptional regulators in the developing pancreas.

CONCLUSIONS—The precise temporal resolution of this model defines the narrow window of neurogenin 3 expression in islet progenitor cells and permits sequential analyses of sorted cells as well as the testing of gene regulatory models for the differentiation of pancreatic islet cells. Diabetes 58:1863−1868, 2009

The mature pancreas is composed of exocrine (acinar and duct cells) and endocrine (α, β, δ, ε, and PP-cells) compartments. The differentiation of these distinct cell types is regulated by the coordinated expression of numerous transcription factors (1−3). Among these transcription factors, neurogenin 3 (Neurog3), a member of the basic helix-loop-helix transcription factor family, plays essential roles in initiating endocrine differentiation during embryonic development, regeneration, and transdifferentiation into functional insulin-producing cells (4−9). In addition, the transient nature of Neurog3 expression makes it a useful marker for uniquely identifying endocrine progenitor cells—cells that have committed to the endocrine lineage but have not yet differentiated into hormone-producing endocrine cells (10,11).

Mouse models expressing fluorescent reporter proteins have been used to sort specific cell populations. For example, cells sorted from Ngn3-eGFP mouse lines generated by different groups have been used to examine gene expression profiles during pancreatic endocrine differentiation (12,13). However, because of the long half-life (14), fluorescent reporter proteins persist after the Neurog3 gene itself has shut off; thus, the fluorescent cell population includes cells at different stages of differentiation. Destabilized fluorescent proteins have shorter half-lives but lower fluorescence (15). In addition, sorting cells at earlier time points may decrease the overlap with more differentiated cells described previously (13); however, this approach cannot be used at later time points or for distinguishing more mature cells.

To solve this problem, we developed a novel transgenic mouse model (Ngn3-Timer) in which human NEUROG3 upstream and downstream sequences were used within a bacterial artificial chromosome (BAC) to drive expression of DsRed-E5, a variant of the *Discosoma sp.* red fluorescent protein that shifts its fluorescence emission peak from green to red in a time-dependent manner (16). Using fluorescence microscopy, green fluorescence could be detected in developing pancreata of Ngn3-Timer embryos as early as embryonic day 9.5 (E9.5) (data not shown). Both green and red fluorescent signals were readily detected in developing pancreata of Ngn3-Timer embryos from E12.5 to E18.5, whereas predominantly red fluorescence was observed at postnatal day 7 (P7) (supplemental Fig. 1, available in the online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0390/DC1), consistent with previous reports that few Neurog3-expressing cells persist after birth (5). At E17.5, histological analyses detected green-dominant and green/red double-positive fluorescent cells in close apposition with the ductal lumen, whereas red-dominant cells appeared in islet-like clusters (Fig. 1A), supporting a model whereby cells in the endocrine lineage emerge from ductal regions as Neurog3-positive cells and migrate away from the ductal region as differentiation progresses. Consistent with this model, staining for Neurog3 protein overlapped with predominantly green-dominant cells and some green/red double-positive cells (supplemental Fig. 2), whereas insulin staining overlapped with green/red double-positive cells and red-dominant cells (supplemental Fig. 3).

To quantitatively shift the fluorescence during endocrine cell differentiation, flow cytometric analyses were performed with dissociated cells isolated from Ngn3-Timer mice. Labeled cells isolated from E13.5 pancreata emitted fluorescence predominantly in the green channel; however, a progressive increase in red fluorescence was observed in older embryos until P0, when the number of

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green-dominant cells dramatically declined compared with the earlier time points and most labeled cells fluoresced in the green/red double-positive area (Fig. 1B). These data support the hypothesis that the DsRed-E5 protein initially expressed in the Neurog3 lineage emits in the green spectrum and that as the cells turn off Neurog3 expression, age, and differentiate, it shifts to the red emission spectrum.

FIG. 1. Expression of the DsRed-E5 fluorescent protein in developing pancreas. A: The pancreas was dissected from an Ngn3-Timer embryo at E17.5 and stained with DBA lectin, a marker of pancreatic duct (blue). Green-dominant cells detected within ducts are labeled with white arrows; green/red double-positive and red-dominant cells (yellow or orange in color, right panel) are labeled with orange arrowheads. Scale bar: 50 μm. B: Pancreata from wild-type (A–C) and Ngn3-Timer mice (D–F) were dissociated at E13.5, E17.5, and P0 and analyzed using flow cytometry. Green fluorescence is shown on the vertical axis and red fluorescence on the horizontal axis. (A high-quality digital representation of this figure is available in the online issue.)
To verify this hypothesis and estimate the temporal resolution of this model, fluorescent cells were sorted by a fluorescence-activated cell sorter (FACS) into four different populations, placed in culture, and then reanalyzed by flow cytometry at various time points following culture. Green-dominant cells, sorted from gate A in Fig. 2, con-
confirmed the sequential maturation of these cells. The
green/red double-positive cells in gate B converted to the
lower green/red ratio (red dominant) of gate C cells within
12 h. Therefore, the green-dominant cells were within a 6-h
time window after initial DsRed-E5 expression and green/
red double-positive cells within a 12-h time window. On the
other hand, the fluorescent cells sorted using gate C
changed little over 12 h, presumably because of the long
half-life of DsRed-E5.

