PLOS BIOLOGY

RESEARCH ARTICLE

Pax6 limits the competence of developing cerebral cortical cells to respond to inductive intercellular signals

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Citation: Manuel M, Tan KB, Kozic Z, Molinek M, Marcos TS, Razak MFA, et al. (2022) Pax6 limits the competence of developing cerebral cortical cells to respond to inductive intercellular signals. PLoS Biol 20(9): e3001563. https://doi.org/10.1371/journal.pbio.3001563

Academic Editor: Bassem A. Hassan, ICM, FRANCE

Received: January 21, 2022

Accepted: July 8, 2022

Published: September 6, 2022

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: https://doi.org/10.1371/journal.pbio.3001563

Data Availability Statement: Data are either contained within the paper or are available at the European Nucleotide Archive (accession numbers:

Abstract

The development of stable specialized cell types in multicellular organisms relies on mechanisms controlling inductive intercellular signals and the competence of cells to respond to such signals. In developing cerebral cortex, progenitors generate only glutamatergic excitatory neurons despite being exposed to signals with the potential to initiate the production of other neuronal types, suggesting that their competence is limited. Here, we tested the hypothesis that this limitation is due to their expression of transcription factor Pax6. We used bulk and single-cell RNAseq to show that conditional cortex-specific Pax6 deletion from the onset of cortical neurogenesis allowed some progenitors to generate abnormal lineages resembling those normally found outside the cortex. Analysis of selected gene expression showed that the changes occurred in specific spatiotemporal patterns. We then compared the responses of control and Pax6-deleted cortical cells to in vivo and in vitro manipulations of extracellular signals. We found that Pax6 loss increased cortical progenitors' competence to generate inappropriate lineages in response to extracellular factors normally present in developing cortex, including the morphogens Shh and Bmp4. Regional variation in the levels of these factors could explain spatiotemporal patterns of fate change following Pax6 deletion in vivo. We propose that Pax6's main role in developing cortical cells is to minimize the risk of their development being derailed by the potential side effects of morphogens engaged contemporaneously in other essential functions.

Introduction

Gene regulatory networks (GRNs) modulated by intercellular signals control the generation of the specialized cell types that compose multicellular organisms [1,2]. These control

PRJEB5857, PRJEB6774, PRJEB27937, PRJEB32740 and PRJEB21105).

Funding: This research was funded by grants from the Medical Research Council UK (Mr/J003662/1, D.J.P and J.O.M.; Mr/N012291/1, D.J.P.), the Biotechnology and Biological Sciences Research Council UK (Bb/N006542/1, D.J.P. and J.O.M.), the Muir Maxwell Epilepsy Centre (http://www.muirmaxwellcentre.com/; M.I.D.) and Simons Initiative for the Developing Brain (https://sidb.org.uk/; grant number 529085, J.O.M. and D.J.P.), and Principal's Career Development and Edinburgh Global Research Scholarships from the University of Edinburgh (https://www.ed.ac.uk/institute-academic-development/postgraduate/doctoral/career-management/principals-scholarships; K.B.T.) and a Scholarship from the Ministry of Higher Education, Malaysia (https://www.mohe.gov.my/en; M.F.A.R.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: aCSF, artificial cerebrospinal fluid; AHP, afterhyperpolarization; AP, action potential; aP, atypical progenitor; aRGP, atypical RGP; BrdU, Bromodeoxyuridine; CP, cortical plate; CRC, Cajal–Retzius cell; CSF, cerebrospinal fluid; DAB, diaminobenzidene; DAPI, diamidino-2-phenylindole; dcKO, double conditional KO; DE, differentially expressed; DEA, differential expression analysis; DIG, digoxigenin; dLGE, dorsal LGE; DLN, deep layer neuron; DNP, dinitrophenol; EBSS, Earle's Balanced Salt Solution; EdU, ethynyldeoxyuridine; eGC, ectopic GABAergic cell; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FDR, false discovery rate; GABA, gamma aminobutyric acid; GE, ganglionic eminence; GFP, green fluorescent protein; GO, gene ontology; GRN, gene regulatory network; IP, intermediate progenitor; LFC, log2 fold change; MAST, Model-based Analysis of Single-cell Transcriptomics; MEM, minimum essential medium; Pax6 cKO, Pax6 conditional knockout; PCA, principal component analysis; PFA, paraformaldehyde; qRT-PCR, quantitative real-time polymerase chain reaction; RGP, radial glial progenitor; RMP, resting membrane potential; SAG, signaling agonist; scRNAseq, single-cell RNAseq; SLN, superficial layer neuron; SNN, shared nearest neighbor; TSA, Tyramide Signal Amplification; TSS, transcription start site; TTX, tetrodotoxin; UMAP, uniform manifold approximation and projection; UMI, unique molecular identifier.

