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Efficient Generation of NKX6-1$^+$ Pancreatic Progenitors from Multiple Human Pluripotent Stem Cell Lines

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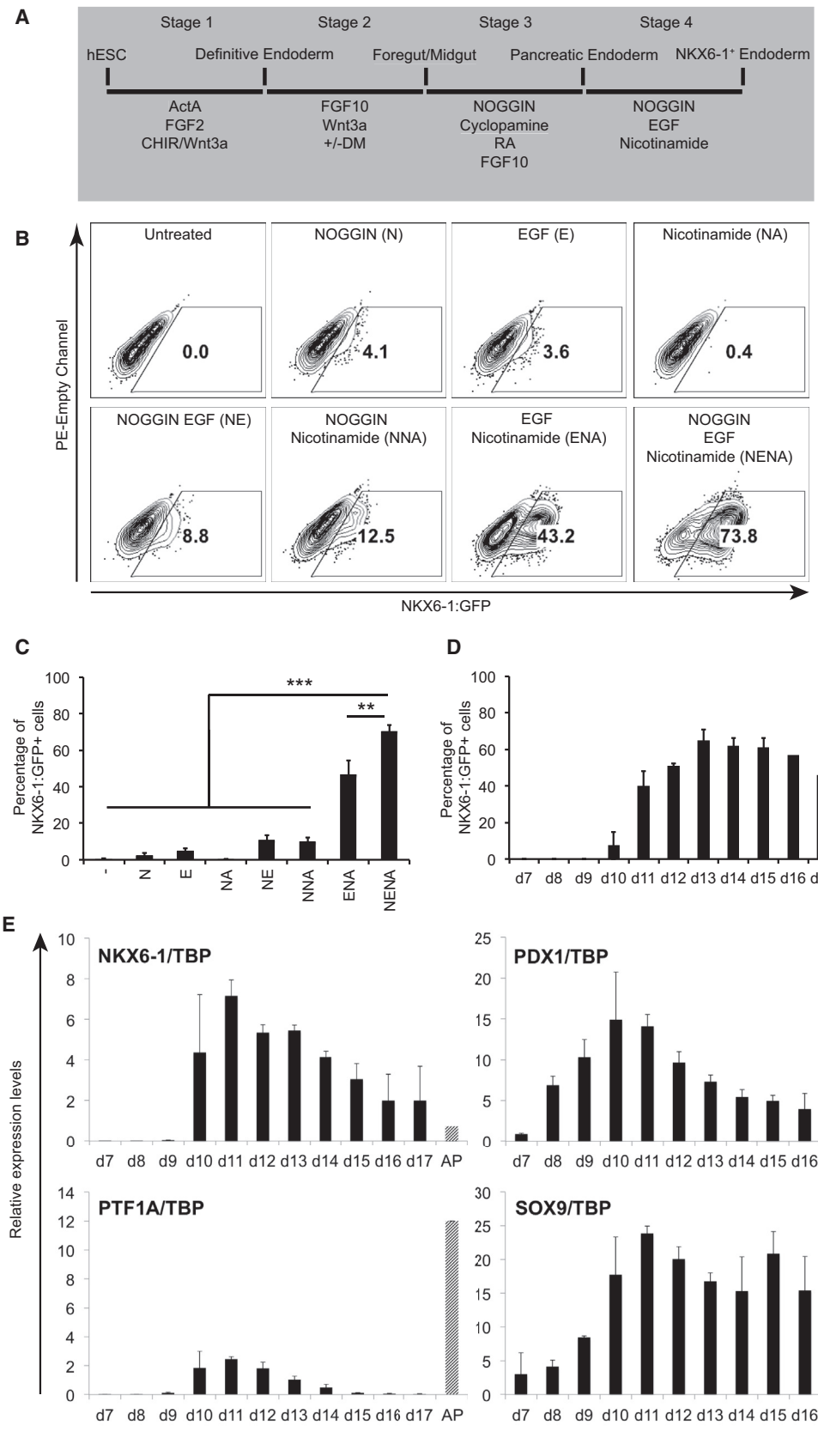
SUMMARY

Human pluripotent stem cells (hPSCs) represent a renewable source of pancreatic beta cells for both basic research and therapeutic applications. Given this outstanding potential, significant efforts have been made to identify the signaling pathways that regulate pancreatic development in hPSC differentiation cultures. In this study, we demonstrate that the combination of epidermal growth factor (EGF) and nicotinamide signaling induces the generation of NKX6-1$^+$ progenitors from all hPSC lines tested. Furthermore, we show that the size of the NKX6-1$^+$ population is regulated by the duration of treatment with retinoic acid, fibroblast growth factor 10 (FGF10), and inhibitors of bone morphogenetic protein (BMP) and hedgehog signaling pathways. When transplanted into NOD scid gamma (NSG) recipients, these progenitors differentiate to give rise to exocrine and endocrine cells, including monohormonal insulin$^+$ cells. Together, these findings provide an efficient and reproducible strategy for generating highly enriched populations of hPSC-derived beta cell progenitors for studies aimed at further characterizing their developmental potential in vivo and deciphering the pathways that regulate their maturation in vitro.

INTRODUCTION

To generate functional beta cells from human pluripotent stem cells (hPSCs), it is necessary to accurately model the key stages of pancreatic development in the differentiation cultures used. Studies in the mouse have shown that the exocrine, endocrine, and ductal lineages of the adult pancreas derive from multipotent progenitor cells (MPCs) that are specified between embryonic day 9.5 (E9.5) and E12.5 of development and are characterized by the co-expression of a combination of transcription factors, including *Pdx1*, *Ptf1a*, *Nkx6-1*, *Cpa*, *Myc*, and *Sox9* (Burlison et al., 2008; Gu et al., 2002; Haumaitre et al., 2005; Henseleit et al., 2005; Kawaguchi et al., 2002; Kopp et al., 2011; Seymour and Sander, 2007; Solar et al., 2009; Zhou et al., 2007). Development of the ductal/endocrine lineages from MPCs is associated with loss of *Ptf1a* and maintenance of *Nkx6-1* expression, whereas the downregulation of *Nkx6-1* and sustained expression of *Ptf1a* are required for specification of the exocrine lineage (Schaffer et al., 2010). Expression of *Nkx6-1* is required for development of the beta cell lineage from endocrine progenitors (Sander et al., 2000).

The generation of MPCs in the mouse fetus is preceded by the emergence of an independent population of insulin-expressing cells that is distinguished from the adult beta cell population by the fact that the cells are polyhormonal and non-glucose responsive, and do not express *Nkx6-1* (Jørgensen et al., 2007; Teitelman et al., 1993). Lineage-tracing studies have shown that these polyhormonal cells do not give rise to adult beta cells, leading to the hypothesis that they represent an embryonic/fetal lineage that is distinct from MPCs (Herrera, 2000; Herrera et al., 1994). The observation that deletion of *Nkx6-1* inhibits the development of adult beta cells, but not polyhormonal cells (Sander et al., 2000), further supports the interpretation that these two populations of endocrine cells arise from separate lineages that are established through different developmental programs. The program that gives rise to polyhormonal cells is known as the first transition, whereas the one that generates the MPCs and adult endocrine cells is referred to as the second transition. Immunohistochemical analyses of human fetal pancreata suggest that endocrine specification differs somewhat between humans and mice, as the first cells to be detected in humans are monohormonal insulin$^+$ cells (Jennings et al., 2013). Polyhormonal cells have been observed in the human pancreas between gestational week 9 (G9w) and G16w; however, they appear to arise after the formation of monohormonal cells (Jennings et al., 2013; Pan and Brissova, 2014). These findings suggest that if two distinct programs exist in humans, commitment to the monohormonal lineage (the human equivalent of the second-transition population) would occur faster in humans than in mice.



