Misexpression of Ptf1a in Cortical Pyramidal Cells *In Vivo* Promotes an Inhibitory Peptidergic Identity

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The intracellular transcriptional milieu wields considerable influence over the induction of neuronal identity. The transcription factor Ptf1a has been proposed to act as an identity “switch” between developmentally related precursors in the spinal cord (Glasgow et al., 2005; Huang et al., 2008), retina (Fujitani et al., 2006; Dullin et al., 2007; Nakhai et al., 2007; Lelièvre et al., 2011), and cerebellum (Hoshino et al., 2005; Pascual et al., 2007; Yamada et al., 2014), where it promotes an inhibitory over an excitatory neuronal identity. In this study, we investigate the potency of Ptf1a to cell autonomously confer a specific neuronal identity outside of its endogenous environment, using mouse *in utero* electroporation and a conditional genetic strategy to misexpress Ptf1a exclusively in developing cortical pyramidal cells. Transcriptome profiling of Ptf1a-misexpressing cells using RNA-seq reveals that Ptf1a significantly alters pyramidal cell gene expression, upregulating numerous Ptf1a-dependent inhibitory interneuron markers and ultimately generating a gene expression profile that resembles the transcriptomes of both Ptf1a-expressing spinal interneurons and endogenous cortical interneurons. Using RNA-seq and *in situ* hybridization analyses, we also show that Ptf1a induces expression of the peptidergic neurotransmitter nociceptin, while minimally affecting the expression of genes linked to other neurotransmitter systems. Moreover, Ptf1a alters neuronal morphology, inducing the radial redistribution and branching of neurites in cortical pyramidal cells. Thus Ptf1a is sufficient, even in a dramatically different neuronal precursor, to cell autonomously promote characteristics of an inhibitory peptidergic identity, providing the first example of a single transcription factor that can direct an inhibitory peptidergic fate.

Key words: cortex; inhibitory interneuron; neuronal identity; peptidergic; Ptf1a; subtype specification

Introduction

The functional nervous system depends on the correct wiring of a diverse array of neuronal subtypes, which in turn demands that

the identity of each neuronal subtype is appropriately specified during development. While the many influences that impact neuronal identity have yet to be fully understood, it is becoming clear that the intracellular transcriptional environment plays a critical role in dictating a neuron's subtype-specific features. As a result, developmentally expressed transcription factors implicated in neuronal identity specification are being explored for their ability to induce the development of particular neuronal subtypes *in vitro* and *in vivo* (Amamoto and Arlotta, 2014). However, the full complement of transcription factors that confer subtype-specific identities has yet to be elucidated, and their abilities to cell autonomously direct neuronal identity have yet to be fully characterized.

One prominent subtype-specifying transcription factor that has recently been explored is Fezf2, which is required for the development of corticofugal projection neurons (CFuPN; Molyneux et al., 2005). Fezf2 is a powerful identity-specifying transcription factor sufficient to induce CFuPN characteristics in post-mitotic layer IV pyramidal cells (De la Rossa et al., 2013) and callosal projection neurons (Rouaux and Arlotta, 2013). Furthermore, even in a different region of the CNS, misexpression of Fezf2 in striatal progenitors of medium spiny neurons is sufficient to overcome foreign intracellular and extracellular cues and

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alter the transcription factor expression, cellular morphology, axonal projection, and neurotransmitter status of these neurons to resemble CFuPNs (Rouaux and Arlotta, 2010). These studies indicate that the expression of *Fezf2* alone is capable of cell autonomously driving a CFuPN identity, even in a dramatically different neuronal subtype.

In this study, we examine the abilities of the subtype-specifying transcription factor, pancreas transcription factor 1a (*Ptf1a*), to transform neuronal identity *in vivo*. It has been shown that *Ptf1a* is required for an inhibitory interneuronal identity over the identity of excitatory counterparts in the spinal cord (Glasgow et al., 2005), cerebellum (Hoshino et al., 2005; Pascual et al., 2007), and retina (Fujitani et al., 2006; Nakhai et al., 2007), and corresponding gain-of-function studies have demonstrated that excitatory neurons in each of these regions can adopt inhibitory features with the expression of *Ptf1a* (Dullin et al., 2007; Huang et al., 2008; Lelièvre et al., 2011; Yamada et al., 2014). In the dorsal spinal cord, subpopulations of inhibitory peptidergic interneurons in particular have been shown to require *Ptf1a* expression (Bröhl et al., 2008). Previous misexpression studies have also suggested that *Ptf1a* may be sufficient to induce developmental changes that resemble inhibitory interneurons (Hoshino et al., 2005; Nishida et al., 2010). However, a thorough analysis of the ability of *Ptf1a* to cell autonomously induce the identity of a specific neuronal subtype *in vivo* has not yet been undertaken.

Materials and Methods

Mouse strains. *Emx1-Cre* homozygous or heterozygous males (Gorski et al., 2002) were crossed with CD1 females (Charles River) to be used for *in utero* electroporation. *Ptf1a^{Cre}* mice (Kawaguchi et al., 2002) were used to generate *Ptf1a* knock-outs, as described previously (Borromeo et al., 2014). CD1 mice were selected because this strain yields relatively large litters, has a comparably more translucent uterus than other strains, and tolerates electroporation, as evidenced by high embryonic survival rates.

