The cellular regulators PTEN and BMI1 help mediate NEUROGENIN-3–induced cell cycle arrest

Received for publication, April 30, 2019, and in revised form, June 25, 2019. Published, Papers in Press, July 24, 2019, DOI 10.1074/jbc.RA119.008926

R. Sergio Solorzano-Vargas‡, Matthew Bjerknes†, S. Vincent Wu‡, Jiafang Wang‡, Matthias Stelzner‡, James C. Y. Dunn**, Sangeeta Dhawan††, Hazel Cheng‡, Senta Georgia§§, and Martin G. Martin†††

From the ‡Department of Pediatrics, Division of Gastroenterology and Nutrition, Mattel Children’s Hospital and the David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California 90095, the **Department of Medicine, Medical Sciences Building, University of Toronto, Toronto, Ontario M5S 1A8, Canada, the †Veterans Affairs Greater Los Angeles Healthcare System, and Department of Medicine, University of California, Los Angeles, Los Angeles, California 90073, the ††Division of General Surgery, Department of Surgery, University of California, Los Angeles, Los Angeles, California 90095, the ‡‡Division of Pediatric Surgery, Stanford University School of Medicine, Stanford, California 94305, the †§Division of Translational Research and Cellular Therapeutics, City of Hope, Duarte, California 91010, and the †††Department of Pediatrics, Division of Endocrinology, Children’s Hospital of Los Angeles, University of Southern California, Los Angeles, Los Angeles, California 90027

Edited by Joel M. Gottesfeld

Neurogenin-3 (NEUROG3) is a helix-loop-helix (HLH) transcription factor involved in the production of endocrine cells in the intestine and pancreas of humans and mice. However, the human NEUROG3 loss-of-function phenotype differs subtly from that in mice, but the reason for this difference remains poorly understood. Because NEUROG3 expression precedes exit of the cell cycle and the expression of endocrine cell markers during differentiation, we investigated the effect of lentivirus-mediated overexpression of the human NEUROG3 gene on the cell cycle of BON4 cells and various human nonendocrine cell lines. NEUROG3 overexpression induced a reversible cell cycle exit, whereas expression of a neuronal lineage homolog, NEUROG1, had no such effect. In endocrine lineage cells, the cellular quiescence induced by short-term NEUROG3 expression required cyclin-dependent kinase inhibitor 1A (CDKN1A)/p21CIP1 expression. Expression of endocrine differentiation markers required sustained NEUROG3 expression in the quiescent, but not in the senescent, state. Inhibition of the phosphatase and tensin homolog (PTEN) pathway reversed quiescence by inducing cyclin-dependent kinase 2 (CDK2) and reducing p21CIP1 and NEUROG3 protein levels in BON4 cells and human enteroids. We discovered that NEUROG3 expression stimulates expression of CDKN2a/p16INK4a and BMI1 proto-oncogene polycomb ring finger (BMI1), with the latter limiting expression of the former, delaying the onset of CDKN2a/p16INK4a-driven cellular senescence. Furthermore, NEUROG3 bound to the promoters of both CDKN1a/p21CIP1 and BMI1 genes, and BMI1 attenuated NEUROG3 binding to the CDKN1a/p21CIP1 promoter. Our findings reveal how human NEUROG3 integrates inputs from multiple signaling pathways and thereby mediates cell cycle exit at the onset of differentiation.

NEUROGENIN-3 (NEUROG3) is a basic helix-loop-helix (HLH) transcription factor involved in gastrointestinal endocrine (GIE) cell lineage determination (1). In the mouse intestine, some NEUROG3+ cells are in cycle, but the majority are postmitotic and beginning to express early GIE cell markers (2). Similarly, in the developing mouse pancreas, mitotically active progenitor cells exit the cell cycle after the initiation of Neurog3 expression, in a process involving the cyclin-dependent kinase inhibitor 1a (Cdkn1a/p21CIP1) (3). Furthermore, ectopic expression of Neurog3 in early postnatal β-cells attenuates their proliferation, and results in a reduction of total β-cell mass leading to diabetes mellitus. Neurog3 expression seems to drive mouse pre-endocrine lineage cells from the cell cycle, initially inducing cellular quiescence. Cellular senescence sets in as the exit from cell cycle becomes irreversible.

The polycistronic gene Bmi1 (4) is also involved in cell cycle exit during mouse endocrine cell lineage development. Proliferating β-cells from young mice express high levels of Bmi1, and low levels of Cdkn2a/p16INK4a (5–7). As mice age, Bmi1 is displaced from the p16INK4a locus, resulting in increased p16INK4a expression and the consequent withdrawal of proliferating β-cells from the cell cycle. Similarly, β-cells in Bmi1-null mice

This work was supported by National Institutes of Health NIDDK Grants DK083762 and DK41301, and NIAID Grant U01DK085535. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This article contains Figs. S1–S3 and Tables S1–S3.

1 To whom correspondence should be addressed. Tel.: 310-794-5532; Fax: 310-206-0855; E-mail: mmartin@mednet.ucla.edu.

2 The abbreviations used are: HLH, helix-loop-helix; bHLH, basic helix-loop-helix; BMI1, BMI1 proto-oncogene polycomb ring finger; CDK, cyclin-dependent kinase; CHGA, chromogranin A; cNEUROG1, cNEUROG3, and cCONTROL, constitutively expressing lentiviruses; Cum, cumeate; Dox, doxycycline; GAST, gastrin; GIE, gastrointestinal endocrine; HES, hair/enhancer of split; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; NEUROD1, neurogenic differentiation factor 1; NEUROG3, neurogenin-3; NLS, nuclear localization signal; p16INK4a, Cdkn2a cyclin-dependent kinase inhibitor 2a; p21CIP1, Cdkn1a cyclin-dependent kinase inhibitor 1a; p53IP3, Cdkn1c cyclin-dependent kinase inhibitor 1c; PCSK1, proprotein convertase subtilisin/kexin type 1; PTEN, phosphatase and tensin homolog; qPCR, quantitative PCR; SA-β-Gal, senescence-associated β-galactosidase; ANOVA, analysis of variance; PFA, paraformaldehyde.