Real-time PCR analyses performed on RNA from cells
sorted by FACS into the four populations defined in Fig. 2
confirmed the sequential maturation of these cells. The
expression level of Neurog3 was highest in green-domin-
inant cells and dramatically decreased in green/red double-
positive cells (Fig. 3A), reflecting the rapid downregulation
of Neurog3 once it has initiated endocrine differentiation.
Expression of the islet hormones peaked later (Fig. 3B–D),
with the mRNA encoding insulin and glucagon highest in
cells from gate C, the most mature population.

To gauge the expression of known pancreatic transcrip-
tional regulators (13,17) in these differentiating cell popu-
lations, TaqMan RT-PCR assays were performed with RNA
from cells isolated by FACS from E17.5 Ngn3-Timer pan-
creata. The expression levels of 145 genes were normal-
ized to β-glucuronidase, and the relative level of mRNA
from each gate was determined with respect to the value
in nonfluorescent cells (gate N). For 21 genes, expression,
in the earliest progenitor cells (gate A) exceeded expres-
sion in the nonfluorescent cells by more than 10-fold (gate
[A]/gate [N] > 10; Fig. 3E–J and supplemental Table).

Among these genes, three (Neurog3, Pax4, and Mycl1)
demonstrated a sharp expression peak in gate A, with a
subsequent decline of more than 10-fold from gate A to
gate C (Fig. 3J)—which is consistent with the conclusion
that these genes have expression restricted to the islet
progenitor cells. These data confirm previous evidence
that Neurog3 directly activates transient Pax4 gene ex-
pression followed by repression by Pax4 (18). The profile
of Mycl1 paralleled those of Neurog3 and Pax4; therefore,
it may also play a specific role in the transient endocrine
progenitor cells.

Several of the other genes, including NeuroD, Nkx2.2,
Myt1, and Ins6m1, are also known targets of Neurog3 but
persist in mature islet cells (1–3). In addition, this ap-
proach identified several factors with known expression
in the pancreas (13,17) but without known roles in the
Neurog3 pathway, including Mycl1, Ntf4, Pou2f2,
Sim1, and Fev. Fev, which encodes an ETS transcription
factor and showed the highest relative induction in the
gate A cells (Fig. 3E), and we have recently confirmed its
expression and function in the islet lineage (Y. Ohta and
M.S.G., unpublished data). Finally, Rfx6, a member of the
Rfx family of winged-helix transcription factors, has not
been studied in the pancreas previously, but these data
place it in the Neurog3 pathway and islet lineage.

Of the 145 genes only three, Mafa, Hopx, and Myt11,
showed a robust increase from gate A to gate C (Fig. 3K)
that paralleled the expression profiles of Ins1 and Ins2
(Fig. 3B and C). Mafa appears late in pancreatic develop-
ment specifically in mature β-cells (19) and directly trans-
activates the insulin gene in conjunction with NeuroD1
and Pdx1, which are expressed earlier. Therefore, Mafa
may play a critical role in the final maturation of β-cells. It
follows that Hopx and Myt11 potentially also contribute to
the maturation of the endocrine cells.

As expected, Ptf1a and Rbpjl, known exocrine genes,
were confined to gate N (Fig. 3L). On the other hand,
mRNA encoding Hes1, Notch2, and Onecut1 (Hnf6),
which regulate the expression of Neurog3 (4,20,21), persisted
in gate A but rapidly declined as cells matured (Fig. 3M).

In summary, the Ngn3-Timer mouse provides a useful in
vivo tool for temporal dissection of the differentiation
processes. The model allowed us to accurately isolate
cells from distinct narrow time points along the differenti-
ation pathway and also to study the characteristics of	hose cells. We have used this tool to identify genes that
likely play unique roles at these different steps of differ-
entiation. The availability of this tool also permits rela-
tively facile methods for studying signals that impact the
initiation or completion of differentiation, the rate of
differentiation, or the proliferation or death of differenti-
ating cells. Likewise, similar transgenic models could be
used to study the temporal features of differentiation in
other cell populations.