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A number of different studies over the past decade have demonstrated that it is possible to generate pancreatic cells, including both polyhormonal and monohormonal insulin-expressing cells from hPSCs (Nostro and Keller, 2012; Pagliuca et al., 2014; Rezania et al., 2014). Polyhormonal cells display characteristics of the murine first-transition endocrine population, as they are NKX6-1^{-} and non-glucose responsive. They likely represent the polyhormonal population found in the human fetal pancreas after G9w. Monohormonal insulin^{+} cells express NKX6-1, are glucose responsive, and are comparable to the second-transition-derived endocrine cells in mice and the monohormonal cells detected in humans as early as G7.5w (Jennings et al., 2013; Pan and Brissova, 2014).

As the presence of NKX6-1 is one of the distinguishing features of these two lineages, expression of this transcription factor is used to monitor the emergence of the beta cell lineage in hPSC differentiation cultures. Populations enriched in NKX6-1^{+} cells will give rise to functional beta cells following transplantation into immunodeficient mice, indicating that they do indeed represent progenitors of adult monohormonal lineages (Kelly et al., 2011; Kroon et al., 2008). Currently, the signaling pathways that regulate the generation of the NKX6-1^{+} progenitors are not well defined. hPSC-line heterogeneity appears to be one factor that can influence the generation of the beta cell progenitors, as Kelly et al. (2011) reported the development of populations consisting of 6%–45% NKX6-1^{+} cells from different cell lines with the same protocol. More recently, Rezania et al. (2012) showed that activation of PKC can enhance the generation of the NKX6-1^{+} progenitors, whereas inhibition of the transforming growth factor β (TGF-β) pathway blocks their development in H1 and ESI49 differentiation cultures. The effect of PKC activation, however, has not been tested on a broad range of hPSC lines.

In this report, we show that the combination of epidermal growth factor (EGF) and nicotinamide signaling together with inhibition of the bone morphogenetic protein (BMP) pathways promotes the efficient development of NKX6-1^{+} progenitors from all of the hPSC lines tested. Additionally, we demonstrate that the duration of retinoic acid/fibroblast growth factor 10 (RA/FGF10) induction and inhibition of BMP and hedgehog signaling influences endocrine lineage development, with a short duration favoring the specification of NKX6-1^{+} progenitors, and an extended duration promoting the development of polyhormonal cells. Following transplantation into NOD scid gamma (NSG) recipient mice, the NKX6-1^{+} progenitors induced with NOGGIN, EGF, and nicotinamide (NENA) mature and give rise to the different endocrine lineages, including beta-like cells.

RESULTS

EGF and Nicotinamide Signaling Induces the Development of NKX6-1^{+} Progenitors

Our current differentiation protocol optimized for the generation of the pancreatic lineages (Nostro et al., 2011) supports the development of low numbers of NKX6-1^{+} cells along with polyhormonal insulin-expressing cells from both H1 and INS$^{GFP/w}$ human embryonic stem cells (hESCs). However, these cell types emerge as distinct populations in cultures (Figures S1A and S1B), consistent with the interpretation that they represent separate lineages. Based on the observations that EGF signaling is required for branching morphogenesis during endocrine lineage development, and that both EGF-family ligands and receptors are widely expressed in the mouse and human fetal pancreas (Gittes, 2009; Miettinen et al., 1995; Miettinen and Heikinheimo, 1992; Miettinen et al., 2000), we speculated that this pathway could be instrumental in the generation of hESC-derived NKX6-1-expressing pancreatic progenitors. To test this hypothesis, we generated hESC-derived pancreatic endoderm using a modified version of our previously described protocol (Nostro et al., 2011) and then cultured the cells for an additional 10 days with different combinations of EGF, NOGGIN, and nicotinamide to specify the NKX6-1 progenitor fate (Figure 1A). We then analyzed the populations for the presence of

Figure 1. NOGGIN, EGF, and Nicotinamide Induce NKX6-1 Expression

(A) Schematic of the protocol used to differentiate hESCs toward pancreatic endoderm. At the final stage of differentiation, cells were treated with NOGGIN, EGF, and nicotinamide, singly or in combinations.

(B) Flow-cytometric analysis for GFP was carried out at d13 of differentiation.

(C) Average percentage of NKX6-1:GFP^{+} cells as measured by flow-cytometry analysis at d13 of differentiation in the different treatment groups (−, untreated; N, NOGGIN; E, EGF; NA, nicotinamide; NE, NOGGIN+EGF; NNA, NOGGIN+nicotinamide; NENA, NOGGIN+EGF+nicotinamide). Error bars represent SD of the mean (n = 3 independent experiments).

(D) Kinetic analysis of NKX6-1:GFP expression as measured by flow cytometry between d7 and d17 of differentiation. Error bars represent SD from the mean of three experiments. **p < 0.05, ***p < 0.001 using Student's t test for statistical analysis.

(E) qPCR analysis for *NKX6-1*, *PDX1*, *PTF1A*, and *SOX9* expression during pancreatic differentiation of the NKX6-1$^{GFP/w}$ hESC line (d7–d17). NKX6-1$^{GFP/w}$ cells were differentiated as described in (A). Expression levels are normalized to the housekeeping gene *TBP* and compared with adult pancreas (AP, dashed bar). Error bars represent SD of the mean (n = 3 independent experiments).

See also Figures S1 and S2.

NKX6-1$^+$ cells. NOGGIN was included because several studies have demonstrated that BMP signaling is dispensable for normal pancreatic development (Bardeesy et al., 2006; Wandzioch and Zaret, 2009), and nicotinamide was tested because it has been shown to promote endocrine differentiation from human fetal explants (Otonkoski et al., 1993). To be able to monitor NKX6-1 expression quantitatively, we used an hESC reporter line in which the *GFP* cDNA had been targeted to the *NKX6-1* locus (A.H., A.G.E., and E.G.S., unpublished data).

NOGGIN, EGF, or nicotinamide alone did not promote the development of a significant number of NKX6-1:GFP$^+$ cells (Figures 1B and 1C). In contrast, the combinations of EGF and nicotinamide or EGF, nicotinamide, and NOGGIN induced the generation of sizable populations of NKX6-1:GFP$^+$ cells, representing more than 40% and 70% of the total culture, respectively (Figures 1B and 1C). Kinetic analysis of the population induced with NENA showed that the first NKX6-1:GFP$^+$ cells were detected by day 10 (d10) of differentiation and the size of the population increased rapidly to peak at d13 (Figure 1D). To validate the fidelity of the NKX6-1$^{GFP/w}$ hESC reporter line, we performed intracellular flow-cytometric analyses on NKX6-1:GFP-derived d13 cells using an anti-NKX6-1 antibody. As shown in Figures S1C–S1E, the antibody stained the majority of the GFP$^+$ population, indicating that GFP expression is indicative of NKX6.1 expression. To further characterize the NENA-induced population, we analyzed it for the expression of markers indicative of MPCs, including *PDX1*, *PTF1a*, *SOX9*, and *NKX6-1*. The kinetics of *NKX6-1* mRNA expression correlated well with that of NKX6-1:GFP, further confirming that GFP reflects endogenous NKX6-1 activity (Figures 1D and 1E). *PTF1a*, *SOX9*, and *PDX1* (Figure 1E) were all expressed during the d7–d17 time frame in temporal patterns that overlapped with that of *NKX6-1*. Together, these findings strongly suggest that the combination of EGF and nicotinamide signaling together with inhibition of the BMP pathway promotes the efficient development of progenitors that display expression patterns indicative of MPCs.

