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Research Article **Development**

Sex-determining region Y (SRY) box 2 (*SOX2*) haploinsufficiency causes a form of hypopituitarism in humans that is characterized by gonadotrophin deficiency known as hypogonadotrophic hypogonadism. Here, we conditionally deleted *Sox2* in mice to investigate the pathogenesis of hypogonadotrophic hypogonadism. First, we found that absence of SOX2 in the developing Rathke pouch of conditional embryos led to severe anterior lobe hypoplasia with drastically reduced expression of the pituitary-specific transcription factor POU class 1 homeobox 1 (POU1F1) as well as severe disruption of somatotroph and thyrotroph differentiation. In contrast, corticotrophs, rostral-tip POU1F1-independent thyrotrophs, and, interestingly, lactotrophs and gonadotrophs were less affected. Second, we identified a requirement for SOX2 in normal proliferation of periluminal progenitors; in its absence, insufficient precursors were available to produce all cell lineages of the anterior pituitary. Differentiated cells derived from precursors exiting cell cycle at early stages, including corticotrophs, rostral-tip thyrotrophs, and gonadotrophs, were generated, while hormone-producing cells originating from late-born precursors, such as somatotrophs and POU1F1-dependent thyrotrophs, were severely reduced. Finally, we found that 2 previously characterized patients with *SOX2* haploinsufficiency and associated hypogonadotrophic hypogonadism had a measurable response to gonadotropin-releasing hormone (GnRH) stimulation, suggesting that it is not the absence of gonadotroph differentiation, but rather the deficient hypothalamic stimulation of gonadotrophs, that underlies typical hypogonadotrophic hypogonadism.

SOX2 regulates the hypothalamic-pituitary axis at multiple levels

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Introduction

Heterozygous mutations in sex-determining region Y (SRY) box 2 (*SOX2*) have been associated with major eye abnormalities (i.e., anophthalmia, microphthalmia, and coloboma), hypopituitarism characterized by pituitary hypoplasia on imaging, and gonadotrophin deficiency (hypogonadotrophic hypogonadism [HH]) (1). In addition to hypoplasia of the corpus callosum, hypothalamic hamartoma, and hippocampal malformation, patients carrying inactivating *SOX2* mutations frequently manifest other defects, such as esophageal atresia, sensorineural hearing loss, pituitary masses, and learning difficulties. Numerous nonsense, frameshift, and missense mutations, leading to the expression of SOX2 proteins with compromised function, as well as chromosomic deletions encompassing *SOX2* have been identified (2).

SOX2 is a member of the SRY-related HMG box B1 (SOXB1) subfamily of transcription factors, which also includes SOX1 and SOX3 (3). In both humans and mice, SOX2 is widely expressed during embryonic development throughout the entire epithelium of the brain and posterior neural tube, including the cortex, hippocampus, cerebellum, and spinal cord (4). In addition, persistent SOX2 expression is observed in the developing eye, in particular in the lens, neural retina, and optic nerve, and also in the prosensory region of the cochlea (5, 6). Endoderm-derived organs such as the trachea, esophagus, stomach, and posterior gut also express SOX2 during embryogenesis (4, 7). Of relevance, both the hypothalamus and Rathke pouch (RP) (the primordium of the anterior and intermediate pituitary lobes) normally express SOX2, but expression is not observed in the infundibulum (a recess at the floor of the hypothalamus) and the posterior pituitary (PP) lobe in humans (4, 8).

The pituitary gland is of dual embryonic origin. The anterior pituitary (AP) derives from RP, a region of the oral ectoderm underlying the developing diencephalon, and comprises the anterior and intermediate lobes. The PP is of neural origin and develops from a recess in the ventral midline of the diencephalon (the infundibulum). By late gestation, the anterior lobe contains 5 hormone-producing cell types; these include somatotrophs, lactotrophs, thyrotrophs, gonadotrophs, and corticotrophs, which secrete growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone (TSH), gonadotrophins (follicle-stimulating hormone [FSH] and luteinizing hormone [LH]), and ACTH, respectively (1, 9). The intermediate lobe contains melanotrophs, which express melanotrophin (MSH). Finally, the PP contains no endocrine cell types, but is instead richly endowed with axonal projections from hypothalamic neurons. Two independent birth-dating studies in mice have highlighted that the vast majority of AP cell types exit the cell cycle concurrently between 11.5 and 13.5 dpc (10, 11). At 13.5 dpc, a transitional zone composed of noncycling undifferentiated precursors appears immediately ventral to the periluminal area and is marked by the expression of CDK1NC ($p57^{Kip2}$) and cyclin E. These cells do not express any markers of terminal differentiation (12). It is now believed that this transitional zone contains a finite pool of