In all, ~50 pregnant dams underwent *in utero* electroporation surgery, with an average of 10–12 embryos per dam. All embryos in both uterine horns were injected with the misexpression or control constructs and electroporated, excluding only the two most proximal embryos. All dams and ~90% of electroporated embryos survived postoperatively until prenatal harvest, comparable to previously reported numbers (Saito, 2006). Postnatal survival of electroporated pups was lower, with ~10–20% of electroporated embryos successfully reaching P21. Expression of the GFP reporter was confirmed in ~80–90% of surviving electroporated embryos, comparable to previously reported numbers (Saito, 2006). Those embryos lacking expression were more likely the result of unsuccessful injection and electroporation rather than underexpression of the transgene. For a given immunohistochemistry or *in situ* hybridization experiment, three or four embryonic cortices for each condition were analyzed. For RNA-seq, three to six embryonic cortices were analyzed per condition per replicate. All experiments have been approved by and conform to the regulatory standards of the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center.

Constructs. For the generation of the *Ptf1a*-misexpression vector, full-length *Ptf1a* cDNA (pENTR2B-p48, generously provided by D. Huangfu, Sloan Kettering Institute, New York) was subcloned into the multiple cloning site of a *pCAG-lsl-IRES-EGFP* backbone (generously provided by S. Anderson, University of Pennsylvania). The *pCAG-lsl-IRES-EGFP* backbone without *Ptf1a* was used as the control GFP-only expression plasmid. For postnatal neuronal morphology experiments, a *pCAG-mCherry* plasmid (generously provided by S. Anderson, University of Pennsylvania) was coelectroporated into embryonic cortices. Plasmids were purified using the Qiagen Miniprep or Maxiprep kits.

In utero electroporation surgery and tissue harvesting. *In utero* electroporation was performed as described previously (Saito and Nakatsui, 2001; Saito, 2006). Pregnant CD1 dams were anesthetized with isoflurane and the uterus exposed. Less than 1 μ l of purified plasmid (2 μ g/ μ l)

spiked with Fast Green (Sigma) was injected through the uterus into the lateral ventricle of E12.5 embryos using beveled glass micropipettes (Drummond Scientific). Five 38–42 V pulses of 50 ms duration at 950 ms intervals were given across the head using round, 7 mm platinum-plated tweezer electrodes connected to a BTX ECM830 Electro Square Porator. Throughout the procedure, embryos were kept moist by constant bathing in 37°C PBS. The uterus was returned to the abdominal cavity, the wound was sutured, and wound clips placed over the incision. Embryonic brains were harvested at the desired developmental stage and fixed in 4% PFA for 6 h. For postnatal harvesting, animals were anesthetized and transcardially perfused with 4% PFA before excision and post-fixation of the brain. Brains were cryoprotected in sucrose and processed for immunohistochemistry and *in situ* hybridization.

Transcriptome profiling and bioinformatics. For RNA-seq experiments, E12.5 electroporated cortices from three to six embryos were collected at E15.5 for each sample and dissociated using a papain dissociation kit (Worthington). GFP-only control samples were processed in duplicate and *Ptf1a*-misexpressing samples were processed in triplicate. GFP^{ON} cells from either *Ptf1a*-misexpressing or GFP-only control cortices were then isolated using FACS. Total RNA from ~58,000–106,000 cells was extracted using TRIzol RNA Isolation Reagents (Life Technologies). Quality of RNA was ensured before amplification by analyzing 20–50 pg of each sample using the RNA 6000 Pico Kit and a Bioanalyzer (Agilent). High-quality (RIN > 8) total RNA (2.1–7.8 ng) was subsequently amplified using the SMARTer Universal Low Input RNA Kit for Sequencing (Clontech Laboratories) according to instructions provided by the manufacturer. The sequencing library was prepared using KAPA HTP Library Preparation Kit (Kapa Biosystems), adaptors were diluted 1/30-fold, and amplification was set for 10 cycles. Barcoded samples were then run on a HiSeq 2500 in a 50 bp/50 bp paired end run, using the TruSeq SBS Kit v3 (Illumina). An average of 53 million paired reads was generated per sample and the percentage of mRNA bases was close to 52%, on average. Data from RNA-seq results were processed according to Borromeo et al. (2014). In short, sequence reads were aligned to the mm9 genome using TopHat, and an expression-level model was built with the FPKM method of Cuffdiff (Trapnell et al., 2009, 2010, 2013). Genes were considered differentially expressed with a *q* value < 0.05. All RNA-seq samples from *Ptf1a*-misexpression and GFP-only control replicates can be found on the GEO database (GSE59481). Data used for *Ptf1a* ChIP-seq (Meredith et al., 2013) and neural tube RNA-seq (Borromeo et al., 2014) comparisons can also be found in the GEO database (GSE55841).

Defining gene signatures for gene set enrichment analysis. For *Ptf1a* neural tube-activated and -suppressed gene signatures, we used differentially expressed genes found in the E11.5 *Ptf1a*^{−/−} versus wild-type samples described by Borromeo et al. (2014) (GSE55841). To define gene signatures for GABAergic cortical interneurons or glutamatergic cortical pyramidal cells, we used expression data from isolated cingulate cortical GABAergic (GIN, *Gad1* inhibitory, GSM63018–20) and glutamatergic (YFPH, Layer 5 pyramidal cells, GSM63036–38) neurons obtained from the GEO database (GSE2882). The microarray data were preprocessed using the robust multi-array average function for background correction and the quantile method for normalization of data in the “affy” R package (Gautier et al., 2004). Genes upregulated or downregulated were identified in these two cell types by performing linear models for microarray data (Smyth, 2004).