© 2019 Solorzano-Vargas et al. Published under exclusive license by The American Society for Biochemistry and Molecular Biology, Inc.
become quiescent prematurely, reducing β-cell numbers with consequent failure of glucose homeostasis (5–7).

The role of BMI1 as a mediator of senescence and quiescence is further highlighted by the recent observation that the population of Bmi1 1+ cells in the mouse intestinal crypt is enriched with quiescent early GIE lineage cells (8), likely related to Neurog3-expressing cells known to be concentrated in the same crypt domain (2). Since Neurog3 expression is weak in the Bmi1 1+ population (8), Bmi1 expression is likely downstream of Neurog3. Similar quiescent cells, identified by mTert expression, respond to post-fasting refeeding by repressing Phosphatase and tensin homolog (Pten) expression, and re-entering the cell cycle (9). These observations suggest that in mice, Neurog3’s cell cycle effects occur in early GIE lineage cells and are mediated downstream by Bmi1 and Pten.

The phenotype of loss of NEUROG3 function is similar in humans and mice, however, there are clinically significant differences. Notably, whereas loss of NEUROG3 function in both humans and mice abolishes production of intestinal endocrine cells, β-cell development is less impacted in humans than in mice at birth, but results in severe diabetes in early childhood (10, 11). Therefore, it is of interest to establish whether NEUROG3 expression in human pre-endocrine cells similarly induces exit from the cell cycle, and if so identify the mechanisms involved.

Here, we introduce the use of the BON4 cell line as a model for investigating the response of the human endocrine cell lineage to NEUROG3. We report that NEUROG3 initially induces quiescence in a p21CIP1-dependent manner. Furthermore, if NEUROG3 expression is prolonged, then the initially reversible cycle arrest (cellular quiescence) gradually becomes permanent (cellular senescence). The early Neurog3-induced cellular quiescence is reversible by inhibition of PTEN, due to reduction in steady-state NEUROG3 levels in BON4 cells and human enteroids. We also report that the onset of NEUROG3-induced senescence is slowed due to concomitant induction of BMI1 expression, which acts by attenuating p16INK4 expression.

Results

**NEUROG3 inhibits the cell cycle in an endocrine cell line, but NEUROG1 does not**

BON cells are a well-characterized human endocrine lineage cell line (12), from which a homogenous appearing BON4 subline was isolated; it expresses very low levels of NEUROG3 and NEUROG1 (Fig. S1, A and B). BON4 cells are readily transduced with constitutively active lentiviruses-encoding NEUROG3 (cNEUROG3), NEUROG1 (cNEUROG1), or Control sequence (cControl); expression is detectable within 1 day of transduction (Fig. S1, A and B). NEUROG1 is a close homolog of NEUROG3, not involved in GIE specification (13). In an unrelated study, 3 to be published separately, we confirmed that the cNEUROG3 lentivirus drives levels of NEUROG3 expression similar to that seen in untransduced primary cultures of human intestinal epithelium undergoing active endocrine cell production.

Cumulate (Cum)-inducible cell lines, denoted $BON_{Cum→NEUROG3}$, $BON_{Cum→NEUROG1}$, and $BON_{Cum→Control}$ were generated by transducing BON4 cells with Cum-inducible lentiviruses encoding NEUROG3, NEUROG1, or a Control sequence, respectively. Expression and protein were detectable within 1 day of adding Cum to the culture medium (Fig. S1, B and E).

We were unable to establish similar cell lines from BON4 cells successfully transduced with cNEUROG3 because the cells quickly ceased proliferating, as assessed by the MTT assay (Fig. 1A), and loss of expression of the active cell cycle marker Ki67 (Fig. 1B, Fig. S1C). Analysis of the DNA content of the transduced cells by flow cytometry confirmed that the majority were in G0-phase of the cell cycle, confirming that the cells exited the cell cycle (Fig. 1C). In contrast, control cells transduced with cNEUROG1 proliferated actively and were indistinguishable from those transduced with control lentivirus (cControl) (Fig. 1, A–C).

The nuclear localization signal was deleted from the cNEUROG3 construct (cNEUROG3:ΔNLS), to test the effect of reducing NEUROG3 translocation to the nucleus. MTT assays show that cNEUROG3:ΔNLS has a largely impaired ability to induce cell cycle exit (Fig. S1D). These results demonstrate that NEUROG3, but not NEUROG1, induces human endocrine lineage cells to exit the cell cycle, via mechanisms requiring translocation to the nucleus.

**NEUROG3 inhibits the cell cycle in most nonendocrine cell lines, but NEUROG1 does not**

We also found that transduction with cNEUROG3 induces cell cycle exit in cell lines derived from mesodermal tissues (macrophages, fibroblasts, and kidney) or endodermal tissues (stomach, colon, liver, and insulinoma), but transduction with cNEUROG1 had no effect (Fig. 1D).