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precursors, which will initiate commitment into specific AP cell lineages. Rostral-tip thyrotroph, corticotroph, and gonadotroph precursors exit the cell cycle first from 11.5 dpc. This is closely followed by the POU class 1 homeobox 1–dependent (POU1F1-dependent or Pit1-dependent) lineage, comprising thyrotrophs, lactotrophs, and somatotrophs. Finally, melanotroph precursors, which compose the intermediate lobe, have been shown to exit the cell cycle last, after all the anterior lobe cell types (11).

The functioning of the pituitary gland is primarily regulated by the parvocellular and magnocellular neurons, which reside in distinct nuclei in the hypothalamus. Specifically, the parvocellular axonal projections remain within the brain, where they terminate at the median eminence, a central "hub" allowing the release of parvocellular products into the portal vasculature for delivery to the AP. For example, synthesis and secretion of gonadotropins is controlled by gonadotropin-releasing hormone (GnRH) parvocellular neurons whose axons project to the median eminence where GnRH is transported by the portal system to the AP to regulate gonadotroph function. In contrast, the magnocellular neurons of the paraventricular and supraoptic hypothalamic nuclei project their axonal terminals to the PP lobe through the pituitary stalk, where they secrete their hormone products, oxytocin (OXT) and arginine vasopressin (AVP), directly into the general circulation, bypassing in this way any intermediate portal system (13, 14). Of note, all neuroendocrine hypothalamic neurons are born within the brain, with the exception of the GnRH neurons, which arise from the embryonic olfactory pit epithelium and migrate through the nose into the brain to reach the hypothalamus.

The ablation of *Sox2* in a straightforward knockout mouse results in early embryonic lethality soon after implantation, preventing the study of its function during organogenesis (15). Subsequent studies using hypomorphic and *Sox2* conditional alleles have revealed that severe reduction or complete removal of SOX2 within the developing neural tube does not cause gross morphological defects in the brain or spinal cord, possibly due to redundancy with SOX1 and SOX3 (16–19). Despite this, these genetic approaches have revealed an essential role for this transcription factor in the maintenance of neurons in specific brain regions, proliferation and/or maintenance of neural stem cells, and neurogenesis (5, 7, 17, 18). In the pituitary, haploinsufficiency of *Sox2* leads to an overall reduction in gland size with no compromise of any hormone-producing cell type and only subtle morphological defects, suggesting a likely role for *Sox2* either in the hypothalamus, developing pituitary, or both (20). Here, we have used a conditional genetic approach to gain insights into its function during normal pituitary development and in the pathogenesis of the hypopituitarism associated with *SOX2* haploinsufficiency in humans. Our research has revealed the action of *Sox2* at multiple levels within the hypothalamic-pituitary axis.

Results

Severe anterior lobe hypoplasia and neonatal lethality in Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$ mutants. We have previously shown that the *Hesx1-Cre* mouse line drives robust Cre-mediated activity in the developing pituitary gland (21, 22). We crossed *Hesx1$^{Cre/+}$* mice with *Sox2$^{fl/fl}$* animals carrying a *loxP-Sox2-loxP* conditional allele to reveal the function of *Sox2* during pituitary organogenesis (5). Genotypic analysis of 9.5- to 18.5-dpc embryos derived from crosses between *Hesx1$^{Cre/+}$;Sox2$^{fl/+}$* mice and either *Sox2$^{fl/+}$* or *Sox2$^{fl/fl}$* animals showed no statistically significant variation from the expected Mendelian ratios (Supplemental Tables 1 and 2; supplemental material available online with this article; doi:10.1172/JCI64311DS1). In contrast, genotyping of postnatal mice from birth to 3 weeks failed to identify any viable *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mice (Supplemental Tables 1 and 2), demonstrating that deletion of *Sox2* using the *Hesx1-Cre* line causes neonatal/perinatal death.