To investigate whether this approach for generating NKX6-1$^+$ cells is broadly applicable to other hPSCs, we tested combinations of EGF, NOGGIN, and nicotinamide on H1 and H9 hESC lines, and analyzed the development of NKX6-1$^+$ cells by intracellular flow cytometry. Similar to our observations with the NKX6-1$^{GFP/w}$ line, the addition of NOGGIN, EGF, or nicotinamide alone had little effect, whereas the combination of all three pathway regulators induced populations consisting of up to 60% NKX6-1$^+$ cells (Figure 2A). Titration experiments revealed that the proportion of NKX6-1$^+$ cells increased with increasing concentrations of EGF, up to a maximum of 80% following induction with 100 or 300 ng/ml of the factor (Figure S2A). Additionally, they established optimal concentrations of NOGGIN between 10 and 100 ng/ml and of nicotinamide at 10 mM (Figures S2B and S2C).

The NENA-induced, H1-derived population also expressed the spectrum of transcription factors indicative of MPC development, including *PDX1*, *PTF1a*, and *SOX9* (Figure 2B). The temporal pattern of expression of these transcription factors was similar to that observed with the NKX6-1$^{GFP/w}$-derived population and overlapped with that of *NKX6-1* (Figures 1E and 2B). Some of the hESC-derived pancreatic cells appeared to be undergoing commitment to the endocrine lineages, as expression of the endocrine-specific transcription factors *NEUROD1* and *NGN3* was also upregulated during this time frame (Figure 2B). To further characterize the NENA-induced populations, we analyzed them for the presence of the NKX6-1, PDX1, and SOX9 proteins by immunofluorescence. The differentiated culture contained cells that co-expressed NKX6-1 and PDX1, and NKX6-1 and SOX9 (Figure 2C), consistent with the interpretation that they represent MPCs or endocrine/ductal progenitors.

Given that Rezania et al. (2012) showed that activation of PKC enhanced the generation of NKX6-1$^+$ progenitors, we were interested in determining whether direct manipulation of the PKC pathway would impact the development of this population in the presence of NENA. Interestingly, we did not observe any increase in the percentage of NKX6-1$^+$ cells at d13 of differentiation following addition of the PKC activator TPB (Figure S2D). In contrast, inhibition of PKC with the specific inhibitor Gö6976 (Chen et al., 2009) led to a modest but significant reduction in the percentage of NKX6-1$^+$ cells, suggesting that PKC signaling plays some role in the development of NKX6-1$^+$ progenitors, and that the pathway was already active in our NENA-induced population (Figure S2D). Induction of stage 4 H1-derived cells with TPB and NOGGIN in the absence of EGF and nicotinamide promoted the development of a small NKX6-1$^+$ population (~10%). The size of this population was not influenced by the addition of SB431542 (SB) or nicotinamide (Figure S2E). An alternative PKC activator, PdBu (Rezania et al., 2012), was more effective than TPB, as addition of it together with NOGGIN in the absence of EGF and nicotinamide resulted in the generation of a population consisting of ~30% NKX6-1$^+$ cells (Figure S2F). Higher concentrations of PdBU did not lead to an increase in the size of the NKX6-1$^+$ population (Figure S2F).

The Duration of Stage 3 Impacts the Development of the NKX6.1 Progenitors and the Polyhormonal Population

Because stage 3 is essential for establishing the pancreatic fate in differentiation cultures, we were interested in

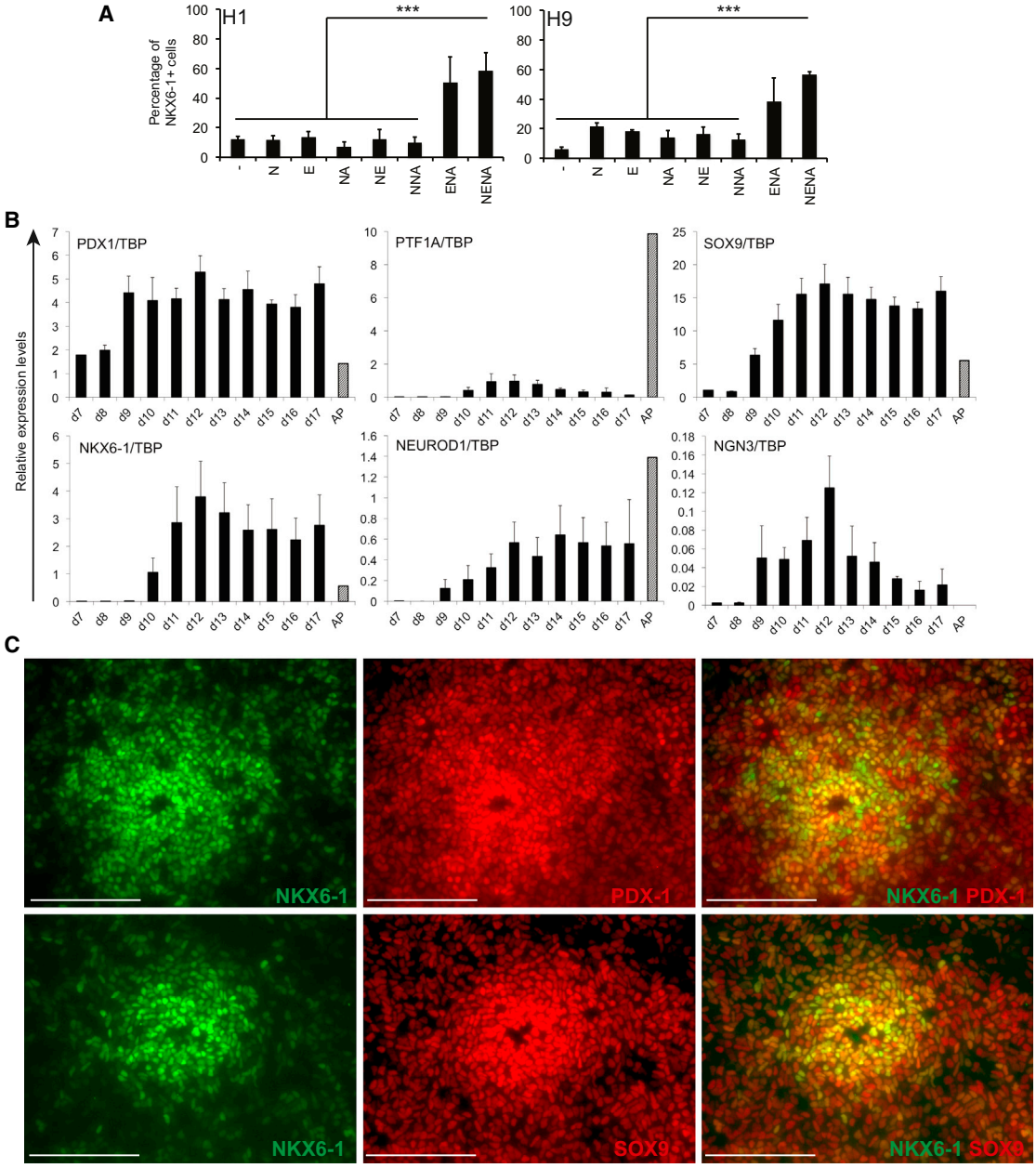

Figure 2. NOGGIN, EGF and Nicotinamide Induce NKX6-1 Expression in Non-Targeted hESC Lines

(A) Flow-cytometric analysis of intracellular NKX6-1. H1 cells and H9 hESCs were differentiated according to a published protocol (Nostro et al., 2011) up to stage 3 and then treated with NOGGIN, EGF and nicotinamide, alone or in combination, for 6 days. Flow-cytometric analysis was performed at d13 of differentiation. Triple treatment was sufficient to induce the highest percentage of NKX6-1$^+$ cells. Error bars represent SD of the mean (n = 3 independent experiments). ***p < 0.001 using Student's t test.