**NEUROG1 was previously reported to induce cell cycle arrest in P19 cells, a mouse teratoma cell line with a well-characterized ability to differentiate into neural cells (14). We confirm that P19 cells exit the cell cycle when transduced with cNEUROG1, whereas cNEUROG3 and cControl transduced cells were unaffected. Furthermore, a Cum-inducible $P19_{Cum→NEUROG1}$ cell line was produced and responded to Cum induction by exiting the cell cycle, whereas a $P19_{Cum→NEUROG3}$ cell line did not (Fig. 1, E and F).**

**NEUROG3 induces the expression of various mature GIE cell lineage markers in BON4 cells**

Transduction of BON4 cells with cNEUROG3 induces both protein and transcript expression of common GIE cell markers including chromogranin A (CHGA), proprotein convertase subtilisin/kexin type 1 (PCSK1), gastrin (GAST), and somatostatin (SST) (Fig. 2, A and B). Thus, NEUROG3 not only induces cell cycle exit, but it also drives GIE cell differentiation.

**NEUROG3-induced cell cycle arrest is p21CIP1-dependent**

Changes in the expression of cyclin-dependent kinase inhibitors during NEUROG3-induced cell cycle exit were measured. $CDKN1A/p21^{CIP1}$ and $CDKN1C/p57^{kip2}$ expression increased dramatically by day 1, and $CDKN2A/p16^{INK4a}$ increased

---

3 R. S. Solorzano-Vargas, M. Bjerknes, J. Wang, S. V. Wu, M. G. Garcia-Careaga, D. Pisit, H. Cheng, M. S. German, S. Georgia, and M. G. Martin, unpublished data.
steadily post-transduction, whereas CDKN1B/p27kip1 expression was essentially constant following transduction of BON4 cells with cNEUROG3 (Fig. 3A).

We transduced BON4CUM→NEUROG3 cells with a doxycycline (Dox)-inducible p21CIP1 shRNA lentivirus, establishing a BON4CUM→NEUROG3 doubly inducible cell line, which

| Lentivirus  | % G1/G0 | % S  | % G2 | % Apo |
|------------|---------|------|------|-------|
| cControl   | 68.0    | 29.2 | 3.5  | 4.0   |
| cNEUROG1   | 65.8    | 29.8 | 4.2  | 4.2   |
| cNEUROG3   | 90.4    | 9.8  | 0.0  | 2.7   |
addition of Cum to the culture medium, due to the induction of NEUROG3 expression (Fig. 3B). In the absence of Cum, proliferation of BON4_Dox→shRNA(p21CIP1)_Cum→NEUROG3 cells were not impacted by depleting p21CIP1 by adding Dox to the medium (Fig. 3B). Importantly, blocking p21CIP1 expression by adding Dox to the medium 3 days before inducing NEUROG3, strongly attenuated the cell cycle arrest and expression of endocrine differentiation markers induced by NEUROG3 (Fig. 3, B and C).

The rate of NEUROG3 degradation is increased if it is phosphorylated by cyclin-dependent kinases (CDKs) (15, 16). Depletion of p21CIP1, a CDK inhibitor, could thereby lead to reduced NEUROG3 protein levels. Indeed, treatment of Cum-induced BON4_Dox→shRNA(p21CIP1)_Cum→NEUROG3 cells with Dox results in a dramatic reduction in steady-state levels of NEUROG3 (Fig. 3D). However, depletion of p21CIP1 expression in control BON4_Dox→shRNA(p21CIP1)_Cum→NEUROG1 cells had no effect on proliferation and NEUROG1 levels (Fig. 3D, data not shown).

Finally, we confirmed that NEUROG3, but not NEUROG1, directly interacts with E box elements within the immediate promoter region of p21CIP1. Direct functional interaction of NEUROG3 with the human p21CIP1 promoter was demonstrated using a luciferase expression reporter assay (Fig. S1F), and a chromatin immunoprecipitation (ChIP) assay of BON4 cells expressing FLAG-tagged NEUROG3 (Fig. S1G). These findings demonstrate that NEUROG3-induced cell cycle exit is mediated by direct interaction with the p21CIP1 promoter.

**NEUROD1 expression is not required for NEUROG3-induced quiescence**

The bHLH transcription factor NEUROD1 is a major downstream NEUROG3 target (17), so we assessed its role in NEUROG3-induced quiescence. NEUROD1 protein is readily detectable in BON4 cells, and its expression increased following transduction with cNEUROG3, but not following cNEUROG1 transduction (Fig. S2A). We transduced BON4_Dox→shRNA(NEUROD1)_Cum→NEUROG3 cells with a Dox-inducible NEUROD1 shRNA lentivirus, establishing a BON4_Dox→shRNA(NEUROD1)_Cum→NEUROG3 doubly inducible cell line. Dox induction of shRNA expression significantly decreased NEUROD1 mRNA and protein levels (Fig. S2B). Importantly, the NEUROG3-induced cell cycle exit was not rescued by blocking the NEUROG3-induced up-regulation of NEUROD1 (Fig. S2C); therefore, NEUROD1 up-regulation is not required for the NEUROG3-induced cell cycle exit.

Actively proliferating BON4 cells express NEUROD1; therefore, it is noteworthy that proliferation continues, but at a significantly slower pace, following NEUROD1 knockdown by Dox-treating BON4_Dox→shRNA(NEUROD1)_Cum→NEUROG3 cells (Fig. S2C). This

---

**Figure 2. NEUROG3 induces several endocrine markers in BON4 cells.**

A. Western blotting of cells transduced with cNEUROG3 and assessed at various days for expression levels of NEUROG3-FLAG and several endocrine targets, including CHGA, GAST, and PCSK1. Western blots were performed 3 times and the figure is representative. B. qPCR assessment of endocrine transcripts over time following transduction with cNEUROG3 and standardized by tubulin. qPCR was performed from RNA isolated from 3 different samples and run in triplicate for each sample. Statistics: B, two-way ANOVA and two-way Tukey’s multiple comparisons with adjusted p values.