H&E staining of *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutant pituitaries at 9.5 and 10.5 dpc revealed no gross morphological defects in the developing RP of these mutants compared with those of control littermates (Figure 1, A and B, and data not shown). The first clear evidence of a morphological defect, typically mild anterior lobe hypoplasia, was observed at 12.5 dpc and further pronounced by 14.5 dpc (Figure 1, C–F). At 18.5 dpc, a fully penetrant phenotype of severe anterior lobe hypoplasia was observed in all embryos analyzed, often with remnants of pituitary tissue embedded within the oropharyngeal ectoderm (Figure 1, G–I). As a consequence, normal development of the basisphenoid cartilage was disrupted in these mutant embryos, so that it remains as 2 separate entities surrounding the ectopically located AP tissue (Figure 1I). However, the posterior and intermediate lobes were present and apparently normal in *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutants (Figure 1H). These results suggest that early induction of RP occurs normally in *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutant embryos, but subsequently there is a failure of the developing AP to expand normally, leading to a very small and partially ectopic anterior lobe at the end of gestation.

Terminal differentiation of somatotrophs and mature thyrotrophs is disrupted in Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$ mutant pituitaries. Specific immunostaining against pituitary hormones at 18.5 dpc revealed that numbers of somatotrophs (GH$^+$) and thyrotrophs (TSH$^+$) in *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutant pituitaries were substantially reduced, accounting for approximately 0.9% and 3.6% of those found in matched control pituitaries (Figure 2, A–D, and Supplemental Figure 1, C and D). However, corticotrophs/melanotrophs (ACTH$^+$) and thyrotrophs/gonadotrophs (αGSU$^+$, where αGSU indicates α–glycoprotein subunit) were less affected and represented approximately 42.0% and 36.2% of the numbers identified in control pituitaries (Figure 2, I–L, and Supplemental Figure 1, A and B). Immunostaining against LH and FSH confirmed the presence of gonadotrophs in the *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutant pituitaries, although these were reduced to 25.8% and 51.5% of the numbers identified in controls (Figure 2, M–P). This finding was further corroborated by in situ hybridization for *Nr5a1* (*Sf1*), a factor required for gonadotroph differentiation that precedes FSH and LH expression (23, 24), which showed an apparent increase in *Nr5a1*$^+$ cell numbers in the absence of SOX2 (Supplemental Figure 1, E and F). Finally, PRL immunostaining and in situ hybridization using specific *Prl* antisense riboprobes confirmed that the differentiation of lactotrophs was not as severely impaired as that of somatotrophs and thyrotrophs in the mutant pituitary, accounting for approximately 31.7% of the PRL$^+$ cells in control pituitaries (Figure 2, E and F, and Supplemental Figure 1, G and H).

The transcription factor *Pou1f1* is required for normal specification of somatotrophs, thyrotrophs, and lactotrophs (*Pou1f1* lineage) (25, 26). POU1F1 immunostaining revealed a dramatic reduction of POU1F1$^+$ cells from 12.5 to 18.5 dpc in the *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutant compared with control pituitaries (Figure 2, G and H, and Figure 3, A–D). At 18.5 dpc, numbers of POU1F1$^+$ cells in the mutant pituitaries represented a mere 1.4% of those found in control pituitaries. The emergence of *Pou1f1* in the developing pituitary gland relies on *Prop1* (prophet of *Pou1f1*) expression (27–29).