Immunohistochemistry and in situ hybridization. Immunohistochemistry on 12 μ m coronal cryosections of embryonic cortex and 20 μ m coronal cryosections of postnatal cortex was performed with the following antibodies: rabbit anti-Cleaved caspase-3 (1:400; Cell Signaling Technology), rat anti-Ctip2 (1:500; Abcam), rabbit anti-Cux1 (1:100; Santa Cruz Biotechnology; M-222), rabbit anti-GABA (1:500; Sigma), rabbit anti-GFP (1:1000; Invitrogen), rabbit anti-Ki67 (1:300; Abcam), guinea pig anti-Lim1 (1:8000; generously provided by S. Morton and T. Jessell, Columbia University), rabbit anti-Pax2 (1:1000; Covance), rabbit anti-Pax6 (1:500; Covance), rabbit anti-*Ptf1a* (1:5000; generously provided by C. Wright, Vanderbilt University, Nashville, Tennessee), and mouse anti-Tuj1 (1:1000; Covance). As Tuj1 exhibited fairly extensive staining, a GFP^{ON} neuron was considered Tuj1 positive if cytoplasmic Tuj1 staining was observed surrounding approximately one-third or greater of the

nucleus with a ring-like pattern, if $>50\%$ of the GFP^{ON} cell body exhibited Tuj1 staining without the ring-like pattern, or if discernable Tuj1-positive processes could be observed definitively extending from the GFP^{ON} cell body.

In situ hybridization of *Calb2*, *Gad1*, *Gad2*, *GlyT2* (Allen Brain Atlas: www.brain-map.org), *Kirrel2* (Minaki et al., 2005; generously provided by Y. Ono, KAN Research Institute), *Nphs1*, *Pnoc* (Open Biosystems), and *Prdm13* (Chang et al., 2013) was performed with DIG-labeled riboprobes, and processed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). Probes for *Gad1* (Betley et al., 2009) and *Gad2* (Betley et al., 2009) were generated from a P10 mouse cDNA library using the following primer sets: *Gad1* (5'-CCA TCT CGC AAG CAA CTA CA-3', 5'-GCG CTA ATA CGA CTC ACT ATA GGG AAT GCA CAG TGT GGG TTT CA-3') and *Gad2* (5'-AAA ATC TCT TGG GCC CTT TC-3', 5'-GCG CTA ATA CGA CTC ACT ATA GGG CCG GAG TCT CCA TAG AGC AG-3'). Probes for *Calb2* and *Nphs1* were generated from P3 and E10.5 mouse cDNA libraries, respectively, using the following primer sets: *Calb2* (5'-TCT GGC ATG ATG TCC AAG AG-3', 5'-GGC GTC CAG TTC ATT CTC AT-3') and *Nphs1* (5'-CTT CAC ACT GAC TGG GCT GA-3', 5'-AAG GCT TGG CGA TAT GAC AC-3').

Morphology tracing and analysis. For morphological analysis of pyramidal cells at P21, 20 μ m coronal cryosections of cortices coelectroporated with *pCAG-lsl-Ptf1a-IRES-EGFP* and *pCAG-mCherry* were imaged using a Leica SP5 confocal microscope and Leica Application Suite Advanced Fluorescence imaging software. The GFP signal was amplified with an anti-GFP antibody. Tracing of pyramidal cells was performed on collapsed images using Adobe Photoshop and encompassed the cell soma and any connected neurites captured in the 20 μ m section. In *Emx1-Cre*-positive and *Cre*-negative cortices, tracing was performed for mCherry^{ON}/GFP^{ON} and mCherry^{ON} cells, respectively. Using software developed in the laboratory, neurite angles were plotted by determining the angle at which proximal neurites intersected the cell soma. Angular distributions were calculated for the dorsal (0–180°) and ventral (181–360°) halves of neurons separately. Neurite branching was calculated by counting the total number of branch points per 20 μ m section for individually traced neurons. Classification of pyramidal cell morphology was determined by having five blind, independent raters determine whether or not traced cellular morphology resembled a stereotypical pyramidal cell (Jones, 1986).

Statistical analysis. Differences in percentage colabeling were calculated using a Wilcoxon rank sum (Mann–Whitney) test. Differences in the distribution of neurite angle projections were calculated using a Kolmogorov–Smirnov test. Differences in neurite branching and pyramidal cell morphology classifications were calculated using a Student's *t* test. The significance threshold was $p \leq 0.05$. Results are reported as mean value $\pm$ SEM.

Results

Misexpression of Ptf1a in developing cortical pyramidal cells

We examined the potency of Ptf1a to transform neuronal identity *in vivo* by misexpressing it in developing cortical pyramidal cells, where Ptf1a is not endogenously used, allowing us to infer which subtype-specific features Ptf1a can drive cell autonomously. We electroporated a conditional Ptf1a-misexpression plasmid, which expresses both full-length Ptf1a and GFP in the presence of *Cre*-recombinase (Fig. 1*A,B*), into the cortices of E12.5 *Emx1-Cre* mice, ensuring spatial specificity of Ptf1a expression exclusively in excitatory pyramidal cells (Gorski et al., 2002). By E15.5, the majority of Ptf1a-misexpressing cells, identified by the conditional expression of GFP (GFP^{ON}), continued to express Ptf1a (71 $\pm$ 6%; n = 541 cells, n = 3 mice; Fig. 1*C',D*). Pyramidal cells electroporated with a conditional GFP-only control plasmid did not colabel with Ptf1a (0.1 $\pm$ 0.1%; n = 620 cells, n = 3 mice; p = 0.046; Fig. 1*A,C,D*). Ptf1a-misexpressing neurons remained viable, as the majority continued to express Tuj1 (88 $\pm$ 4 vs 92 $\pm$ 1% in controls; n = 639 and 502 cells, respectively, n = 3 mice; p = 0.28; Fig. 1*E,F*), and did not express cleaved caspase-3 (data not

