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Thyroid Progenitors Are Robustly Derived from Embryonic Stem Cells through Transient, Developmental Stage-Specific Overexpression of Nkx2-1

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SUMMARY

The clinical importance of anterior foregut endoderm (AFE) derivatives, such as thyrocytes, has led to intense research efforts for their derivation through directed differentiation of pluripotent stem cells (PSCs). Here, we identify transient overexpression of the transcription factor (TF) Nkx2-1 as a powerful inductive signal for the robust derivation of thyrocyte-like cells from mouse PSC-derived AFE. This effect is highly developmental stage specific and dependent on FOXA2 expression levels and precise modulation of BMP and FGF signaling. The majority of the resulting cells express thyroid TFs (Nkx2-1, Pax8, Foxe1, Hhex) and thyroid hormone synthesis-related genes (Tg, Tpo, Nis, Iyd) at levels similar to adult mouse thyroid and give rise to functional follicle-like epithelial structures in Matrigel culture. Our findings demonstrate that Nkx2-1 overexpression converts AFE to thyroid epithelium in a developmental time-sensitive manner and suggest a general methodology for manipulation of cell-fate decisions of developmental intermediates.

INTRODUCTION

Since tissue progenitors, giving rise to all mature cell types within a given tissue, are essential intermediates in embryonic development, their in vitro derivation has important implications for the fields of pluripotent stem cell (PSC) biology and regenerative medicine. Significant advances in anterior foregut endoderm (AFE) progenitor biology in recent years (Ikonomou and Kotton, 2015) have led to derivation of AFE lung and thyroid progenitors (TPs) and their clinically relevant progeny from PSCs (Green et al., 2011; Huang et al., 2014; Kurmann et al., 2015; Longmire et al., 2012; Mou et al., 2012). However, with one notable exception (Huang et al., 2014), efficiencies of progenitor derivation have been relatively low (<40%).

Overexpression of transcription factors (TFs) is a well-established approach to manipulate cellular identities as it results in reconfiguration or emergence of core TF networks, with derivation of induced PSCs from somatic cells being the most prominent example (Takahashi and Yamanaka, 2006). Inducible TF expression in PSCs has been used to potentiate the derivation of specific lineages and facilitate mechanistic understanding of cell specification (Bondue et al., 2008; Mazzoni et al., 2013; Petros et al., 2013; Seguin et al., 2008). For example, Costagliola and coworkers (Antonica et al., 2012) used forced overexpression of the thyroid TFs Nkx2-1/Pax8 in mouse embryonic stem cells (mESCs) to produce thyrocyte-like cells with high efficiency (~60%) that formed in vitro follicular structures and rescued athyroid mice upon transplantation. However, studies that systematically investigate the mechanistic interplay between pulsed heterologous TF expression and developmental stages in directed differentiation of PSCs are lacking. To address this question, we used thyroid-directed differentiation (Kurmann et al., 2015) in combination with transient Nkx2-1 overexpression as our model system.

Here, we report that transient expression of Nkx2-1 functions as an inductive signal during thyroid-directed differentiation to convert AFE-stage cells to thyrocyte-like cells that self-organize to epithelial, follicle-like structures in 3D Matrigel culture. This thyroid conversion effect pertains only to a narrow developmental window of competence contingent on several parameters, including dual bone morphogenetic protein (BMP)/fibroblast growth factor (FGF) signaling and correct anterior patterning of definitive endoderm (DE). We employ emerging computational methods (linear algebra projections; Lang et al., 2014; Pu-suluri et al., 2015) and genome-wide gene expression analysis by RNA-sequencing (RNA-seq) to demonstrate that the resulting cells are similar to mouse embryonic thyrocytes. Finally, we suggest using mathematical modeling that the induction effect is potentially governed by a time-dependent bistable switch.
Figure 1. Stage-Specific Effect of Nkx2-1 Overexpression on Derivation of NKX2-1+ Progenitors