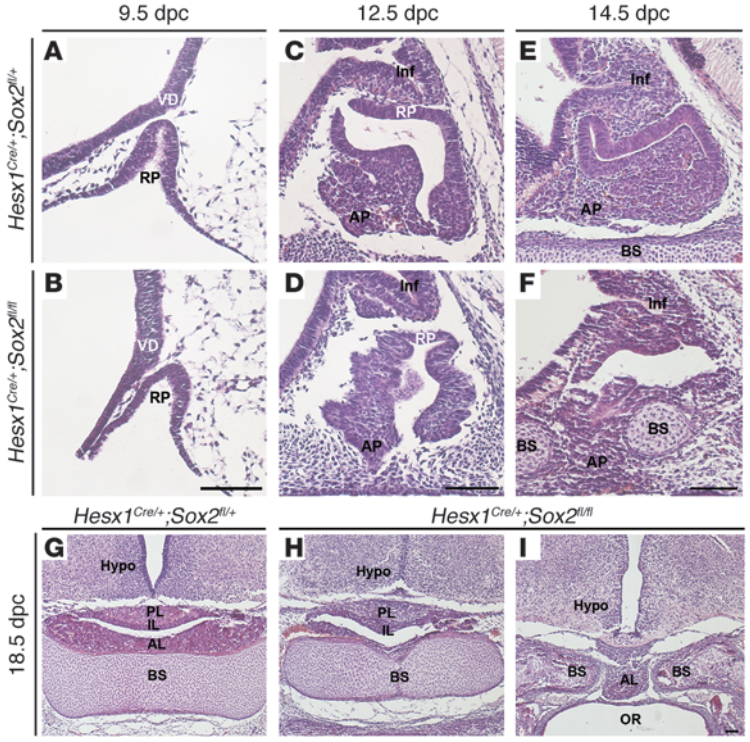

Figure 1
Abnormal pituitary morphogenesis and severe anterior lobe hypoplasia in *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant mice. H&E staining of sagittal (**A**–**F**) or transverse (**G**–**I**) histological sections from *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/+}$ control and *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant embryos. (**A** and **B**) No significant differences in the morphology of RP and ventral diencephalon (VD) are observed between genotypes at 9.5 dpc. (**C** and **D**) By 12.5 dpc, the infundibulum (Inf), a depression of the floor of the ventral diencephalon, RP periluminal epithelium, and the developing AP are clearly distinguishable in a control embryo (**C**). However, RP and developing AP are dysmorphic and slightly hypoplastic in the mutant embryo (**D**). (**E** and **F**) At 14.5 dpc, a substantial AP has developed over the basisphenoid (BS) bone in the control embryo (**E**), but AP is dysmorphic, small, and ectopically located between the developing unfused basisphenoid cartilages in the mutant pituitary (**F**). (**G**–**I**) At 18.5 dpc, the pituitary gland is situated between the hypothalamus (Hypo) and basisphenoid and comprises the posterior, intermediate, and anterior lobes (PL, IL, and AL, respectively) in a control embryo (**G**). In the mutant pituitary, posterior lobes and intermediate lobes are morphologically distinguishable, but only a rudiment of the anterior lobe is observed, often lying between the unfused basisphenoid and in contact with the oropharyngeal cavity (OR). **H** and **I** show sections at different axial levels of the same embryo. Scale bars: 100 μm.

In agreement, in situ hybridization analysis revealed a significant reduction of the *Prop1* expression domain in the mutant pituitaries at 12.5 dpc compared with controls (Supplemental Figure 2, C–F). In contrast to POU1F1$^+$ cells, rostral-tip POU1F1-independent thyrotrophs as well as corticotrophs, the first 2 differentiated cell types that are detectable in the early developing anterior lobe (11, 30, 31), were specified in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant pituitaries, despite the clear reduction in anterior lobe size (Figure 3, E–N). In agreement with this finding, expressions of *NeuroD1* and *Pomc1* (32, 33), which are 2 markers of the corticotroph and melanotroph lineages, were normal or slightly elevated into dorsal regions of RP at 12.5 dpc (Supplemental Figure 2, G–J). *Lhx3*, a marker of pituitary cells, was expressed in the dysmorphic and slightly hypoplastic pituitary of *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutants at this stage (Supplemental Figure 2, A and B).

Together, these results suggest that (a) absence of *Sox2* causes severe impairment of somatotroph and mature thyrotroph differentiation, possibly due to a reduction in *Prop1* and *Pou1f1* expression at early developmental stages; (b) lactotrophs are present in the hypoplastic and dysmorphic pituitary of *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutants to an extent that cannot be accounted for by the sparse POU1F1$^+$ cells observed; and (c) this differentiation phenotype is related to the timing of cell-cycle exit: rostral-tip thyrotrophs, corticotrophs, and gonadotrophs, whose precursors become postmitotic from 11.5 dpc are the least affected cell types. In contrast, mature thyrotrophs and somatotrophs, which differentiate at later developmental stages, are almost absent.