(B) qPCR analysis for *NKX6-1*, *PDX1*, *PTF1A*, *SOX9*, *NEUROD1*, and *NGN3* expression between d7 and d17 of pancreatic differentiation. Expression levels are normalized to the housekeeping gene *TBP* and compared with adult pancreas (AP, dashed bar). Error bars represent SD of the mean (n = 5 independent experiments).

(C) Immunofluorescence image of d13 culture, showing NKX6-1 in green and PDX1 and SOX9 in red. The solid bar is 200 μm.

See also Figures S1 and S2.

determining whether the duration of this induction step could influence the type of progenitors that develop. Additionally, we investigated the role of TGF-β signaling during the NENA step because Rezania et al. (2012) provided evidence that it is required for the generation of the NKX6.1$^+$ progenitors, whereas we previously showed

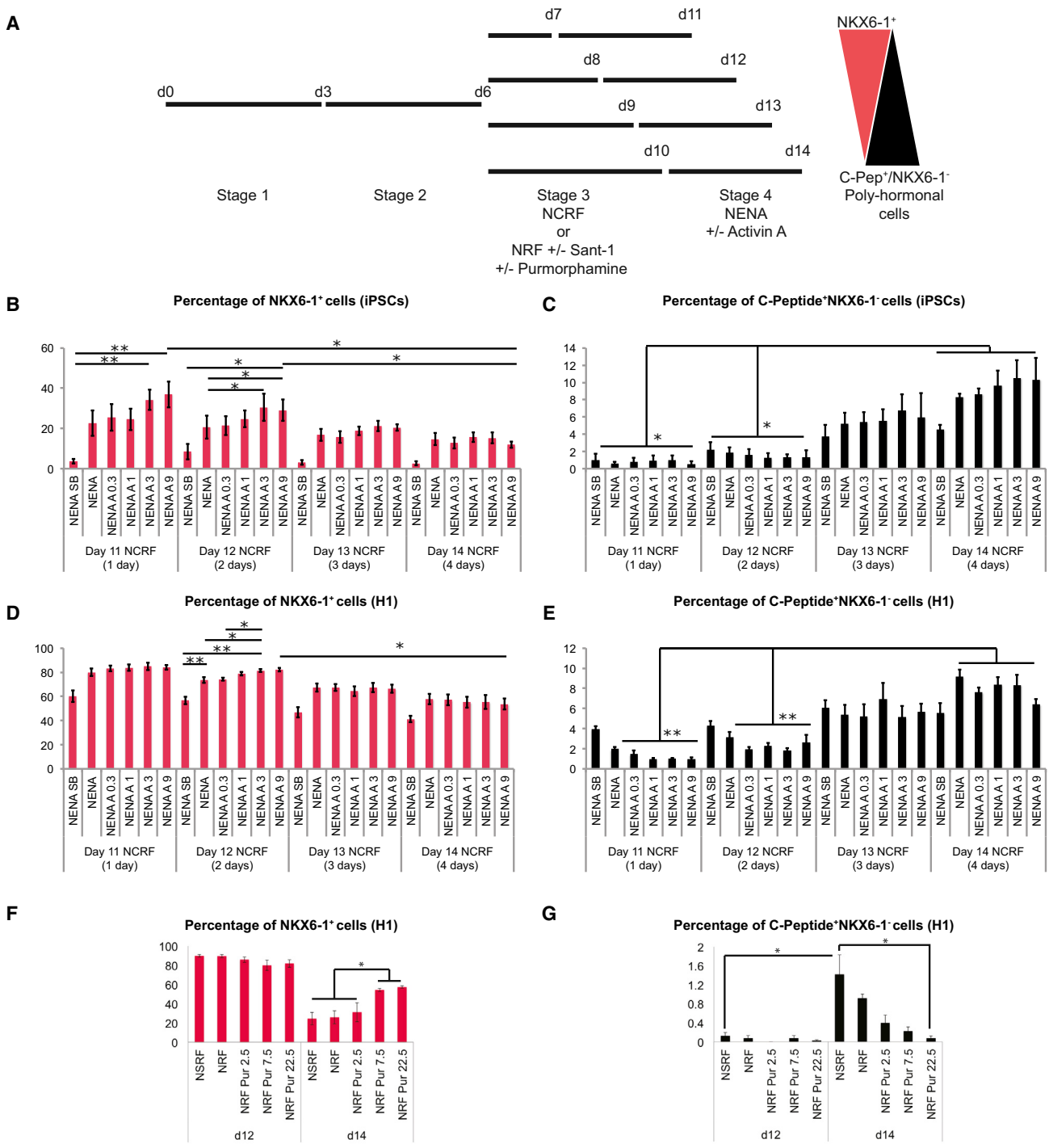

Figure 3. Short RA/Cyclopamine/FGF10 Exposure and Activin Treatment Favor NKX6-1 Development

(A) Schematic of the protocol used to differentiate hPSCs toward first- and/or second-transition progenitors. Stage 3 of differentiation consisted of NOGGIN, cyclopamine, RA, and FGF10 (NCRF). Sant-1 was used as a substitute for cyclopamine and purmorphamine (2.5, 7.5, and 22.5 μM) was used to activate hedgehog signaling in the absence of Sant-1 and cyclopamine.

(B–E) NCRF treatment lasted for 1–4 days. Stage 4 of differentiation was supplemented with SB431542 (SB) or activin A at 0.3, 1, 3, and 9 ng/ml (A 0.3, A 1, A 3, and A 9). Average percentage of NKX6-1:GFP$^+$ cells in red (B and D) and C-peptide$^+$ in black (C and E) as measured by flow-cytometry analysis at d11, d12, d13, and d14 of differentiation. Error bars represent SD of the mean of the percentage of C-peptide$^+$