shown). Furthermore, misexpression of Ptf1a induced a notable shift in neuronal migration, with the vast majority of cells failing to reach the cortical plate and, therefore, not expressing the superficial and deep layer markers Cux1 and Ctip2, respectively (12 $\pm$ 1 vs 48 $\pm$ 2% in controls for Cux1; n = 502 and 551 cells, respectively, n = 3 mice; p = 0.05; 5 $\pm$ 3 vs 31 $\pm$ 7% in controls for Ctip2; n = 563 and 571 cells, respectively, n = 3 mice; p = 0.049; Fig. 1*G–J*). Despite remaining closer to the ventricular zone, Ptf1a-misexpressing neurons exhibited only a modest, though significant, enrichment of the progenitor markers Ki67 (3 $\pm$ 0.3 vs 1 $\pm$ 0.6% in controls; n = 886 and 953 cells, respectively, n = 3 mice; p = 0.049; Fig. 1*K,L*) and Pax6 (16 $\pm$ 11 vs 1 $\pm$ 0.5% in controls; n = 781 and 745 cells, respectively, n = 3 mice; p = 0.05; Fig. 1*M,N*), indicating that overall, they continued to differentiate appropriately. The retention of Pax6 expression in some Ptf1a-misexpressing cells reflected their dorsal rather than ventral telencephalic origin, supporting that Ptf1a was misexpressed in pyramidal cell and not cortical interneuron precursors. Thus, our system can be used to effectively misexpress Ptf1a in pyramidal cell precursors, altering elements of their development, but not their differentiation as post-mitotic neurons.

Ptf1a misexpression induces inhibitory interneuronal gene expression in excitatory cortical pyramidal cells

Upon the successful introduction of Ptf1a into pyramidal cell precursors, we set out to determine the extent to which it could induce an ectopic Ptf1a-specific developmental program in this foreign context. We used RNA-seq to perform a comparative transcriptome analysis between FACS-purified GFP^{ON} cells isolated from Ptf1a-misexpression and GFP-only control cortices (Fig. 2*A*). Misexpression of Ptf1a resulted in a significant ($q < 0.05$) upregulation of 2217 genes and downregulation of 2263 genes, of which 32% of upregulated genes and 20% of downregulated genes are likely to be direct targets of Ptf1a (Fig. 2*A*), as determined through comparison to E12.5 Ptf1a ChIP-seq data from the neural tube (Meredith et al., 2013). Conversely, approximately half (52%) of the direct targets of Ptf1a in the neural tube were upregulated or downregulated in pyramidal cells (Fig. 2*A*; Meredith et al., 2013).

To examine common regulatory actions of Ptf1a across the CNS, we compared our dataset of differentially expressed genes to a dataset of genes regulated by Ptf1a in neural tube (Borromeo et al., 2014). We found that 43% of Ptf1a-activated genes in neural tube are also upregulated in pyramidal cells, while only 12% of Ptf1a-suppressed genes are downregulated in pyramidal cells (Fig. 2*B*). Interestingly, genes that were commonly regulated between pyramidal cells and neural tube were enriched (56%) for direct neural tube targets of Ptf1a (Fig. 2*B*). Thus, while a majority of the impact that Ptf1a has on the pyramidal cell transcriptome is mediated via its indirect actions, the relatively more conserved actions of Ptf1a in these two contexts appear to be mediated directly.

We next sought to assess more comprehensive similarities or differences between the transcriptional profile of our Ptf1a-misexpressing pyramidal cells and those of other endogenous Ptf1a-expressing populations, as well as endogenous inhibitory cortical interneuron populations that do not normally use Ptf1a. To do so, we generated a ranked list from greatest upregulation to greatest downregulation of differentially expressed genes following Ptf1a misexpression. Gene set enrichment analysis (GSEA; Subramanian et al., 2005) was then used to compare

Figure 1. Ptf1a affects neuronal migration but not differentiation. **A–D**, GFP-only control or Ptf1a-misexpression constructs (**A**) were introduced into pyramidal cell precursors of E12.5 *Emx1-Cre* embryos via *in utero* cortical electroporation (**B**) and harvested at E15.5. GFP^{ON} cells in control cortices exclude Ptf1a ($0.1 \pm 0.1\%$) and migrate to the cortical plate, whereas GFP^{ON} cells in Ptf1a-misexpression cortices express Ptf1a ($71 \pm 6\%$) and settle below the cortical plate (CP; **C, D**). GFP^{ON} cells in Ptf1a-

whether gene sets from endogenous Ptf1a-expressing dorsal neural tube interneuron populations or endogenous cortical inhibitory interneuron populations are enriched at one end of the ranked list or the other.

To identify gene signatures for dorsal spinal cord inhibitory and excitatory neurons, we used published RNA-seq data from *Ptf1a*^{−/−} and wild-type neural tubes (Borromeo et al., 2014) and defined 136 Ptf1a-activated genes (enriched in dorsal neural tube inhibitory neurons) and 244 Ptf1a-repressed genes (enriched in dorsal neural tube excitatory neurons). Similarly, for gene signatures representing cortical inhibitory interneurons and excitatory pyramidal cells, we used published microarray data (Sugino et al., 2006) and identified 356 unique genes upregulated in GABAergic neurons from the cingulate cortex (cortical interneuron gene set) and 737 unique genes upregulated in layer 5 pyramidal cells of cingulate cortex (cortical pyramidal cell gene set; adjusted $p \leq 0.05$; fold change ≥ 2). GSEA results demonstrated that genes upregulated in Ptf1a-misexpressing pyramidal cells were significantly enriched for Ptf1a-activated genes identified in dorsal neural tube (Normalized enrichment score (NES) = 2.26; FDR q value < 0.001 ; Fig. 2C). However, genes downregulated by Ptf1a misexpression in pyramidal cells were not significantly enriched for Ptf1a-suppressed genes in dorsal neural tube (NES = 0.86; FDR q value = 0.791; Fig. 2D), which may point to regionally distinct differences between genes available to be suppressed by Ptf1a in pyramidal cells versus dorsal neural tube interneuron precursors. When compared with cortical neuron signatures, genes upregulated in Ptf1a-misexpressing pyramidal cells were enriched for cortical inhib-