(A) Directed differentiation protocol for NKX2-1+ TPs.
(B) Integration schematic of the Nkx2-1 transgene into the HPRT locus.
(C) Schematic of the knockin reporters (BrachyuryGFP and Foxa2hCD4) and rtTA engineered into the iNkx2-1 line.
(D) Intracellular flow cytometry for NKX2-1 in undifferentiated cells with and without 24 hr of Dox treatment.
(E) Immunostaining of undifferentiated iNkx2-1 cells post-24-hr Dox; nuclear counterstain with propidium iodide (PI). Scale bars represent 100 μm.
(F) Intracellular NKX2-1 flow cytometry plots from D14 following 24-hr pulses of Dox added at indicated intermediate stages. Representative of three differentiations.
(G) Kinetics of endogenous Nkx2-1 expression by RT-qPCR following 24-hr staged pulses of Dox. Fold changes relative to undifferentiated cells, error bars represent SD (n = 3 wells from same differentiation). Representative of three independent experiments.
(H) Representative flow cytometry plot of NKX2-1 expression directly post-24-hr Dox.

(legend continued on next page)
RESULTS

Efficient Temporal Control of Dox-Inducible Nkx2-1 Transgene Expression

We hypothesized that transient, temporally regulated Nkx2-1 overexpression during directed differentiation of mESCs would lead to distinct differentiation outcomes due to differential competence of in vitro developmental stages. We created an mESC line (iNkx2-1) with a doxycycline (Dox)-inducible Nkx2-1 transgene (Figures 1B, 1C, S1A, and S1B) (Ting et al., 2005). This line was previously targeted with knockin reporters for Foxa2 (Foxa2^{GFP}) and T (Bry^{GFP}) (Gadue et al., 2006). The iNkx2-1 line displayed rapid on/off kinetics in response to Dox (Figures 1D, 1E, and S1C–S1E). Neither the transgene addition nor Dox affected pluripotency or standard directed differentiation (Figures S1F and S1G). This system allowed for efficient manipulation of Nkx2-1 expression, resulting in robust induction of the Nkx2-1 transgene (>95% Nkx2-1+ cells by flow cytometry) following 24-hr exposure to Dox (1 μg/ml).

Stage-Specific Effect of Transient Nkx2-1 Overexpression at the AFE Stage Results in Efficient Lineage Conversion

To test the temporal effects of Nkx2-1 overexpression on thyroid differentiation, we employed our iNkx2-1 cell line in combination with our thyroid differentiation protocol (Kurmann et al., 2015) (Figure 1A), which recapitulates key stages of thyroid development. In short, DE is induced by Activin A (Kubo et al., 2004), followed by further patterning to AFE (anteriorization) (Green et al., 2011; Longmire et al., 2012) using brief dual BMP/transforming growth factor β (TGF-β) inhibition through Noggin/ SB431542 (NS) treatment, and specification to NKX2-1+ cells (Longmire et al., 2012) and adult mouse thyroid tissue (Figures 2A and 2B). In addition, we observed extensive coexpression of Nkx2-1 and Pax8 (Figures 2C, upper panel, 2D, and S2E) with the increasing presence of TG to D22/30 (Figures 2B–2D and S2E).

AFE-Stage Nkx2-1 Overexpression Leads to Efficient Thyroid Derivation

Next we asked: does stage-specific, transient Nkx2-1 overexpression potentiate the thyroid lineage or the derivation of non-thyroid Nkx2-1+ lineages? Early (Pax8, Hhex, Foxe1) and more mature (Tg, Tpo, Nis, Tshr, Iyd, Dio1) thyroid markers showed distinct activation kinetics along the differentiation course (Figure S1H) and were upregulated at D14 and D22 in the Dox D6–D7 induced cultures at levels comparable with in vivo purified Nkx2-1^{GFP} E13.5 thyrocytes (Longmire et al., 2012) and adult mouse thyroid tissue (Figures 2A and 2B). In addition, we observed extensive coexpression of Nkx2-1 and Pax8 (Figures 2C, upper panel, 2D, and S2E) with the increasing presence of TG to D22/30 (Figures 2B–2D and S2E).

To test the functionality of our thyrocyte-like cells, we cultured cells in 3D Matrigel to induce organoid formation (Kurmann et al., 2015; Martin et al., 1993), which promoted expansion and maturation of the thyroid-like cells (Figure S2G) and organization into follicle-like structures (Figure S2F). These structures expressed Nkx2-1, Pax8, E-cadherin, TG secreted into the follicle lumen, and importantly basolateral NIS, indicating maturation (Figure 2C). Following culture to D50 in maturation media (Figures 2E and S2H), organoids expressed thyroid produced T4 (Figures 2F and 2G), indicating full functional capability.