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that it is not necessary for specification of the polyhormonal population (Nostro et al., 2011). For these analyses, we used H1 hESCs and the iPSC line 38-2. Day 6 cells were induced for 1, 2, 3, or 4 days with a combination of NOGGIN, cyclopamine, RA, and FGF10 (NCRF; stage 3 media) and then cultured for 4 days in NENA in the presence of either the TGF-β inhibitor SB or different concentrations of activin A (0–9 ng/ml; Figure 3A). The percentage of NKX6-1$^+$ and C-peptide$^+$ cells was evaluated at d11, d12, d13 and d14 of differentiation, respectively (Figures 3B–3E and S3). With this staining strategy, C-peptide$^+$NKX6-1$^-$ cells are considered as polyhormonal cells equivalent to the murine first-transition population, whereas C-peptide$^-$NKX6-1$^+$ cells represent putative MPC progenitors (equivalent to the murine second-transition progenitors). Double-positive C-peptide$^+$NKX6-1$^+$ cells were not detected under any of the tested conditions (Figure S3A). Several interesting patterns emerged from these experiments. First, the duration of the stage 3 induction step dramatically influenced the development of the polyhormonal population, as significantly more C-peptide$^+$NKX6-1$^-$ cells were detected in both H1- and 38-2-derived populations induced for 4 days than in those induced for 1 or 2 days (Figures 3C, 3E, and S3A). In contrast, 1 or 2 days of induction was optimal for development of the NKX6-1$^+$ population from both hPSC lines, as extended stage 3 induction resulted in a decrease in the proportion of NKX6-1$^+$ cells that developed in vitro (Figures 3B, 3D, and S3A). Second, activin treatment modestly increased the proportion of NKX6-1$^+$ cells generated from H1 and 38-2 cells. Given these findings, it is possible that the size of the NKX6-1$^+$ progenitor population could be increased with higher concentrations of activin A in these hPSC lines (Figures 3B and 3D). Third, addition of the TGF-β inhibitor led to a small but significant reduction in the proportion of NKX6-1$^+$ cells that developed in the H1-derived population, whereas it completely inhibited the emergence of NKX6-1$^+$ cells in the 38-2-derived population (Figures 3B, 3D, and S3A). TGF-β signaling did not have any impact on the differentiation of another hESC line, MEL1, which gave rise to ~60% NKX6-1$^+$ cells when differentiated for 2 days in stage 3 media (Figure S3B). A reduction in the duration of stage 3 from 2 days to 1 day led to a consistent increase in the percentage of NKX6-1$^+$ cells generated from two additional hiPSC lines, MSC-iPSC1 and BJ-iPSC1 (Figure S3C).

Studies in mouse and chick embryos have shown that inhibition of hedgehog signaling creates a permissive environment for pancreatic specification (Apelqvist et al., 1997; Hebrok et al., 1998, 2000). To test whether the hedgehog signaling pathway plays a role in the development of human NKX6-1$^+$ cells, we treated differentiating cultures with NOG, FGF10, and RA alone or in combination with either Sant-1 or cyclopamine (data not shown) to block the hedgehog signaling pathway, or with increasing concentrations of purmorphamine, a hedgehog pathway agonist (Sinha and Chen, 2006). We treated H1-derived cells with these molecules for 2 or 4 days and then with NENA for an additional 4 days, and then evaluated the NKX6-1 and C-peptide profiles by flow cytometry at d12 and d14, respectively. Interestingly, the percentage of NKX6-1$^+$ cells that were generated at d12 of differentiation from the progenitors treated for 2 days with the stage 3 factors was not affected by activation or inhibition of the hedgehog pathway (Figure 3F). In contrast, the size of the d14 NKX6-1$^+$ population that developed from the progenitors treated for 4 days with the stage 3 factors was impacted by manipulation of the hedgehog pathway. Specifically, activation of the pathway with purmorphamine (at 7.5 and 22.5 μM) led to an increase in the percentage of NKX6-1$^+$ cells (Figure 3F). The reverse pattern was observed for the C-peptide$^+$NKX6-1$^-$ population. The highest frequency of C-peptide$^+$NKX6-1$^-$ cells was generated from progenitors treated for 4 days with the hedgehog inhibitor together with the stage 3 factors. Addition of purmorphamine during the 4-day period significantly reduced the proportion of these cells (Figure 3G).

Taken together, these findings clearly indicate that the duration of the stage 3 induction step influences the ratio of polyhormonal cells and NKX6.1$^+$ progenitors that develop within the culture, and demonstrate that a short induction period, independent of hedgehog signaling, promotes the development of populations that consist predominantly of NKX6-1$^+$ expressing cells. They also show that prolonged culture with NOG, FGF10, and RA together with inhibition of hedgehog signaling is required for the formation of polyhormonal cells. Finally, they suggest that the requirement for TGF-β signaling for NKX6-1 induction is cell-line specific.

and NKX6-1$^+$ cells (n = 5 independent experiments, except for C-peptide 38-2: n = 3 independent experiments). **p < 0.01 using Student's t test.

(F and G) The NRF treatment lasted 2 or 4 days and hedgehog signaling was activated during this stage by purmorphamine (2.5, 7.5, and 22.5 μM) or inhibited by treatment with Sant-1 (2.5 mM). Average percentage of NKX6-1:GFP$^+$ cells in red (F) and C-peptide$^+$ in black (G) as measured by flow-cytometry analysis at d12 and d14 of differentiation.

Error bars represent SD of the mean of the percentage of C-peptide$^+$ and NKX6-1$^+$ cells (n = 4 independent experiments); *p < 0.05 using one-way ANOVA and Student's t test for comparisons between d12-d14 NSRF treatments. See also Figure S3.

Table 1. Efficiency of Differentiation

Cell Line	Stage 4 Percentage of NKX6-1$^+$ Cells ± Average Deviation	Stage 1 Percentage of CXCR4$^+$/CD117$^+$	Duration of Stage 3	Stage 4 (EGF) ng/ml	Stage 4 (Act A) ng/ml
H1	83.1 ± 4.2	>93%	2 days	100	–
H9	56.4 ± 2	>93%	3 days	50	–
NKX6-1$^{GFP/w}$	71.0 ± 3.2	>93%	2 days	100	–
MEL-1	67.5 ± 11.5	>93%	2 days	50	3
INS$^{GFP/w}$	82.6 ± 8.8	>93%	2 days	100	–
hiPSCs 38-2	36.9 ± 6.4	>93%	1 day	50	9
MSC-iPSC1	83.8 ± 2.1	>93%	1 day	100	–
BJ-iPSC1	74.6 ± 10.5	>93%	1 day	100	–

Replicates: n = 3 independent experiments for H9, NKX6-1$^{GFP/w}$, MEL1, MSC-iPSC1, BJ-iPSC1; n = 4 independent experiments for H1, INS$^{GFP/w}$; n = 5 independent experiments for hiPSC 38-2.

The efficiencies of differentiation, including endoderm induction and NKX6-1 progenitor specification across the different hPSC lines, are summarized in Table 1.

NENA-Induced Progenitors Generate Islet-like Structures In Vivo

To determine whether the NENA-induced population contains endocrine progenitors, we transplanted cells from d13 H1-derived cultures into the mammary fat pad of immunocompromised NSG mice as previously described (Basford et al., 2012). The grafts were harvested at 1.5, 3, 5, and 6 months post-transplantation and analyzed for the expression of NKX6-1, cytokeratin-19 (CK19), insulin (INS), C-peptide, glucagon (GCG), somatostatin (SST), and pancreatic polypeptide (PP). At the 1.5-month time point, we detected high numbers of NKX6-1$^+$ cells and cells that expressed CK19 (Figure S4A). Low numbers of INS$^+$, C-peptide$^+$, and GCG$^+$ cells were also detected at this time point. Almost all of these INS$^+$ cells were NKX6-1$^+$ and none co-expressed C-peptide and GCG, indicating that they represent monohormonal endocrine cells (Figure S4A). Interestingly, the INS$^+$ cells were also CK19$^-$ but were located in close proximity to CK19$^+$ cells (Figure S4A). Between 3 and 6 months post-transplantation, we observed islet-like structures within the grafts (Figure 4 and data not shown) that contained NKX6-1$^+$, INS$^+$, GCG$^+$, SST$^+$, and PP$^+$ monohormonal cells. Trypsin$^+$ and CK19$^+$ cells were also detected at this time point, suggesting that acinar and ductal lineage cells were present in the graft. Interestingly, at this time point, fewer endocrine cells were found within the CK19$^+$ structures.