misexpression cortices remain neuronal as the majority ($88 \pm 4\%$) express the neuronal marker Tuj1, comparable to GFP-only control cortices ($92 \pm 1\%$; $p = 0.28$; **E–F**). Ptf1a-misexpressing GFP^{ON} cells do not reach the cortical plate, and thus show reduced expression of the layer-specific markers Cux1 ($12 \pm 1\%$; **G', H**) and Ctip2 ($5 \pm 3\%$; **I', J**), which are typically expressed by GFP^{ON} control cells in superficial ($48 \pm 2\%$; $p = 0.05$; **G, H**) and deep ($31 \pm 7\%$; $p = 0.049$; **I, J**) layers, respectively. GFP^{ON} cells in Ptf1a-misexpression cortices exhibit a modest, but significant increase in the expression of the progenitor markers Ki67 ($3 \pm 0.3\%$; **K', L**) and Pax6 ($16 \pm 11\%$; **M', N**) relative to GFP^{ON} cells from control cortices ($1 \pm 0.6\%$; $p = 0.049$ for Ki67; **K, L** and $1 \pm 0.5\%$; $p = 0.05$ for Pax6; **M, N**). $*p \leq 0.05$, $n > 500$ cells from 3 mice. Scale bars: **C, C', E, E', G, G', I, I', K, K', M, M'**, 50 μm ; for higher magnification images, 10 μm . Data represent the mean $\pm$ SEM.

Figure 2. Misexpression of Ptf1a alters the pyramidal cell transcriptome. Comparative transcriptome analysis of purified GFP^{ON} cells from control and Ptf1a-misexpressing cortices showed that Ptf1a was able to activate and suppress a total of 4480 genes in pyramidal cells (PCs), of which 26% are likely direct targets. These direct targets represent approximately half of known Ptf1a binding sites in the neural tube (NT; *A*; Meredith et al., 2013). Twenty-three percent of genes regulated by Ptf1a in the neural tube (Borromeo et al., 2014) were also activated or suppressed by Ptf1a in PCs, of which 56% were direct targets (*B*). *C–F*, GSEA plots comparing differentially expressed genes in Ptf1a-misexpressing pyramidal cells with other interneuron populations. Genes from RNA-seq analysis of Ptf1a-misexpressing pyramidal cells are ranked sequentially along the x-axis from greatest upregulation (red) to greatest downregulation (blue). Individual genes from neural tube or cortical interneuron gene sets are then assessed for position within the ranked list (black tallies), and the running-sum statistic of gene enrichment, calculated by walking down the ranked list of genes, is plotted for each gene signature. Enrichment of differentially expressed genes between Ptf1a-misexpressing and control pyramidal cells for dorsal neural tube Ptf1a-activated gene set (*C*). Enrichment of differentially expressed genes between Ptf1a-misexpressing and control pyramidal cells for dorsal neural tube Ptf1a-suppressed gene set (*D*). Enrichment of differentially expressed genes between Ptf1a-misexpressing and control pyramidal cells for a cortical inhibitory interneuron gene set (*E*). Enrichment of differentially expressed genes between Ptf1a-misexpressing and control pyramidal cells for a cortical excitatory pyramidal cell gene set (*F*). $N \geq 3$ mice for each RNA-seq replicate, run-in triplicate for Ptf1a-misexpression samples, and duplicate for GFP-only control samples.

itory interneuron gene sets (NES = 1.72; FDR q value < 0.001; Fig. 2*E*), and genes that are downregulated in these cells were enriched for cortical excitatory pyramidal cell gene sets (NES = -1.23; FDR q value = 0.014; Fig. 2*F*).

Strikingly, a closer examination of the genes upregulated by Ptf1a in excitatory cortical pyramidal cells showed a number of genes that are typically expressed in subpopulations of inhibitory interneurons. For example, we observed expression of the cell adhesion molecules Kirrel2 and Nphs1, as well as the transcriptional repressor Prdm13 ($n = 3$ mice; Fig. 3*A–D'*), which are

Ptf1a dependent in inhibitory interneurons of the spinal cord (Nishida et al., 2010; Chang et al., 2013). Furthermore, the calcium binding protein calretinin (*Calb2*), expressed in some cortical inhibitory interneuronal populations (Xu et al., 2004), was also upregulated in pyramidal cells ($n = 3$ mice; Fig. 3*E, E'*). The acquisition of an inhibitory interneuronal molecular identity in turn correlated with an alteration in neurotransmitter marker expression in pyramidal cells, which are typically excitatory. Ptf1a-misexpressing pyramidal cells upregulated the GABA-synthesizing enzyme GAD65 (*Gad2*; $n = 3$ mice; Fig. 3*F–G'*), as

Figure 3. Ptf1a induces the expression of inhibitory interneuronal markers. **A–H'**, Electroporated cortical regions (**A, A'**) exhibit expression of *Kirrel2* (**B, B'**), *Nphs1* (**C, C'**), *Prdm13* (**D, D'**), *Calb2* (**E, E'**), *Gad2* (**F–G'**), and *Pnoc* (**H, H'**) in Ptf1a-misexpression cortices, but not GFP-only control cortices. $N \geq 3$ mice for each condition. Scale bars: **A–F', H, H'**, 50 μm ; **G, G'**, 10 μm . CP, cortical plate; IZ, intermediate zone; Isl, lox-STOP-lox; SP, subplate; SVZ, subventricular zone.

well as the Ptf1a-dependent (Bröhl et al., 2008) inhibitory peptide precursor preproenkephalin (*Pnoc*; $n = 4$ mice; Fig. 3 **H, H'**).