Although Nkx2-1 is also critical for the development of the lung and forebrain (Kimura et al., 1996; Xu et al., 2008), we found minimal evidence of derived forebrain

(1) Averaged flow cytometry data (n = 12 independent experiments) comparing untreated (no Dox) and treated (Dox D6–D7) samples. Error bars represent SEM. ****p < 0.0001.

(2) Representative immunostaining of Dox-induced and uninduced cultures at D14. Scale bars represent 100 μm. See also Figure S1.
3D Maturation

PAX8 Merge
NKX2-1
TG

Day 14, Dox D6-D7, 2-Dimensional Culture

Day 30, Dox D6-D7, 3-Dimensional Culture

Day 22, Dox D6-D7, 2-Dimensional Culture

Early Thyroid Markers

Late Thyroid Markers

Key:
- No Dox
- Dox D6-D7
- E13.5 Nkx2-1+ Thyroid
- Adult Thyroid

C

Day 14, Dox D6-D7, 2-Dimensional Culture

Day 22, Dox D6-D7, 2-Dimensional Culture

Day 30, Dox D6-D7, 3-Dimensional Culture

A

B

D14 D22 Thy
D14 D22 Thy
D14 D22 Thy
D14 D22 Thy
D14 D22 Thy
D14 D22 Thy

D14: Dox D6-D7
D14: No Dox

G

T4 Production

no NaI + NaI mThyroid

D14 D22 Thy

(legend on next page)
Figure 2. Nkx2-1 Overexpression at AFE Stage Results in Efficient Thyroid Differentiation

(A) Experimental schematic of the extended culture conditions in (B), (C) (top three panels), and (D).

(B) RT-qPCR for thyroid marker expression. Fold changes calculated relative to undifferentiated cells, error bars represent SEM (n = 5 independent experiments, n = 1 control).

(C) Immunostaining of induced cultures at D14, D22 (gelatin substratum), and D30 (Matrigel embedded). Scale bars represent 100 μm.

(D) Intracellular flow cytometry for populations at D22 (bottom).

(E) Schematic of the maturation culture conditions for (F) and (G).

(F) T4 ELISA from D50 (Dox D6–D7) cells ± NaI from D40 to D50 (n = 3 wells from the same differentiation). Mouse thyroid tissue for reference (n = 2 tissue samples).

(G) D50 immunostaining for +NaI cultures corresponding with (F). Scale bar represents 100 μm.

* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. See also Figure S2.
D14 cells projected strongly on purified E13.5 mouse Nkx2-1GFP+ thyrocytes, while D1 cells projected on mESCs, as expected (Figure 4C). Overall, these data suggest that transient NXK2-1 overexpression in the course of directed mESC thyroid differentiation does not fundamentally alter the identity of the derived TPs.

From two clusters of interest (“early” and “late”) (Figures 4A and S4C), we identified potential transcriptional regulators and cell surface markers (CSMs) of thyroid fate (Tables S1 and S2). These lists contain genes with potentially novel roles in thyroid development, as well as genes previously implicated in foregut and early thyroid development both in vitro and in vivo (Fagman et al., 2011; Longmire et al., 2012; Millien et al., 2008). Of the early cluster CSMs, Ephb2 and SIRPA were identified to be highly expressed in DE (Figures 4D and 4E). Interestingly, Ephb2 expression

### Table 1: NKX2-1 Expression in D14 Cells

| Condition | Dox | NKX2-1+ |
|-----------|-----|---------|
| D5-D6 NS  | No Dox | 0 |
| D6-D7 Dox | **** |

### Figure 3: Efficient Thyroid Specification Is Dependent on Precise Modulation of BMP/FGF Signaling and Derivation of the Thyroid-Competent FOXA2Neg AFE Population

(A) Intracellular D14 flow cytometry for NKX2-1 following D5–D6 NS or Activin treatment (n = 3 independent experiments).

(B) Intracellular D14 flow cytometry plots from varying duration of the anteriorization stage followed by 24 hr of Dox treatment.

(C) Flow cytometry sort schematic of D6 FOXA2+ AFE.

(D) Intracellular D22 flow cytometry from the sorted and induced populations shown in (C). Representative of three independent experiments.