---

**Figure 1. NEUROG3 induces cell cycle arrest.** A. MTT analysis of BON4 cells transduced with constitutive cNEUROG3, cNEUROG1, or cControl lentiviruses assessed daily at the indicated time points after transduction. The experiment was performed on three separate occasions and each by triplicate. B. Representative images of Ki67 and 4′,6-diamidino-2-phenylindole (Dapi) staining of cells transduced 5 days earlier with lentiviruses. Staining was performed in 6 different assays, and images are representative. C. FACS analysis was performed on day 5 of cells transduced with the various lentiviruses. Shown are a representative histogram and result of three separate experiments performed in triplicate. D. cNEUROG3, cNEUROG1, or cControl lentiviruses were transduced into the indicated cells lines, and the MTT assay assessed 5 days later. E. MTT analysis of Cum or vehicle-treated P19_Cum→NEUROG3 and P19_Cum→NEUROG1 cells assessed daily at the indicated time points. F. Edu-treated P19_Cum→NEUROG3 and P19_Cum→NEUROG1 cells following Cum treatment for 5 days and incubated with 1 μM Edu for 24 h. Three separate experiments were performed in triplicate, and the graph represents the data from the three experiments. Statistics: A, D, and E: two-way ANOVA and two-way Tukey’s multiple comparisons with adjusted p values.
observation indicates that baseline NEUROD1 expression promotes BON4 cell proliferation.

**NEUROG3-induced cell cycle exit is initially reversible but becomes irreversible with time**

BON4Cum→NEUROG3 cells undergoing various durations of Cum-induced cell cycle exit differ in the extent of their recovery 2 days after cessation of Cum treatment, by which time NEUROG3 levels are significantly reduced (Fig. S1E, Fig. 4B). The cell cycle exit induced by short-term treatment (<6 days) was reversible, whereas long-term treatment (10 days) was not (Fig. 4A). Similarly, the induction of expression of the endocrine differentiation marker CHGA declined 2 days after a short-term (<6 days), but not after long-term treatment, suggesting that short-term exposure to NEUROG3 is insufficient to "lock in" an endocrine cell fate (Fig. 4B).

**PTEN inhibition reverses NEUROG3-induced quiescence, by decreasing NEUROG3 protein**

The phosphatase PTEN has been implicated in the process of cell cycle re-entry by quiescent mouse intestinal cells (9). PTEN is ubiquitously expressed in human cells (18), prompting us to test if PTEN function is involved in NEUROG3-induced quiescence in human BON4 cells.

A NEUROG3-induced quiescent state was invoked by adding Cum to the culture medium of BON4Cum→NEUROG3. On day 3 of Cum treatment, bisperoxovanadium-pic (bpV(pic), a specific PTEN inhibitor (19); 100 nM final concentration), or an equal volume of vehicle in matched controls, was added to the culture medium. Control BON4Cum→NEUROG3 and BON4Cum→Control cells underwent similar Cum and bpV(pic) treatments. Cell proliferation and NEUROG3 protein levels were measured 1 day later.
PTEN/BMI1 and NEUROG3 cellular arrest

**A**

**BON4**<sub>Cum→NEUROG3</sub>

| days | 5 | 7 | 14 | 16 |
|------|---|---|----|----|
| + Cum | *(pedestal)* | +/− | − | +/− |
| − Cum | | | | |

**Normalized cell population size (MTT assay)**

| Cum (Days of induction) | − | + | +/− | − | +/− |
|-------------------------|---|---|----|---|----|
| 5 | **1.0** | 0.5 | 1.0 | 0.5 | ns |
| 14 | **1.0** | 0.5 | 1.0 | 0.5 | ns |

**B**

**BON4**<sub>Cum→NEUROG3</sub>

Time (days)

| 0 | 2 | 4 | 6 | 8 | 10 | 12 |
|---|---|---|---|---|----|----|
| + Cum | | | | | | |
| − Cum | | | | | | |

**NEUROG3**

| 6 | 8 | 10 | 6+2 | 8+2 | 10+2 |
|---|---|----|-----|-----|------|
| 20 kDa | 50 kDa | 50 kDa |

**C**

**BON4**<sub>Cum→NEUROG3</sub>

| Normalized Cell population size (MTT assay) | Cum | PTEN-Inh | Control |
|--------------------------------------------|-----|---------|---------|
| **1.0** | + | 3 | C |
| 0.5 | + | 1 | C |
| 1.0 | + | 3 | C |
| 0.5 | + | 1 | C |

**D**

**Control**

| CDK2 | CDK4 | AKT-pan | pAKT-pan | p21 | NEUROG3 | TUBULIN |
|------|------|---------|----------|-----|---------|---------|
| 30 kDa | 30 kDa | 60 kDa | 60 kDa | 20 kDa | 20 kDa | 50 kDa |

**PTEN-inh**

| CDK2 | CDK4 | AKT-pan | pAKT-pan | p21 | NEUROG3 | TUBULIN |
|------|------|---------|----------|-----|---------|---------|
| 30 kDa | 30 kDa | 60 kDa | 60 kDa | 20 kDa | 20 kDa | 50 kDa |