Induction of peptidergic neurotransmitter expression over other inhibitory neurotransmitters in Ptf1a-misexpressing cortical pyramidal cells

Interestingly, although Ptf1a is necessary for the development of multiple classes of inhibitory interneurons (Glasgow et al., 2005; Hoshino et al., 2005; Fujitani et al., 2006; Bröhl et al., 2008; Huang et al., 2008), its sufficiency to induce nociceptin expression in pyramidal cells predominated over other potential inhibitory neurotransmitters. With an eightfold increase in expression, *Pnoc* had the highest relative upregulation of any inhibitory neurotransmitter marker. While our transcriptome analysis also identified a significant upregulation of *Gad1*, preproenkephalin (*Penk*), and galanin (*Gal*) in Ptf1a-misexpressing pyramidal cells, all of which are Ptf1a dependent in the spinal cord (Glasgow et al., 2005; Bröhl et al., 2008), expression levels of these neurotransmitter markers were not ultimately detectable by *in situ* hybridization ($n = 3$ mice; Fig. 4 **C, C'**; data not shown). Furthermore, despite high expression of *Gad2*, the paucity of *Gad1* expression, which pro-

duces the majority of cytosolic GABA (Asada et al., 1997), ultimately resulted in a lack of appreciable GABA production ($n = 3$ mice; Fig. 4 **D, D'**). No GABA production was observed despite extending the age of harvest, or by together varying the age of electroporation and of harvest ($n = 3$ mice for E12.5 electroporation and E17.5 harvest, $n = 2$ mice for E14.5 electroporation and P0 harvest; Fig. 4 **E, F**). The observed lack of GABA was further supported by an absence of Pax2 and Lhx1 expression—two Ptf1a-dependent transcription factors that help maintain a GABAergic fate in many subcortical interneuron populations (Glasgow et al., 2005; Pillai et al., 2007; data not shown). Finally, the Ptf1a-dependent (Huang et al., 2008) glycinergic marker *GlyT2* was also not expressed ($n = 3$ mice; Fig. 4 **B, B'**).

Interestingly, Ptf1a-misexpressing pyramidal cells may retain some hybrid characteristics in their neurotransmitter status, as analysis of glutamatergic markers showed no significant downregulation of vesicular glutamate transporters, vGluT1 (slc17a7), vGluT2 (slc17a6), or vGluT3 (slc17a8; data not shown). In addition, we observed a significant downregulation of the glutamate transporter EAAT3 (slc1a1) and the cysteine/glutamate transporter xCT (slc7a11), along with a significant upregulation of

Figure 4. Inhibitory neurotransmitter status in Ptf1a-misexpression cortices. Electroporated regions from serial sections of GFP-only control and Ptf1a-misexpression cortices (**A, A'**) do not show expression of *GlyT2* (**B, B'**), *Gad1* (**C, C'**), or GABA (**D, D'**). Altering the harvest age (**E**) or both the electroporation and harvest ages (**F**) also did not result in an induction of GABA expression. $N = 2$ mice for **F**, $n = 3$ mice for all others. Scale bars: 50 μ m. CP, cortical plate; elec, electroporation age; harv, harvest age; IZ, intermediate zone; Isl, lox-STOP-lox; SP, subplate; SVZ, subventricular zone.

the glutamate transporters EAAT1 (slc1a3) and EAAT4 (slc1a6; data not shown), although the relative change in expression of most of these markers was minute and unlikely to be detectable by *in situ* hybridization. The ongoing expression of some glutamatergic markers indicates that while Ptf1a misexpression is sufficient to induce characteristics of an inhibitory peptidergic neuronal identity, the resultant neurotransmitter phenotype of Ptf1a-transformed pyramidal cells may, in fact, remain somewhat mixed, with retention of some pyramidal cell molecular characteristics. These data demonstrate that, while Ptf1a may not fully suppress all glutamatergic machinery, it does induce

aspects of an inhibitory, primarily nociceptinergic identity in developing pyramidal cells.

Misexpression of Ptf1a alters pyramidal cell morphology

With such significant Ptf1a-induced alterations to the pyramidal cell transcriptome, we next investigated whether the misexpression of Ptf1a altered pyramidal cell morphology. Embryonically, GFP-only control neurons possessed a typical bipolar morphology and were clustered in the cortical plate in parallel dorsoventral arrangements (Fig. 5*A–C'*). In contrast, Ptf1a-misexpressing neurons often elaborated a more complex, multipolar arrangement of neurites along the tangential axis (Fig. 5*E, E'*). We examined this shift in morphology at postnatal stages by coelectroporating E12.5 *Emx1-Cre* cortices with both the Ptf1a-misexpression plasmid and a nonconditional *pCAG-mCherry* plasmid, which allowed us to better visualize the morphology and projections of postnatal neurons. Mice were harvested at P21, and Ptf1a-misexpressing neurons (*mCherry*^{ON}/*GFP*^{ON}) were compared with control neurons from Cre-negative cortices (*mCherry*^{ON}/*GFP*^{OFF}). Pyramidal cells in control cortices exhibited a stereotypical morphology, with a prominent apical dendrite and triangular projection pattern ($n = 80$ neurons; $n = 3$ mice; Fig. 5*F–G', J*). Ptf1a-misexpressing neurons, however, exhibited a radial redistribution of dorsal neurites and a reduced polarity, likely due to loss of the prominent apical dendrite ($n = 66$ neurons; $n = 3$ mice; $p = 0.004$; Fig. 5*H–I', J'*), as well as a 2.7-fold increase in neurite branching ($p = 0.001$; Fig. 5*K*). As a result, Ptf1a-misexpressing neurons were less likely to be morphologically classified as a pyramidal cell ($p = 0.001$; Fig. 5*L*). These data indicate that in conjunction with an altered transcriptome, misexpression of Ptf1a dramatically reshapes pyramidal cell morphology, which persists into postnatal stages.