(E) D22 immunostaining from FOXA2High and FOXA2Neg sorted populations. Top, scale bar represents 100 μm; bottom, scale bars represent 1,000 μm (composite image).

(F) D22 RT-qPCR data from the sorted populations shown in (C), with and without Dox D6–D7. Fold changes relative to undifferentiated cells, statistics from paired t tests, (n = 5 independent experiments).

(G) D14 RT-qPCR from induced and uninduced cells with specification factor variations. Fold changes relative to undifferentiated cells (n = 3 wells from same differentiation). Results are representative of three independent experiments.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Figure S3.
in in vivo foregut endoderm was reported recently (Dravis and Henkemeyer, 2011). From the late cluster, reflective of a differentiated phenotype (Figure S4C), out of several CSMs correlating with the RNA-seq data, Col25a1 and Gabre were found to be enriched in both our induced and purified in vitro Nkx2-1mCherry+ TPs (Figures S4D and S4E).

Figure 4. RNA-Sequencing Data Reveal Thyroid Signature
(A) RNA-seq heatmap of the top 9,088 differentially expressed genes with highest variance (false discovery rate <0.05) among samples, clustered by samples (columns) and genes (rows). Late and early clusters are indicated.
(B) PCA plot of RNA-seq populations.
(C) Projection graph representing the degree of similarity (x axis, exact match = 1) between RNA-seq samples (dots) and reference gene expression datasets (y axis).
(D) RT-qPCR validation data of cell surface markers identified in the early cluster.
(E) Flow cytometry data corresponding with (D).
(F) Bistable model: a protein that cooperatively binds its own promoter leads to a positive feedback-based, bistable switch.
(G) Percentage of high-protein-expressing cells in Monte-Carlo simulations of the bistable switch shown in (F) for three pulse lengths of protein expression. Results were calculated using 1,000 simulations in each condition.
(H) Stochastic trajectories of protein number as a function of time in the absence of pulsing (main figure) and in the presence of a long pulse (inset). Colored lines show stochastic trajectories that successfully activate the Nkx2-1 feedback loop (purple lines) or fail to activate the feedback loop (red lines). The dashed line indicates the time the basal transcription rate was turned off in all simulations.
See also Figure S4.
The Stage-Specific Effect of NKX2-1 Overexpression May Be Governed by a Bistable Switch

The central observation underlying our work is that activation of an Nkx2-1 transgene at different stages during thyroid-directed differentiation results in a wide percentage range of endogenous NKX2-1+ TPs on D14 (Figure 1F). As NKX2-1 is known to bind its own promoter (Boggaram, 2009) and almost all cells are transgene NKX2-1+ post-Dox induction (Figure 1H), the behavior of the system implies the existence of an AFE-stage-specific positive feedback loop at the Nkx2-1 locus. This is further supported by the immediate activation of the endogenous Nkx2-1 expression at the Dox D6–D7 condition (Figure 1G).

Based on these data, we modeled the D6–D7 cell response to the Dox pulse as a bistable switch (Ferrell, 2012), where the two states are “on” and “off” expression of endogenous Nkx2-1 (figures 4F–4H). Overall, this model could qualitatively reproduce our basic experimental observations, suggesting that a time-dependent bistable switch may underlie the effect of transgene overexpression on cell-fate decisions during directed differentiation of PSCs.

DISCUSSION

PSC-based systems hold great promise for the mass production of transplantable, clinically relevant cell types and for in vitro modeling of complex disease states. Currently, a major roadblock to achieving these goals is the poor or variable differentiation efficiency of many differentiation protocols. This study used developmental-stage-specific overexpression of a single TF, NKX2-1, to (1) investigate how cell competence changes in a dynamic, developmentally relevant system and (2) improve the efficiency of TP specification.

Our results demonstrate that the inductive effect of NKX2-1 is restricted to a singular stage of competence contingent on several synergistic parameters (FOXA2 levels, duration of anteriorization, and BMP4/FGF2 signaling), implying that AFE-staged cells possess a unique epigenetic status allowing for robust conversion to thyroid. Respiratory lineages were not derived as indicated by insignificant numbers of SPC+ cells and the only presumed lung marker with high levels of expression (Sftpb) is expressed in both adult mouse (figure S2A) and human thyroid (http://gtxportal.org/home/gene/SFTPB). We presume that the absence of Wnt agonists, an important signal for lung specification (Goss et al., 2009; Harris-Johnson et al., 2009; Huang et al., 2014), is the major reason for the paucity of respiratory lineages following NKX2-1 overexpression in our system.