**E**

**BON4**<sub>Cum→NEUROG1</sub>, **BON4**<sub>Cum→NEUROG3</sub>

| PTEN-Inh | − | + | − | + |
|----------|---|---|---|---|
| FLAG | | | | |
| TUBULIN | | | | |

**F**

| NEUROG3 | p21<sup>CP</sup> | TUBULIN |
|---------|-----------------|---------|
| 20 kDa | 20 kDa | 50 kDa |
PTEN/BMI1 and NEUROG3 cellular arrest

Quiescent Cum-treated BON4, NEUROG3 cells had resumed proliferating after 1 day of PTEN inhibition, whereas vehicle-treated controls remained quiescent (Fig. 4C). Furthermore, PTEN inhibition activated phospho-AKT and CDK2 (Fig. 4D), whereas NEUROG3 and p21cip1 levels were significantly decreased, compared with vehicle-treated controls (Fig. 4E). In contrast, NEUROG1 levels in Cum-induced BON4, NEUROG1 cells were unaffected by PTEN inhibition (Fig. 4E). Similarly, PTEN inhibition of human enteroids attenuated NEUROG3 and p21cip1 levels (Fig. 4F).

Prolonged NEUROG3 expression leads to cellular senescence in a p21cip1-dependent manner

Prolonged exposure of BON4, NEUROG3 cells to Cum gradually induces strong p16ink4a expression (Fig. 3A). Such cells fail to re-enter the cell cycle following Cum withdrawal (Fig. 4A), indicating that they have become senescent. To confirm their senescence, we stained a time series of such cells for senescence-associated β-Gal (SA-β-Gal), a marker of cellular senescence (20). SA-β-Gal activity was undetectable in BON4, NEUROG3 cells during the first 5 days of Cum exposure (Fig. 5A) despite the detection of low and slowly increasing levels of p16ink4a expression at that time (Fig. 3A). However, SA-β-Gal activity was discernible after 6 days of Cum exposure, and by 8 days most cells were SA-β-Gal positive (Fig. 5A), confirming their senescence. Control BON4, control and BON4, NEUROG1 cells treated with Cum continued proliferating (Fig. 1A) and remained negative for SA-β-Gal (data not shown).

Robust expression of p21cip1 is necessary to institute NEUROG3-induced quiescence (Fig. 3, A and B), but it remains unclear whether prolonged NEUROG3 expression, in the absence of up-regulation of p21cip1, is sufficient on its own to eventually drive cells into quiescence and senescence. We tested this by the simultaneous exposure of BON4, shRNA(p21cip1) cells to Cum and Dox, which prevented quiescence and senescence for as long as we tested (15 days). In contrast, BON4, shRNA(p21cip1) Cum alone rapidly became quiescent and eventually senescent. This demonstrates that NEUROG3-induced quiescence and senescence requires p21cip1 (Fig. 5, B and C). It should be noted, however, that in the absence of p21cip1, the resultant Cum-induced NEUROG3 protein level is diminished (Fig. 3D), which may have weakened its impact.

For comparison, we examined the cell cycle state induced by treating BON4, shRNA(NEUROD1) cells with Dox, which represses baseline NEUROD1 expression, greatly attenuating proliferation (Fig. S2, A–C). These cells remained in a slowly cycling quiescent state for a prolonged period without becoming senescent, as indicated by their minimal growth and lack of SA-β-Gal staining even after 15 days (Fig. S2D). Thus, prolonged NEUROG3 exposure in the absence of p21cip1-induced quiescence, or prolonged quiescence in the absence of NEUROG3-induced p21cip1 up-regulation, are both insufficient to induce senescence in BON4 cells.

NEUROG3 induces BM1 expression, which attenuates the induction of p16ink4a, delaying the onset of senescence

The polycomb group protein BM1 is involved in regulating pancreatic β-cell senescence by repressing p16ink4a expression (see Introduction). This led us to suspect that BM1 may similarly be involved in limiting p16ink4a expression in the early quiescent phase of NEUROG3 expression in BON4 cells (Fig. 3A), thereby delaying the onset of senescence. Indeed, Cum treatment of BON4, NEUROG3 cells led to increased BM1 mRNA and protein levels within hours, whereas BM1 did not change in Cum-treated control BON4, NEUROG1 cells (Fig. 6A; Fig. S3A; data not shown). Direct functional interaction of NEUROG3 with the human Bmi1 promoter was demonstrated using a luciferase expression reporter assay (Fig. 6B), and a ChIP assay of BON4 cells transfected with FLAG-tagged NEUROG3 (Fig. 6C) (21).

We assessed whether Bmi1 impairs NEUROG3 binding to the E box in the p21cip1 promoter (22). We examined the loss of BM1 function with specific Bmi1 inhibitor PTC-209 (1.25 μM). The addition of Bmi1 inhibitor significantly enhanced NEUROG3 induction of the human p21cip1 promoter using a luciferase expression reporter assay (Fig. 6D), and by ChIP assay in BON4 cells (Fig. 6E).

The impact of loss of BM1 in the context of NEUROG3-induced quiescence was determined by simultaneous treatment of BON4, NEUROG3 Cells with Cum and PTC-209. The cells exhibited a dramatic increase in expression of p16ink4a, p19ink4d, and CHGA expression (Fig. 6F), accompanied by an accelerated onset of cellular senescence as assessed by SA-β-Gal (Fig. 6G). In contrast, inhibition of BM1 in cells not treated with Cum (and hence not up-regulating NEUROG3) did not undergo senescence. Taken together, these data indicate that within this in vitro system, short-term NEUROG3 induces p21cip1 and BM1 expression, the latter acting to attenuate the up-regulation of p16ink4a and p19ink4d levels, thereby delaying the onset of NEUROG3-induced cellular senescence.