mechanisms affect the developmental trajectories of cells in a variety of ways to guide the production of particular cell types and prevent the emergence of alternatives. Transcription factors whose levels vary among developing cells in precise, reproducible spatiotemporal patterns are essential components of GRNs. In some cases, their regional activation in response to inductive signals drives the production of region-specific cell types, but there are many other ways in which they can operate. For example, they can determine whether, and if so how, cells respond when confronted by inductive signals, i.e., their competence [3,4]. Restricting the competence of cells as they develop is likely to maximize the probability of them following reproducibly their stereotypical developmental trajectories, e.g., by mitigating the effects of biochemical noise in the signals they encounter or in the intracellular pathways processing those signals [5] and by preventing them responding in inappropriate ways to signaling molecules surrounding them.

The cerebral cortex is a complex amalgamation of 2 major neuronal cell classes generated by developmental cell lineages expressing different sets of transcription factors [6–9]. One cell class uses the excitatory neurotransmitter glutamate to propagate neuronal activity through cortical circuits and is produced by progenitors located in the developing cerebral cortex itself. It develops from cell lineages that express transcription factors including Pax6, Neurog2, and Eomes. The other cell type uses the inhibitory neurotransmitter gamma aminobutyric acid (GABA) to refine and elaborate patterns of cortical neuronal activity and is produced by progenitors located subcortically. It develops from cell lineages that express substantially different sets of transcription factors. Pax6 is one of the first transcription factors to be expressed differentially between the progenitors of excitatory and inhibitory cortical neurons [10–12], making it a good candidate to be involved in regulating the likelihood of cortical progenitors adopting an excitatory neuronal fate.

The *Pax6* gene emerged 500 to 700 million years ago and has been conserved through all triproblastic animal lineages, where it is involved in many neural and nonneural processes [13,14]. Its expression in the developing brain of extant vertebrates and invertebrates indicates that it acquired important functions very early in this organ's evolution. In mammalian embryos, it is activated prior to neural tube closure in the anterior neuroectoderm where brain forms [15]. Its importance for the production of cortical excitatory neurons is demonstrated by the phenotypes of constitutively mutant mouse embryos unable to make functional Pax6. These embryos show reduced cortical expression of genes involved in excitatory neuron production and increased cortical expression of genes involved in the development of subcortically derived cell types including inhibitory interneurons [16–24]. We set out to discover what *Pax6* does in cortical progenitors to help govern their normal production of excitatory neurons.

We began by examining the effects of inducing cortex-specific *Pax6* loss-of-function in cortical progenitors using population and single-cell transcriptomics followed by expression analysis of selected genes in tissue sections. The response was dichotomous: Many *Pax6*-null progenitors continued to generate excitatory neurons that made cortical layers relatively normally, while others adopted abnormal developmental trajectories, the nature of which varied with age and cortical location. Subsequent in vivo and in vitro experiments revealed that Pax6 blocks the deviant trajectories by reducing the ability of cortical cells to react abnormally to substances normally present—and carrying out other essential functions—around them. We propose that the main function of Pax6 in cortical development is to imbue the process with stability and reproducibility by protecting it from potentially destabilizing signals in the cortical environment.