To determine whether the site of transplantation impacted differentiation, we transplanted a comparable H1-derived progenitor population under the kidney capsules of NSG mice. We observed a similar pattern of development within the grafts, as they contained monohormonal endocrine cells and TRYP$^+$ cells (Figure S4B). Glucose challenge tests were performed at 6, 12, and 18 weeks post-transplantation. Significantly higher levels of human C-peptide were detected after glucose challenge in the sera of the transplanted mice at 18 weeks in four out of five experiments (Figure S5). The average percentage of NKX6-1$^+$ cells transplanted per experiment was 64% ± 2.4% (range: 60.5%–70%). Each transplantation was successful, and we did not observe any correlation between the level of C-peptide in the sera and the percentage of transplanted NKX6-1$^+$ cells (data not shown). These findings are in agreement with previous reports (Kroon et al., 2008; Rezania et al., 2011). Overall, these data indicate that NENA treatment generates a population that contains progenitors that can give rise to beta-like cells in vivo.

DISCUSSION

The efficient generation of functional cell types from hPSCs in vitro is dependent on precise manipulation of the appropriate signaling pathways at key developmental stages within the lineage of interest. Efforts to identify the pathways that regulate pancreatic development in vitro are complicated by the existence of two developmental programs that give rise to distinct endocrine cell types. Our findings in this study uncover important differences in the regulation of the two programs and show that NKX6-1$^+$ progenitors are specified within 24 hr from foregut patterned endoderm with the combination of NOGGIN, RA, and FGF10. Hedgehog signaling does not

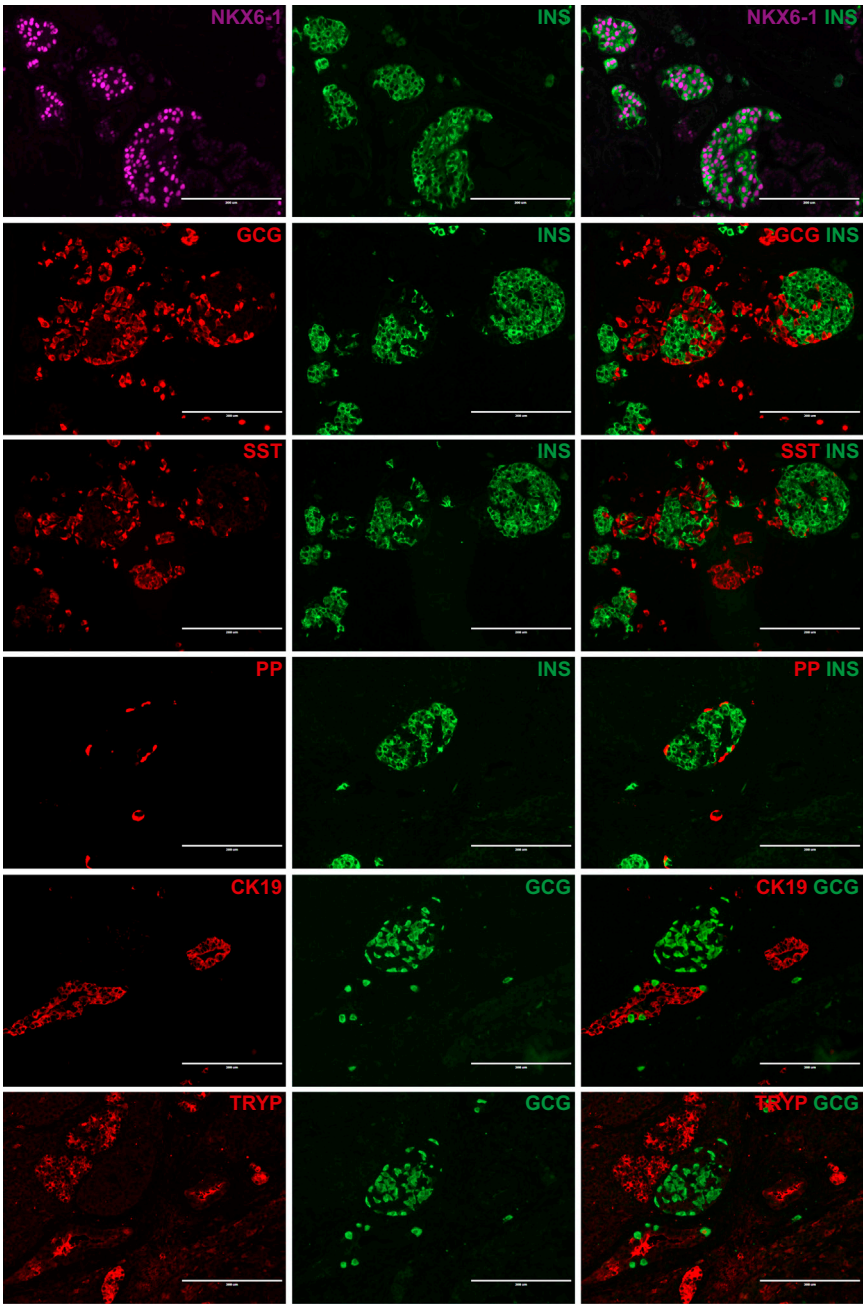

Figure 4. NKX6-1 Cells Generated Using NOGGIN, EGF, and Nicotinamide Have the Potential to Generate Islet-like Structures

Immunohistochemical analysis of NKX6-1, INS, GCG, SST, PP, CK19, and TRYP expression in the graft recovered from the mammary fat pad at 6 months post-transplantation of H1 d13 differentiated cells. Scale bar, 200 μm. See also Figures S4 and S5.

appear to play a role in this specification step. The generation of NKX6-1$^+$ progenitors from this pancreatic endoderm is dependent on EGF and nicotinamide signaling (Figure 5). In contrast, specification of the polyhormonal program requires 72–96 hr of treatment with stage 3 factors and is dependent on inhibition of the hedgehog pathway. Expansion of the derivative lineages is not dependent on the addition of exogenous EGF and nicotinamide (Figure 5). By exploiting these differences, it is now possible to design differentiation strategies that selectively promote the generation of populations of NKX6-1$^+$ progenitors, with minimal contamination of the polyhormonal lineage.

Immunohistochemical analyses of fetal pancreata revealed that the very first hormone$^+$ cells to arise in the human are insulin$^+$ monohormonal cells (G7.5w). Their development is followed by the emergence of other monohormonal cells and a small population of polyhormonal cells (G8w–G9w). These observations suggest that human pancreatic development lacks the two waves of endocrine commitment found in the mouse (Jennings et al., 2013).