Discussion

We have found that the human endocrine-derived BON4 cell line responds to the pro-endocrine transcription factor NEUROG3 by rapidly exiting the cell cycle and expressing markers of endocrine differentiation as outlined in Fig. 6H.
Interestingly, we also found that NEUROG3 drives a similar cell cycle exit in a wide variety of cell lines, but that its homolog NEUROG1 does not. P19 cells is a singular exception. We found that NEUROG3 expression has little effect on P19 cells, whereas NEUROG1 stimulates p27KIP1 expression and induces cell cycle exit (Fig. 1, E and F, data not shown), consistent with the cell line’s neurogenic response to NEUROG1 (14). The failure of NEUROG3 to act in P19 cells, despite P19 cell’s well-characterized pluripotency, warrants future investigation.

We also found that if p21CIP1 expression is repressed, then NEUROG3 fails to induce BON4 cell quiescence and endocrine differentiation (Fig. 3, B and C), as others have shown in mouse pancreas (3, 23). This dependence of NEUROG3 on p21CIP1 is due to the direct action of NEUROG3 at the p21CIP1 promoter (Fig. S1, F and G), explaining why deleting the nuclear localization signal impairs NEUROG3’s ability to induce cell cycle arrest (Fig. S1D).

Phosphorylation of NEUROG3 has recently been shown to increase its degradation (15, 16). This explains our observation that NEUROG3 protein levels were diminished after p21CIP1 repression (Fig. 3D) or PTEN inhibition (Fig. 4, E and F), both of which regulate protein phosphorylation (24–26).

In mouse pancreas, newly differentiating endocrine cells require a short burst of high levels of NEUROG3 exposure to induce differentiation, whereas adult mouse β-cells require tonic low levels of NEUROG3 expression to sustain hormone production (27). We have similarly found that transient NEUROG3 expression in BON4 cells induces cellular quiescence and commencement of endocrine differentiation, both of which are reversible if NEUROG3 is reduced (either by removing Cum-induction, or via increased NEUROG3 phosphorylation and consequent degradation following p21CIP1 or PTEN inhibition). Prolonged expression of NEUROG3 results in senescence and sustained endocrine differentiation.

In BON4 cells, NEUROG3 also drives BMI1 expression, which in turn acts to delay senescence by constraining NEUROG3-induced expression of p21CIP1 and p16INK4a (Fig. 6, A–F and H). Chemical inhibition of BMI1 function accelerates the NEUROG3-induced expression of p16INK4a, p19ARF, endocrine differentiation, and senescence (Fig. 6F). This may explain the pancreatic endocrine maturation strategy, intense short bursts induce cell cycle exit, initiates differentiation, and increases BMI1 expression, which acts to restrain premature p16 expression, facilitating cell cycle re-entry during the postnatal islet growth, and remodeling. As β-cells mature, low-grade expression of NEUROG3 reinforces the endocrine program, but the resultant low BMI1 and high p16 levels render β-cells more refractory to cell cycle re-entry.

It is important to note that whereas the BON4 cell line provides a tractable model in which to explore the consequences of a NEUROG3-induced endocrine state in human cells, its use has caveats. The parental BON cell line was generated from a metastatic human carcinoid tumor of pancreas (12), and hence displays abnormalities including continuous proliferation and high basal levels of hair/enhancer of split-1 (HES1) and NEUROD1 expression (Fig. S2A, data not shown). Hes1 is a direct target of Notch and is a potent transcriptional inhibitor...
of bHLH transcription factors, as well as CDK inhibitors (28, 29). Therefore, it is plausible that the high basal levels of HES1 in BON4 cells represses endogenous expression of NEUROG3 and the CIP/KIP genes. Robust HES1 expression in BON4 cells would be expected to inhibit NEUROD1 expression; but in fact, NEUROD1 is surprisingly abundant in these cells, and as we have shown here, is required to drive their baseline proliferation. In contrast, NEUROD1 is known to induce cell cycle arrest when expressed in various cell lines or in secretin-expressing GIE cells (30). Interestingly, we also found that BON4 cells express high levels of RFX6 mRNA, a transcription factor that functions both up- and downstream of NEUROG3 (data not shown). Although not assessed here, RFX6 may have a role in driving NEUROD1 expression in the absence of NEUROG3 (31).
Experimental procedures

Statistics

The data throughout the manuscript are presented as the mean ± S.E. Multiple t tests were performed using GraphPad Prism software, and significant p values (*, <0.01; **, <0.001; ***, <0.0001) are indicated in all the figures.

Antibodies, oligonucleotides, and vectors

The various antibodies, primers used for quantitative PCR (qPCR), vectors and viruses used are listed in Tables S1–S3. Oligonucleotides used for ChIP, and site-directed mutagenesis will be made available upon request.

Cell lines

Most established cell lines where obtained from ATCC and propagated and maintained as suggested. The BON4 cell line used in this study was generated from the BON cell line, obtained from Dr. Townsend (University of Texas Medical Branch, Galveston, TX). The parental BON that had a more heterogeneous appearance and growth characteristics were maintained in Dulbecco’s modified Eagle’s medium/F-12 media supplemented with 10% FBS (12).