RESEARCH DESIGN AND METHODS

Generation of Ngn3-Timer transgenic mouse. Starting with a BAC con-
taining 134 kb upstream and 30 kb downstream of the human NEUROG3 gene
(clone RP11-3433J3 [Sanger Institute, U.K.], the NEUROG3 coding sequence
was replaced by the DsRed-E5 coding region (pTimer; Clontech, Palo Alto,
CA) via homologous recombination. The modified BAC was purified using
celose chloride gradient ultracentrifugation, dialyzed, and microinjected into
the pronuclei of B6SLF1 oocytes to generate the Ngn3-Timer transgenic
mice. The mice were genotyped by PCR using the forward primer 5’-
cgctgctcatcgctctcta-3’ in the 5′-flanking sequence of NEUROG3 and
the reverse primer 5’-GGTTTGGTGCCCTCGTAG-3’ in the coding region
of DsRed-E5. A total of six lines of Ngn3-Timer mice were generated and
analyzed by means of flow cytometry and microscopy, and two highly
fluorescent lines were maintained for further analyses. Both high-expressing
lines gave the same expression pattern. One of these two lines was used for
all of the studies reported here.

Mice were housed on a 12-h light/dark cycle in a controlled climate. Timed
matings were carried out with E0.5 being set as midday of the day of discovery
of a vaginal plug. All studies involving mice were approved by the University
of California San Francisco Institutional Animal Care and Use Committee.

Whole-mount observation and histological analyses. Transgenic Ngn3-
Timer embryos were killed from E12.5 to P7, and macroscopic appearance
and fluorescence of the Ngn3-Timer mice were examined using the fluorescent
dissecting microscope. For histological analyses, tissues were fixed in 4% paraformaldehyde in PBS at 4°C, washed in PBS alone, and then immersed in
sucrose solution in PBS overnight at 4°C. The next day, the tissues were
embedded and frozen in Tissue-Tek (OCT Compound, Sakura). Tissues were
sectioned at 6 μm, permeabilized with 0.1% Triton X-100, blocked with 5% goat
serum, incubated with biotinylated lectin Dolichos biflorus agglutinin (DBA; Vector Laboratories) diluted 1:200 in PBS, and then visual-
ized using Alexa Fluor 350–conjugated streptavidin ( Molecular Probes, Eu-
geone, OR).

Pancreatic cell dispersion and flow cytometry. The Ngn3-Timer trans-
genomic mice were distinguished from control littermates using the fluorescent
dissecting microscope, and the whole pancreata were manually dissected
from other organs. Pancreata were treated with 0.05% trypsin/0.5 mM EDTA (Invitrogen, Carlsbad, CA) at 37°C for 5 min, and the diges-
tion was inactivated by addition of FBS. The dissociated cells were resuspended in
FACS buffer (% FBS in PBS) and then analyzed using an LSR II flow cytometer
(PeRkinElmer) or sorted using a MoFlo cell sorter (Dako Cytomation, Carp-
ineria, CA). Dead cells were excluded with DNA dye TO-PRO-3 (Molecular Probes, Eugene, OR).

Cell culture. The Ngn3-Timer pancreata were dissociated at E17.5 and sorted
by FACS into the different populations shown. The sorted cells were incu-
ated in growth medium (DME H-16 50%/F-12 50% with 10% FBS, antibiotics,
and insulin-transferrin-selenium) at 37°C in 5% CO2. After 2–12 h of culture,
the cells were dissociated with 0.05% trypsin/0.53 mmol/l EDTA, transferred
into FACS tubes, and then analyzed using an LSR II flow cytometer
(PeRkinElmer).

Real-time quantitative PCR. Total RNA was extracted from cells sorted by
FACS from three independent groups of E17.5 Ngn3-Timer embryos using the
RNeasy Plus micro kit (Qiagen, Valencia, CA) according to the manufac-
turer’s protocol. The quality and quantity of extracted RNA were assessed with
the Agilent 2100 Bioanalyzer using the RNA 6000 Pico Assay kit
FIG. 3. Temporal transcriptome analysis in the pancreata of Ngn3-Timer embryos. A–D: Ngn3-Timer pancreata were dissected at E17.5 and sorted by FACS into four gates (gates A, B, C, and N). The sorted cell populations were analyzed by real-time RT-PCR for mRNA-encoding transcriptional regulators and endocrine hormones. All expression levels were normalized to β-glucuronidase. Neurog3 (A), insulin1 (B), insulin2 (C), glucagon (D). E–M: TaqMan array was performed for 145 transcription factors. Expression levels are shown relative to the level in gate N. Each data point represents the mean of three independent experiments.
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