Our study also achieved the practical goal of highly efficient thyroid differentiation. Our data indicate that the majority of the derived progenitors acquired a thyroid identity comparable with their in vivo counterparts, and importantly, their progeny gave rise to follicular-like structures in a 3D environment, expressed genes of thyroid hormone biosynthesis at levels comparable with adult thyroid, and produced high levels of T4 hormone. Overall, it appears that brief NKX2-1 exogenous expression during a well-defined window of maximum thyroid competence is sufficient to dramatically increase the yield and robustness of PSC thyroid specification and differentiation. Although a similar end-stage result has been previously reported through direct reprogramming of mESCs (Antonica et al., 2012), our approach delves into the mechanistic aspects of thyroid fate decisions and competence at a developmentally relevant stage. Further work is needed to define whether the thyrocyte-like cells produced downstream in our system are functionally equivalent to the reprogrammed cells and to the progeny of purified bona fide TPs (Kummann et al., 2015).

This system can also be useful as a discovery tool in the absence of lineage-specific reporters. Analysis of the RNA-seq has identified both CSMs as well as potential regulators of thyroid fate in AFE and early TPs. Some of the TFs identified (Irx5, Hoxb8, Isl1) have been involved in mouse foregut and thyroid development (Millien et al., 2008; Westerlund et al., 2008), while others, such as PROX1, have been implicated in human thyroid disease (Ishii et al., 2016). Future PSC-based and in vivo studies will unravel the thyroid-related function of select TFs during thyroid specification and development.

Our theoretical model proposes a bistable positive feedback loop as the underlying mechanism to describe the endogenous Nkx2-1 locus activation at the AFE stage leading to subsequent stabilization of the core TF network, establishing thyroid identity. Future work will focus on experimental validation of the model and investigate the possibility that bistable switches controlling bifurcation dynamics in cell-fate decisions (Loh et al., 2014) can lead to the development of highly efficient and robust protocols of general applicability.

EXPERIMENTAL PROCEDURES

All mouse work was approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine.

Generation and Characterization of Inducible Line

The Ainv15 mESC line (Kyba et al., 2002) was a kind gift from Dr. Paul Gadue. As previously described (Ting et al., 2005), this line was engineered with a constitutively expressed rtTA from the Rosa26 locus and a promoter with tetO sites upstream of a loxP site at the HPRT locus. The Nkx2-1 transgene was inserted via co-electroporation of the plox-Nkx2-1 and pSalk-Cre plasmids.
Colonies with restored neomycin resistance were screened for efficient Dox-mediated Nkx2-1 induction.

**mESC-Directed Differentiation**

Directed differentiation was performed in serum-free media as previously described (Kurmann et al., 2015; Longmire et al., 2012) (see Supplemental Experimental Procedures for detailed description).

**Statistical Analysis**

Error bars in graphs represent SD or SEM as indicated in the figure legends. Biological sample replicates (N) are also indicated in the legends. Statistically significant differences between conditions (ΔCt values used for RT-qPCR calculations) were determined using two-tailed unpaired Student t tests or as specified in figure legends. Significance is represented as \(^*p < 0.05\), \(^{**}p < 0.01\), \(^{***}p < 0.001\), \(^{****}p < 0.0001\).

See Supplemental Experimental Procedures for additional methods.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.12.024.

**AUTHOR CONTRIBUTIONS**

L.I. and D.N.K. conceived the work and D.N.K. provided critical intellectual input. L.I. and K.D. designed experiments and wrote the manuscript. S.C. and R.S. performed experiments. A.L. performed computational analysis. J.C. performed the T4 ELISA with supervision. S.M. supervised the RNA-seq and additional RNA-seq computational analysis and generated projection scores. I.Z. performed and L.K. supervised the RNA-seq. L.I. and D.N.K. conceived the work and D.N.K. provided critical intellectual input. L.I. and K.D. designed experiments and wrote the manuscript. All authors reviewed, edited, and approved the manuscript.

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