Lentiviral development and transduction

The single exon NEUROG1 was PCR amplified from human genomic DNA with a C-terminal FLAG tag oligonucleotide. Sequence encoding a C-terminal FLAG tag was also added to a previously described human NEUROG3 cDNA and along with NEUROG1 the constructs were cloned into a lentivirus-based vector to generate constitutively active lentivirus (10). To generate the NEUROG1- and NEUROG3-inducible lentiviruses, both were subcloned into SparQ-IRE5-GFP lentivector. Attenuation of various transcripts was performed using lentiviral shRNA that were purchased from a commercial vendor and are displayed in Table S3. Cells’ containing an inducible system were supplemented with either Cum (30 µg/ml) or Dox (1 µg/ml) and replaced every other day.

Cell proliferation analysis by MTT and FACS

Cell proliferation was assessed by standard MTT assay (32). By FACS analysis, cells were harvested and fixed with 4% PFA. After fixation, cells were resuspended in PBS containing propidium iodine and RNase A, and analyzed for DNA content by flow cytometry using ModFitLt V3 (pMAC). Data are representative of at least 3 separate experiments performed in triplicate.

Luciferase reporter assay

Luciferase assay was performed as previously described (10). Various constructs were transfected using Lipofectamine, and lysates were used to measure luciferase that was normalized by Renilla luciferase.

Western blot analysis

Cells were lysed and 10 µg of protein was loaded onto a 10% polyacrylamide gel and subjected to SDS-PAGE before blotting onto an immune blot from Amersham Biosciences, Hybond. Membranes were blotted using standard procedures using the antibodies shown in Table S1. Western blots were visualized using the ECL plus Western blotting kit and scanned on an imager.

qPCR

qPCR with cDNA synthesized from 5 µg of total RNA was performed either with TaqMan assay, or using Perfecta SYBR® Green SuperMix, Low ROX™ in a real-time Thermocycler. The results obtained for each individual gene were normalized to β-tubulin expression in the sample. The primers used were designed with Primer3, or as suggested by TaqMan, and are shown in Table S2.

SA-β-Gal staining

SA-β-Gal staining was used to determine replicative senescence (33). Cells were washed with PBS and fixed with 4% PFA, and SA-β-Gal staining solution was incubated, and observed every 4 h for 12 h, and then every 12 h. The optimal incubation period was determined based on the visibility of stained cells in the test sample, but not in the control sample.

Chromatin immunoprecipitation (ChIP)

ChIP experiments were carried out using modifications to the micro-ChIP protocol (34). Briefly, BON4 cells were treated with 1% PFA to cross-link the DNA with bound proteins. After washing, the cells were lysed in SDS-based lysis buffer with protease inhibitors, sonicated to shear chromatin to an average size of 500 bp, and cellular debris was removed by centrifugation. Anti-FLAG-M2 antibody was bound to protein G-coated magnetic beads, and diluted chromatin lysate was incubated overnight with antibody-bound magnetic beads and washed in RIPA buffer and TE. Cross-links were reversed and the DNA was purified and quantified by qPCR using SYBR Green PCR. DNA enrichment data were estimated as the percentage bound/input ratio, presented relative to the un-induced control.

Immunofluorescent staining

BON4 cells transduced with one of several lentiviruses and were grown on glass coverslips and subsequently fixed in 4% PFA and washed with PBS before treating with blocking buffer and incubating with primary and secondary antibodies listed in Tables S1–S3.

Statistics

For all other multiple comparisons, one-, or two-way ANOVA was followed by Tukey’s or Dunnett’s multiple comparisons test. For comparisons involving one independent variable with two groups, we performed Student’s t tests. Statistical significance was set at: *, p < 0.05; **, 0.01; and ***, 0.005, with respect to final (adjusted) p values. Variation is reported as S.E.

Author contributions—R. S. S.-V. and M. G. M. conceptualization; R. S. S.-V., M. B., and M. G. M. data curation; R. S. S.-V., M. B., S. V. W., S. G., and M. G. M. formal analysis; R. S. S.-V., J. W., S. D., H. C., and M. G. M. investigation; M. B., H. C., S. G., and M. G. M. writing-original draft; M. B., H. C., S. G., and M. G. M. writing-review and editing; M. S., J. C. D., and M. G. M. funding acquisition; M. G. M. project administration.
**PTEN/BMI1 and NEUROG3 cellular arrest**

**References**

1. Gu, G., Dubauskaite, J., and Melton, D. A. (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129, 2447–2457 Medline

2. Bjerknes, M., and Cheng, H. (2006) Neurogenin 3 and the endoendocrine cell lineage in the adult mouse small intestinal epithelium. Dev. Biol. 300, 722–735 Medline

3. Miyatsuka, T., Kosaka, Y., Kim, H., and German, M. S. (2011) Neurogenin3 inhibits proliferation in endocrine progenitors by inducing Cdkn1a. Proc. Natl. Acad. Sci. U.S.A. 108, 185–190 CrossRef Medline

4. Alkema, M. J., Bronk, M., Verhoeven, E., Otte, A., van ’t Veer, L. J., Berns, A., and van Lohuizen, M. (1997) Identification of Bmi1-interacting proteins as constituents of a multimammalian polycomb complex. Genes Dev. 11, 226–240 CrossRef Medline

5. Tsenh, S. I., Georgia, S., Dhawan, S., and Bhushan, A. (2011) Skp2 is required for incretin hormone-mediated beta-cell proliferation. Mol. Endocrinol. 25, 2134–2143 CrossRef Medline

6. Tsenh, S. I., Dhawan, S., Gurlo, T., and Bhushan, A. (2009) Age-dependent decline in beta-cell proliferation restricts the capacity of beta-cell regeneration in mice. Diabetes 58, 1312–1320 CrossRef Medline