Discussion

In this study, we demonstrate that the transcription factor Ptf1a is capable of cell autonomously redirecting cortical pyramidal cell identity toward an inhibitory peptidergic fate. Despite a dramatically different intracellular and extracellular milieu, Ptf1a is capable of commandeering the pyramidal cell transcriptome to instruct an inhibitory interneuronal molecular expression pattern and induce the inhibitory neuropeptide nociceptin. These alterations in gene expression resemble the transcriptional signatures of both endogenous Ptf1a-expressing spinal interneurons and endogenous cortical interneurons. In addition, Ptf1a misexpression also induces a transformation of pyramidal cell morphology to a more radial, branched projection pattern, which persists postnatally.

Our transcriptome analysis allowed us to distinguish which genes Ptf1a can induce or suppress cell autonomously. Globally, comparing differentially expressed genes in Ptf1a-misexpressing pyramidal cells with a gene set from Ptf1a-expressing interneurons of the dorsal neural tube (Borromeo et al., 2014) demonstrated that the battery of genes upregulated by Ptf1a in our transformed pyramidal cells is significantly enriched for Ptf1a-activated neural tube genes. These data suggest that Ptf1a induces common transcriptional subroutines, independent of the subtype of its neuronal precursor. Even when Ptf1a is expressed ectopically, these subroutines involve the same gene sets as populations of inhibitory interneurons that endogenously use Ptf1a during development. Remarkably, the transcriptional alterations induced by Ptf1a misexpression in cortical pyramidal cells extend beyond the highly specific genetic programs of endogenous Ptf1a-expressing interneurons, as Ptf1a is able to induce more

Figure 5. Ptf1a alters pyramidal cell morphology. **A–E'**, Prenatally, at E15.5, GFP^{ON} cells in GFP-only control cortices migrate to the cortical plate and exhibit a dorsoventral, bipolar morphology (**A–C'**), while GFP^{ON} cells in Ptf1a-misexpression cortices reside below the cortical plate and exhibit a tangential, multipolar morphology (**D–E'**). Boxes in **A** show location of superficial (**B, B', D, D'**) and deep (**C, C', E, E'**) higher magnification images. **F–I'**, Postnatally, at P21, control pyramidal cells exhibit a stereotypical triangular projection pattern and a prominent apical dendrite, while Ptf1a-misexpressing pyramidal cells exhibit a radial, branched projection pattern (**F–I'**). Primary neurite projection angles for Ptf1a-misexpressing neurons have an altered distribution between 0 and 180°, $p = 0.004$, Kolmogorov–Smirnov test (**J, J'**), and 2.7-fold increased branching ($p = 0.001$; **K**). As a result, Ptf1a-misexpressing pyramidal cells were 19 ± 4% less likely to be classified as having a pyramidal cell morphology ($p = 0.001$; **L**). ** $p \leq 0.01$, *** $p \leq 0.001$, $n = 80$ control and 66 Ptf1a-misexpression cells from 3 mice for postnatal quantification. Scale bars: **A**, 50 μ m; **B–I'**, 25 μ m. Ctrl, control; Isl, lox-STOP-lox. Data represent the mean ± SEM.

general characteristics of inhibitory interneurons that do not express Ptf1a during development. In this regard, we observed that the subset of genes upregulated in Ptf1a-misexpressing cortical pyramidal cells is also enriched for gene sets that are differentially expressed in cingulate cortical inhibitory interneurons over excitatory pyramidal cells (Sugino et al., 2006). The results of our GSEA analyses therefore indicate that Ptf1a can cell autonomously direct the expression of gene signatures that are both specific to Ptf1a-expressing interneurons and that more broadly resemble generic inhibitory interneurons.

Interestingly, a closer examination of the Ptf1a-regulated genes across regional datasets revealed that, despite highly correlated gene signatures, Ptf1a-dependent genes in Ptf1a-misexpressing pyramidal cells ultimately only overlap with Ptf1a-dependent neural tube genes by 23%. The regional discrepancies in gene regulation highlight how, outside of its cell-autonomous actions, Ptf1a may still rely on other cooperative factors and environmental conditions for full neuronal identity specification. As evidenced by our GSEA analyses, for example, the genes that Ptf1a suppresses in cortical pyramidal cells and neural tube interneurons appear

quite distinct, as Ptf1a-suppressed genes in cortical pyramidal cells are not significantly enriched for Ptf1a-suppressed neural tube genes. Furthermore, comparison to ChIP data from Borromeo et al. (2014) showed that in the cortex, Ptf1a regulates only approximately half of its direct neural tube targets. Thus, it does not appear that Ptf1a misexpression induces pyramidal cells to adopt the full identity of a highly specific endogenous subpopulation, transforming them, for example, into exact replicas of spinal Ptf1a-expressing interneurons. Rather, it likely induces elements of a generic inhibitory interneuronal transcriptional subroutine with Ptf1a-specific features, thus providing a genetic backdrop on which other regional and precursor-specific intracellular and extracellular cues assist to finalize neuronal identity.