Early induction of RP is not affected in Hesx1$^{Cre/+}$;*Sox2*$^{fl/fl}$ *mutant embryos.* Next we sought to assess the possible contribution of aberrant hypothalamic signaling to the observed pituitary hypoplasia. *Bmp4* is one of the earliest signaling molecules known to be expressed in the presumptive infundibulum and is necessary for the invagination of RP (34, 35). *Fgf8* and *Fgf10* are also both expressed in a domain similar to that of *Bmp4* at this embryonic stage and are essential for the maintenance of RP proliferative progenitors through the induction of the LIM (zinc finger–containing protein-protein interaction domain) homeodomain transcription factors *Lhx3* and *Lhx4* (36–39). At 9.5–10.5 dpc, *Bmp4*, *Fgf8*, and *Fgf10* expression in ventral diencephalon and *Lhx3* induction in RP were comparable between genotypes (Supplemental Figure 3, A–F, I, and J). In addition, immunostaining against the phosphorylated form of MAPK1 (ERK1/2), a readout of active FGF signaling, revealed similar staining in the developing RP of *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutants and control littermates (Supplemental Figure 3, G and H). Together, this analysis demonstrates that the initial steps of pituitary development are not affected in *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutants.

At 12.5 dpc, expression of both *Bmp4* and *Fgf8* in the hypothalamus is thought to be required for the maintenance of RP progenitors and, specifically *Fgf8*, for the inhibition of ventral (i.e., thyrotrophs) and promotion of dorsal (i.e., corticotrophs) fates (34–37). No evident differences were observed in the expression domains of these 2 signaling molecules within the hypothalamus of *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutants and controls (Supplemental Figure 3, K–N). However, the *Lhx3* expression domain was clearly reduced in the mutant pituitaries (Supplemental Figure 2, A and B), suggesting a failure of the RP periluminal progenitors to proliferate, despite the presence of hypothalamic signals.

The relatively late onset of pituitary defects observed in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ embryos prompted us to evaluate the temporal and spatial efficiency of Cre-mediated excision of the *Sox2* conditional allele in these embryos. Immunostaining revealed comparable levels of SOX2 in the developing RP and overlying diencephalon in *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/+}$ control and *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant embryos at 10.5 dpc, demonstrating persistence of SOX2 protein at this early stage (Supplemental Figure 4, A and B). However, at 12.5 dpc, there was a drastic reduction in SOX2 within RP peri-