7. Dhawan, S., Tsenh, S. I., and Bhushan, A. (2009) Bmi-1 regulates the Ink4a/Arf locus to control pancreatic beta-cell proliferation. Genes Dev. 23, 906–911 CrossRef Medline

8. Yan, K. S., Gevaert, O., Zheng, G. X. Y., Anchang, B., Probert, C. S., Larkin, K. A., Davies, P. S., Zheng, C. F., Kaddis, J. S., Han, A., Roelf, K., Calderon, R. J., Cynn, E., Hu, X., Mandleymbala, K., et al. (2017) Intestinal endocrine lineageline cells possess homeostatic and injury-inducible stem cell activity. Cell Stem Cell 21, 78–90.e76 CrossRef Medline

9. Richmond, C. A., Shah, M. S., Deary, L. T., Trotier, D. C., Thomas, H., Gamper, I., Ali, F., McCracken, L., Hindley, C., McDuff, F., Nestorowa, S., et al. (2017) Links endocrine differentiation to the cell cycle in pancreatic progenitors. Proc. Natl. Acad. Sci. U.S.A. 114, 129–142.e6 CrossRef Medline

10. Wang, J., Cortina, G., Wu, S. V., Tran, R., Cho, J. H., Tsai, M. J., Bailey, T. J., Tian, R., Liao, D., He, X., and Chen, H. (2017) Mechanism of delayed islet maturation. Proc. Natl. Acad. Sci. U.S.A. 114, 722–735 CrossRef Medline

11. Wang, S., Jensen, M. T., and Smith, F. (2007) In vitro senescence of human beta cells. Diabetologia 50, 368–377 CrossRef Medline

12. Evers, B. M., Townsend, C. M., Jr., Upp, J. R., Allen, E., Hurlbut, S. C., Kim, D. L. (2000) Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. Dev. Biol. 226–240 Medline

13. Zou, D., Silvius, D., Fritzsch, B., and Xu, P. X. (2004) Eya1 and Six1 are required for the development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes 49, 163–176 CrossRef Medline

14. Chung, J. H., and Eng, C. (2005) Nuclear-cytoplasmic partitioning of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) differentially regulates the cell cycle and apoptosis. Cancer Res. 65, 8096–8100 CrossRef Medline

15. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linksens, M., Rubelj, L., and Pereira-Smith, O. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. U.S.A. 92, 9363–9367 CrossRef Medline

16. Wang, M. H., Hsu, D. S., Wang, H. W., Wang, H. J., Lan, H. Y., Yang, W. H., Huang, C. H., Kao, S. Y., Tseng, C. H., Tai, S. K., Chang, S. Y., Lee, O. K., and Wu, K. J. (2010) Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition. Nat. Cell Biol. 12, 982–992 CrossRef Medline

17. Prabhhu, S., Ignatova, A., Park, S. T., and Sun, X. H. (1997) Regulation of the expression of cyclin-dependent kinase inhibitor p21 by E2A and Id proteins. Mol. Cell. Biol. 17, 8888–8896 CrossRef Medline

18. Jensen, J., Heller, R. S., Funder-Nielsen, T., Pedersen, E. E., Lindsell, C., Weinmaster, G., Madsen, O. D., and Serup, P. (2000) Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes 49, 163–176 CrossRef Medline

19. Wang, S., Jensen, M. T., Seymour, P. A., Hsu, W., Dor, Y., Sander, M., Magnuson, M. A., Serup, P., and Gu, G. (2009) Sustained Neurog3 expression in hormone-expressing islet cells is required for endocrine maturation and function. Proc. Natl. Acad. Sci. U.S.A. 106, 9715–9720 CrossRef Medline

20. Dimri, G. P., Pedersen, E. E., Galli, P., Hald, J., Heller, R. S., Ishibashi, M., Kageyama, R., Guillenmot, F., Serup, P., and Madsen, O. D. (2000) Control of endodermal endocrine development by Hex-1. Nat. Genet. 24, 36–44 CrossRef Medline

21. Murata, K., Hattori, M., Hirai, N., Shinouzaka, Y., Hirata, H., Kageyama, R., Sakai, T., and Minato, N. (2005) Hex1 directly controls cell proliferation through the transcriptional repression of p27Kip1. Mol. Cell. Biol. 25, 4262–4271 CrossRef Medline

22. Mutoh, H., Naya, F. J., Tsai, M. J., and Leiter, A. B. (1998) The basic helix-loop-helix protein BETAlA interacts with p300 to coordinate differentiation of secretin-expressing endoendocrine cells. Genes Dev. 12, 820–830 CrossRef Medline

23. Smith, B. S., Qu, H. Q., Taleh, N., Kishimoto, N. Y., Scheel, D. W., Lu, Y., Patch, A. M., Grabs, R., Wang, J., Lynn, F. C., Miyatsuka, T., Mitchell, J., Seerke, R., Desir, J., Vanden Eijnden, S., et al. (2010) Rfx6 directs islet formation and insulin production in mice and humans. Nature 463, 775–780 CrossRef Medline

24. Mowsmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63 CrossRef Medline

25. Itahana, K., Kusunoki, S., and Itahana, Y. (2001) Colorimetric detection of senescence-associated β-galactosidase. Methods Mol. Biol. 965, 143–156 CrossRef Medline

26. Dahì, G., and Collins, P. (2008) A rapid micro chromatin immunoprecipitation assay (microChIP). Nat. Protocols 3, 1032–1045 CrossRef Medline