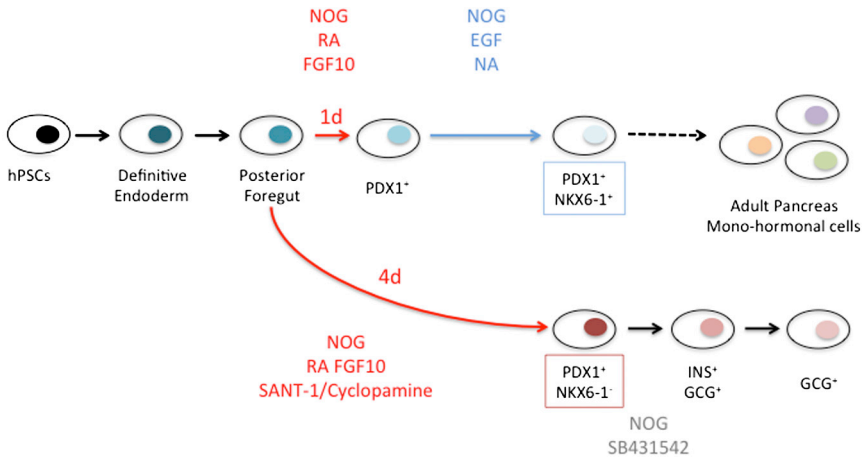

Figure 5. Model of Pancreatic Specification from hPSCs

hPSCs can be efficiently differentiated into definitive endoderm, which can be patterned to a posterior-foregut-like population that has the potential to upregulate PDX1 expression upon treatment with RA and FGF10, and inhibition of SHH (using Sant-1 or cyclopamine) and BMP (using NOGGIN). PDX1-expressing cells have the potential to generate two distinct pancreatic lineages: (1) a multi-potent pancreatic progenitor expressing NKX6-1 with the potential to give rise to adult-like monohormonal cells, and (2) a polyhormonal population that lacks expression of NKX6-1 and the ability to generate functional beta cells, but generates glucagon$^+$ cells (Basford et al., 2012). Our findings in this study uncover important differences in the regulation of the two programs and show that the monohormonal cell progenitor is specified within 24 hr by treatment with a combination of stage 3 agonists and the antagonists RA, FGF10, and NOGGIN, independently of hedgehog signaling, and that the generation of the NKX6-1$^+$ progenitor population from this specified pancreatic endoderm is dependent on EGF and nicotinamide signaling. In contrast, specification of the polyhormonal program requires 72–96 hr of treatment with RA, FGF10, and NOGGIN. Crucially, hedgehog inhibition and expansion of the derivative lineages are not dependent on the addition of exogenous EGF and nicotinamide.

It has been speculated that the absence of an early first-transition endocrine population in humans is due to the lack of direct contact of the developing pancreatic bud with the notochord (Jennings et al., 2013). In the mouse, expression of *Pdx1* is induced by E8.5, when the endoderm is in contact with the notochord, whereas in humans it is first expressed when the gut tube has been separated from the notochord by the dorsal aorta. Given that the notochord is a known source of hedgehog inhibitors, these observations suggest that the duration of hedgehog inhibition may play a role in the development of the polyhormonal population in vivo. Our findings are consistent with this hypothesis and clearly demonstrate that the extended induction period with stage 3 media containing a hedgehog inhibitor promotes the development of polyhormonal cells.

Our observation that EGF signaling is required for the generation of the equivalent of second-transition progenitors in hPSC differentiation cultures is in line with findings from targeting studies in the mouse that showed that *EgfR/ErbB1*$^{-/-}$ and *ErbB3*$^{-/-}$ deficient animals display impaired branching and islet morphogenesis, and delayed beta cell differentiation (Erickson et al., 1997; Miettinen et al., 2000). Although our findings suggest an absolute dependency on EGF signaling, *EgfR*-deficient animals develop beta cells, possibly due to functional redundancy of the other EgfRs expressed in the pancreatic epithelium and mesenchyme. Two previous studies have reported on the sequential addition of EGF and nicotinamide to hPSC-derived pancreatic differentiation cultures (Jiang et al., 2007; Zhang et al., 2009). Although increased levels of *NKX6-1* expression were observed over a period of time in these populations, it is unclear whether these changes were due to the addition of EGF and nicotinamide, as no comparisons were made with populations generated without these factors. As we found that the sequential addition of these factors is not effective for inducing NKX6-1$^+$ progenitors (data not shown), it is quite possible that the observed increase in NKX6-1 expression in these studies was not due to signaling through these pathways. NKX6-1$^+$ progenitors can develop in hPSC differentiation cultures without the addition of EGF and nicotinamide (Kelly et al., 2011; Kroon et al., 2008). However, under these conditions, the efficient development of these progenitors is hPSC-line dependent. The combination of exogenous EGF and nicotinamide signaling largely eliminates this cell line bias and promotes the generation of NKX6-1$^+$ progenitors from all of the hPSC lines that we tested, providing a differentiation platform that can be widely used.

The binding of EGF ligands to the corresponding receptors induces homo- or heterodimerization with the structurally related family of ErbB homologs, resulting in activation of the MAPK, Src, PI3-kinase, and PKC signaling pathways (Citri and Yarden, 2006). PKC may be one intermediate that is responsible for inducing NKX6-1 progenitors following activation of the EGF/EgfR pathway, as Rezania et al. (2012, 2013) demonstrated that TPB and PdBU (both PKC activators) can induce these progenitors. Additionally, we found that addition of the PKC inhibitor

Gö6976 (Chen et al., 2009) in the presence of EGF and nicotinamide resulted in a modest reduction in the percentage of NKX6-1$^+$ cells. Further studies will be required to determine whether other pathways are also involved in the EGF/EgfR-induced effect, given that the addition of the PKC inhibitor resulted in only a 10% reduction in progenitor frequency. Under our conditions, the combination of TPB or PdBU and Noggin led to the induction of only a small NKX6-1$^+$ population in the absence of EGF and nicotinamide. We found that PdBU was more effective than TPB for generating NKX6-1-expressing cells. The reason for the discrepancy between our findings and those of Rezania et al. (2012) is currently not known, but it could stem from differences in the methods used to grow and differentiate the hESCs. In recent studies, both Rezania et al. (2014) and Pagliuca et al. (2014) initiated TPB or PdBU treatment at stage 3 rather than stage 4, as described in the original protocol (Rezania et al., 2012). It is possible that earlier treatment with these molecules induces the NKX6-1$^+$ progenitor population more efficiently.

Conclusions

In conclusion, we have identified key signaling pathways that regulate the development of polyhormonal cells and NKX6-1$^+$ progenitors from hPSCs. With these new insights, we are now able to establish differentiation protocols that selectively induce either one of these populations, enabling studies focused on elucidating the pathways that regulate their proliferation and maturation in vitro. The ability to efficiently generate NKX6-1$^+$ monohormonal progenitors from many different hPSC lines will provide a platform for modeling pancreatic development and pathobiology in vitro, as well as for developing scalable technologies for future cell-based therapy for the treatment of diabetes.