The complexity of the Ptf1a-dependent and independent factors that ultimately shape the final identity of Ptf1a-misexpressing pyramidal cells is encapsulated by their altered neurotransmitter expression. The primary neurotransmitter system impacted by Ptf1a misexpression is a peptidergic one, with induction of the “prepro” form of a neurotransmitter itself. The neuropeptide nociceptin, which is known to be a Ptf1a-dependent neurotransmitter in inhibitory interneurons of the dorsal spinal cord (Bröhl et al., 2008), is the inhibitory neurotransmitter marker most highly upregulated in Ptf1a-misexpressing pyramidal cells. However, despite the upregulation of nociceptin and numerous other inhibitory interneuron markers, we do not observe a major induction of additional inhibitory neurotransmitter systems. Other than the minor GAD isoform, *Gad2*, there is no detectable increase in the expression of other inhibitory neuropeptides, GABAergic markers, or glycinergic markers. In addition, not all glutamatergic machinery used by excitatory cortical pyramidal cells is significantly downregulated, including the vesicular glutamate transporters vGluT1–3, suggesting that while Ptf1a predisposes toward inhibitory peptidergic neurotransmission, additional factors continue to shape pyramidal cell identity, potentially resulting in an incomplete transformation and the retention of a hybrid neurotransmitter phenotype.

Regarding the Ptf1a-dependent changes in pyramidal cell neurotransmission, it is worth noting that our results are in contrast with those of Hoshino et al. (2005), who suggested that Ptf1a promotes a GABAergic phenotype in the cortex. Differences between the experimental methods of these two studies may account for this discrepancy. Hoshino et al. (2005) induce Ptf1a misexpression with a broadly expressed, nonconditional construct at E14.5, when endogenous interneurons have begun to migrate into the cortex. Furthermore, they assess GABA expression only in the minority of Ptf1a-expressing cells that reach the cortical plate. Therefore, one cannot rule out the possibility that their analysis included endogenous GABAergic interneurons, while leaving unaddressed the neurotransmitter phenotype for the majority of Ptf1a-expressing cells that settle below the cortical plate. We used genetic methods to conditionally restrict Ptf1a misexpression to pyramidal cell precursors only and use RNA-seq to show definitively that Ptf1a is insufficient to significantly induce expression of the major GABA-synthesizing GAD isoform, *Gad1*. The minor isoform, *Gad2*, was the only GABAergic marker that we found to be upregulated at detectable levels, and production of GABA was not detectable.

Given the potency of Ptf1a to induce extensive inhibitory interneuron-like features in pyramidal cells and the requirement of Ptf1a for a GABAergic phenotype in spinal interneurons (Glasgow et al., 2005), how does nociceptin emerge as the major neurotransmitter affected by Ptf1a misexpression in the cortex? One possibility is that Ptf1a is in competition with other powerful

fate-specifying transcription factors in cortical pyramidal cells that counteract some of the transcriptional effects of Ptf1a. For example, the CFuPN fate-specifying transcription factor *Fezf2* acts directly to promote an excitatory glutamatergic neurotransmitter status in this subset of cortical pyramidal cells (Lodato et al., 2014). Among its actions, it directly binds the promoters of *vGluT1* and *Gad1*, upregulating the former and downregulating the latter (Lodato et al., 2014). The suppression of *Gad1* by *Fezf2* in these cells may therefore be favored over induction by ectopic Ptf1a. Another possibility is that the actions of Ptf1a will necessarily be limited by the region-specific epigenetic landscape that it encounters within pyramidal cell precursors. Epigenetic modification of Ptf1a binding sites in cortical precursors may make some elements of an identity-specifying genetic program inaccessible to Ptf1a regulation. In this regard, the dependence of cell fate specification on the lineage-specific chromatin accessibility of Ptf1a regulatory regions in progenitor cells has been demonstrated previously (Meredith et al., 2013). Thus, although Ptf1a is sufficient to cell autonomously induce a substantial portion of an inhibitory interneuronal transcriptional subroutine, the selection of certain genes, including those that determine neurotransmission, is likely also dependent on other predetermined transcriptional and epigenetic regulatory mechanisms. In cortical pyramidal cells, prepro-nociceptin therefore appears to be more transcriptionally accessible than other inhibitory neurotransmitter markers.

The functional consequences that result from the transformation of pyramidal cell identity after Ptf1a misexpression are an intriguing area of speculation, particularly regarding the way the changes in neurotransmission and cellular morphology potentially affect circuit connectivity. Although, to our knowledge, a stereotypical cortical peptidergic interneuron morphology has not been described, the transformation of pyramidal cells away from their highly stereotyped projection pattern toward a more branched radial pattern, nevertheless, suggests that their incorporation into the surrounding circuitry, the extent to which they form synapses, and their choice of postsynaptic targets may all be impacted by Ptf1a misexpression. Addressing the degree to which Ptf1a-transformed cells can recapitulate the properties of endogenous inhibitory peptidergic neurons and successfully merge with existing circuitry is an intriguing area of future exploration, raising the exciting possibility of incorporating Ptf1a into therapeutic strategies that may one day use neuronal reprogramming and transplantation to reconstitute damaged neuronal circuits.

In summary, our study illustrates the power of a single transcription factor, Ptf1a, to induce inhibitory interneuronal characteristics in developing cortical pyramidal cells. To our knowledge, it is also the first to describe a transcription factor that can cell autonomously promote features of an inhibitory peptidergic interneuronal identity. We propose a model wherein Ptf1a sits atop a transcriptional cascade that controls a variety of processes that determine a neuron's subtype-specific identity, including its molecular expression pattern, neurotransmitter status, and morphology. Ptf1a likely directs the specification of these properties by promoting Ptf1a-dependent transcriptional programs and providing an internal landscape over which other transcription factors, epigenetic modifiers, and region-specific extracellular cues assist to complete identity formation. While future studies will be required to unravel the mechanisms and downstream effectors by which Ptf1a directs neuronal identity, the results of our study implicate Ptf1a as a potent regulator of an inhibitory peptidergic identity and will guide future

studies of neuronal reprogramming for circuit repair after disease and injury.

Notes

Supplemental material for this article is available at <http://publicationdata.mskcc.org/Kaltschmidt>. This material has not been peer reviewed.

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