EXPERIMENTAL PROCEDURES

hPSC Culture and Differentiation

H1 and H9 hESC lines were obtained from WiCell; Mel-1, INS$^{GFP/w}$, and NKX6-1$^{GFP/w}$ were provided by Drs. E. Stanley and A. Elefanty (Micallef et al., 2012); hiPSC 38-2 and MSC-iPSC1 were provided by Drs. H. Park and G. Daley (Nostro et al., 2011); and BJ-iPSC1 was provided by Drs. T. Araki and B. Neel (Witty et al., 2014). Undifferentiated cells were maintained as previously described (Kennedy et al., 2007). Differentiation was started when hESCs and hiPSCs reached 80% confluence. Briefly, after removal of hESC media, cultures were treated with RPMI media containing 100 ng/ml of hActivin A and 25 ng/ml mWnt3a for 1 day (d0–d1). mWnt3a can be substituted by 2 μM CHIR 99021 (Tocris). Cells were treated with RPMI containing 100 ng/ml of hActivin A and 5 ng/ml hbFGF for 2 days (d1–d3). At day 3 of differentiation, cultures were treated with stage 2 media consisting of serum-free differentiation (SFD) media (Gouon-Evans et al., 2006) containing 50 ng/ml of hFGF10, in combination with 3 ng/ml mWnt3a and 0.75 μM dorsomorphin (Sigma). Dorsomorphin was not required for MEL-1 differentiation. NOGGIN (50 ng/ml) could be used to replace dorsomorphin during stage 2 of differentiation. At d6 of differentiation, the medium was changed to DMEM with 1% vol/vol B27 supplement (without vitamin A) (Life Technologies), ascorbic acid (50 μg/ml; Sigma), KAAD-cyclopamine (0.25 μM; Toronto Research Chemical), all-*trans* RA (2 μM; Sigma), hNOGGIN (50 ng/ml), and hFGF10 (50 ng/ml) for 1–4 days, with the medium changed every day. SANT-1 (0.25 μM; Tocris) could be used to replace KAAD-cyclopamine. At d7–d10, the medium was changed to DMEM with 1% vol/vol B27 supplement (Life Technologies), ascorbic acid (50 μg/ml; Sigma), hNOGGIN (50 ng/ml), hEGF (50–100 ng/ml), nicotinamide (10 mM; Sigma) with or without SB (6 μM; Sigma), and hActivin A at the indicated concentrations. TPB was used at 50 and 500 nM (Calbiochem), and PdBU was used at 10, 30, and 100 nM (Tocris). All cytokines were purchased from R&D Systems unless otherwise stated. Cultures were maintained in a 5% CO_2, 5% O_2, 90% N_2 environment. At the indicated time points, cells were harvested and analyzed by real-time qPCR, immunocytochemistry, and flow cytometry, or harvested for transplantation.

Flow Cytometry

Monolayer cultures generated from hPSC differentiation experiments were dissociated by incubation with trypsin (0.25% trypsin-EDTA) and the cells were stained for the presence of the appropriate markers. NKX6-1$^{GFP/w}$ was analyzed for GFP expression by flow cytometry without fixation. Day 7–17 cells were stained for NKX6-1 (1:2,000; F55A10-c, developed by Dr. Ole Madson and distributed by the Developmental Studies Hybridoma Bank [DSHB]), C-peptide (1:300; GN-ID4-c, developed by Dr. Ole Madson and distributed by the DSHB), and anti-mouse immunoglobulin G (IgG)-phycoerythrin, IgG-Alexa 488, and anti-rat IgG-APC (Jackson ImmunoResearch). Mouse IgG (Jackson ImmunoResearch) and rat IgG (Santa-Cruz Biotechnologies) were used for isotype controls. Intracellular protein staining was carried out on cells fixed with 4% paraformaldehyde (Electron Microscopy Sciences) in PBS. Staining was performed in PBS with 4% fetal calf serum and 0.5% saponin (Sigma). Stained cells were analyzed on an LSRII flow cytometer (BD). Data were analyzed using FlowJo software (Treestar).

Immunostaining

Immunostaining of monolayer cultures was performed as previously described (Nostro et al., 2011). The following primary antibodies were used: goat anti-PDX1 (1:10,000; a gift from Dr. Chris Wright), NKX6-1 (1:2,000; F55A10-c; DSHB), and SOX9 (1:400; Millipore). The stained cells were visualized using a digital inverted fluorescence microscope (AMG EVOS).

Animal Experiments

All animal experiments were approved by the University Health Network Animal Research Committee. Approximately 5–10 million H1-derived d13 cells were mechanically dislodged and resuspended in 20–40 μl Matrigel (BD) and then transplanted into the mammary fat pad of NOD.*Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) mice as previously described (Stewart et al., 2011). Grafts were harvested at 1.5, 3, 5, and 6 months post-transplantation. For kidney

capsule transplantation, approximately 5–10 million H1-derived d13 cells from five different experiments were transplanted into the kidney capsules. Five mice were transplanted per experiment, for a total of 25 mice. The cultured cells were mechanically dislodged and resuspended in 1.5 ml of NENA in 1.5 ml microcentrifuge tubes and shipped overnight at room temperature to the University of Massachusetts Medical School for subrenal transplantation. All animal use was in accordance with the guidelines of the Animal Care and Use Committee of the University of Massachusetts Medical School, and conformed to the recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996). Briefly, the mice were anesthetized and prepared for surgery. The skin and muscle layer over the spleen were incised and the kidney was gently externalized with forceps. H1-derived pancreatic cells (suspended in CMRL plus 1% FBS) were injected into the subrenal capsular space using a Surflo winged infusion set (23 g × 3/4 inch; Terumo Medical). The kidney was then replaced in the abdominal cavity, the muscle was sutured, and the skin was closed with an Autoclip wound closure system (Fisher Scientific).

Glucose-Induced C-Peptide Secretion

Mice were fasted overnight and blood was collected following intraperitoneal injection of glucose (2.0 g/kg body weight). Heparinized blood was collected with protease inhibitor (aprotinin; Sigma) at 6, 12, and 18 weeks post-transplantation. Plasma was stored at −80°C until it was analyzed for C-peptide by human-specific ELISAs (ALPCO Diagnostics).

Immunohistochemistry

hESC-derived grafts were harvested and fixed in 10% buffered formalin, embedded in paraffin, and cut into 3 μm sections by the Pathology Research Program Laboratory at Toronto General Hospital. For immunohistochemistry, sections were dewaxed in xylene and transferred to absolute alcohol. The sections were then rehydrated through a graded series of alcohols prior to microwave-based antigen retrieval. The sections were incubated in preblock solution (10%, serum in PBS). Normal donkey, rabbit, and goat serums (Jackson ImmunoResearch) were used. Incubation with primary antibodies (diluted in PBS, 0.3% Triton X, 0.3% BSA) was performed overnight at 4°C in a humidified chamber. The following primary antibodies were used: C-peptide (DSHB, 1:1,000), insulin (Dako, 1:1,000), somatostatin (Beta Cell Biology Consortium, 1:500), pancreatic polypeptide (Peninsula Laboratories, 1:500), glucagon (Sigma, 1:500), cytokeratin 19 (Abcam, 1:800), nkx6-1 (DSHB, 1:2,000), and trypsin (R&D, 1:300). Sections were washed with PBS-Tween and then incubated with fluorescence-conjugated secondary antibody for 45 min at room temperature. The following secondary antibodies were used: anti-rat IgG-Cy3 (Jackson ImmunoResearch), anti-mouse IgG-DyLight 594 (Thermo Scientific), anti-guinea pig IgG-Alexa 488 (Jackson ImmunoResearch), anti-mouse IgG-DyLight 488 (Thermo Scientific), anti-mouse IgG-Alexa 488 (Jackson ImmunoResearch), anti-sheep IgG-Alexa 647 (Life Technologies), and anti-mouse IgG-Alexa 647 (Life Technologies). Slides were counterstained with DAPI (Biotium) prior to mounting with fluorescence mounting medium (DAKO).

Real-Time qPCR

Total RNA was prepared with the RNAqueous-Micro Kit (Ambion) and treated with RNase-free DNase (Ambion). Then, 500 ng to 1 μg of RNA was reverse transcribed into cDNA using random hexamers and Oligo (dT) with Superscript III Reverse Transcriptase (Invitrogen). Real-time qPCR was performed on a MasterCycler EP RealPlex (Eppendorf) using the QuantiFast SYBR Green PCR Kit (QIAGEN). The oligonucleotide sequences are available on request. A 10-fold dilution series of sonicated mouse or human genomic DNA standards ranging from 50 ng/ml to 5 pg/ml was used to evaluate the efficiency of the PCR and calculate the copy number of each gene relative to the housekeeping gene *TBP*.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.02.017.

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