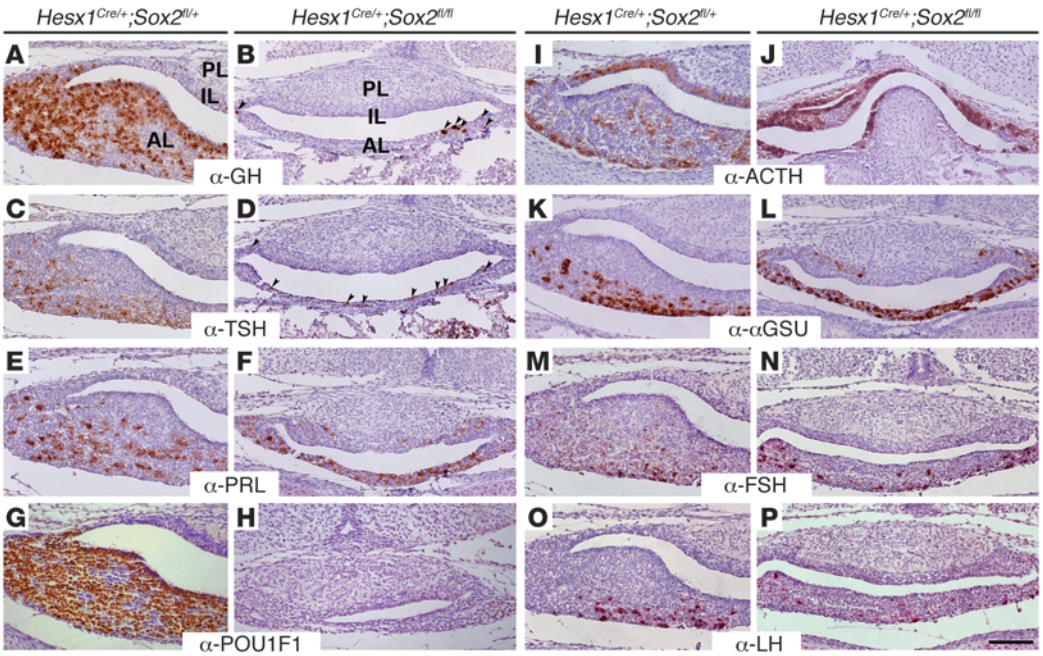

Figure 2

Defective terminal differentiation of specific hormone-producing cell types in *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ pituitaries at late gestation. Immunohistochemistry with specific antibodies against GH, TSH, PRL, POU1F1, ACTH, αGSU, FSH, and LH on transverse histological sections of *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/+}$ control and *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ mutant embryos. Note the severe reduction of somatotrophs (GH$^+$) (**A** and **B**), thyrotrophs (TSH$^+$) (**C** and **D**), and POU1F1$^+$ cells (**G** and **H**) in the mutant pituitary relative to the controls (arrowheads indicate expressing cells in **B** and **D**). Despite the drastic hypoplasia of the anterior lobe, lactotrophs (PRL$^+$) (**E** and **F**), corticotrophs, and melanotrophs (ACTH$^+$) (**I** and **J**) as well as gonadotrophs (FSH$^+$, LH$^+$,and αGSU$^+$) (**K**–**P**) are present. Scale bars: 100 μm.

luminal progenitors in *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ developing pituitaries compared with controls, despite similar levels in the prospective hypothalamus and infundibulum (Supplemental Figure 4, C–F). Absence of SOX2 staining was also confirmed in the anterior and intermediate lobes of the mutant pituitary at 18.5 dpc (Supplemental Figure 4, G and H). This pattern of SOX2 deletion fits with the observed Cre-mediated activity in the *Hesx1*$^{Cre/+}$*;R26*$^{YFP/+}$ embryos, as robust and abundant yellow fluorescent protein (YFP) expression is detected throughout the RP and developing pituitary gland with mosaic YFP reactivity in the infundibulum, ventral hypothalamus, telencephalon, olfactory epithelium (OE), and oral ectoderm (Supplemental Figure 4, I–N, and refs. 21, 22). Together, these data suggest that a significant loss of SOX2 does not occur at the early stages of RP specification in the *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ mutants, which explains the lack of phenotype at 10.5 dpc. In addition, our results suggest that abnormal hypothalamic signaling is not an important contributor to the hypoplastic pituitary phenotype observed at late gestation, but rather, that it is the consequence of the loss of *Sox2* within the developing RP.

Reduced proliferation of RP progenitors in Hesx1$^{Cre/+}$*;Sox2*$^{fl/fl}$ *embryos.* The observed hypoplasia of the AP in the *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ mutants could be due to increased apoptosis of RP progenitors/differentiated cells, decreased proliferation, premature differentiation, and/or a combination of these factors.

Cell death normally occurs within the oral ectoderm closely adjacent to the developing RP, where it is thought to play a role in morphogenesis, to aid the separation of RP from the oral epithelium at 11.5–12.5 dpc (40). Staining against cleaved caspase-3 revealed the presence of apoptotic cells in this area of the oral ectoderm in both mutant and control embryos, but no significant differences in staining were observed within the body of the mutant and control developing pituitaries between 12.5 and 14.5 dpc (Supplemental Figure 5).

Recent studies have demonstrated essential roles for the negative cell-cycle regulators CDK1NC and CDK1NB (p27^{Kip1}) in the control of cell-cycle exit of pituitary cell lineage precursors and terminal differentiation of hormone-producing cells, respectively (12). Specific immunostaining revealed no major differences in the expression pattern of CDK1NC between genotypes at 12.5 and 14.5 dpc (Supplemental Figure 6, I–L). However, a modest but consistent increase in CDK1NB was observed in the *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ mutant pituitaries relative to stage-matched controls (Supplemental Figure 6, M–P). Premature differentiation was further assessed in the mutant pituitaries by analyzing the expression of the early lineage commitment markers ACTH, αGSU, and TBX19 (T-pit) at developmental stages immediately prior to when gene expression can first be detected in normal control embryos. These analyses failed to reveal any difference in the temporal expression of these markers in the developing anterior lobe (Supplemental Figure 6, A–H). Mouse embryos deficient for the Notch downstream target gene *Hes1* display a concomitant increase in both CDK1NC and CDK1NB without relevant changes in differentiation marker expression (41). Therefore, precocious cell-cycle exit may not be indicative of premature cellular differentiation. Together, these data imply that increased cell death or premature differentiation are not important contributors to the AP hypoplasia observed in *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ embryos.

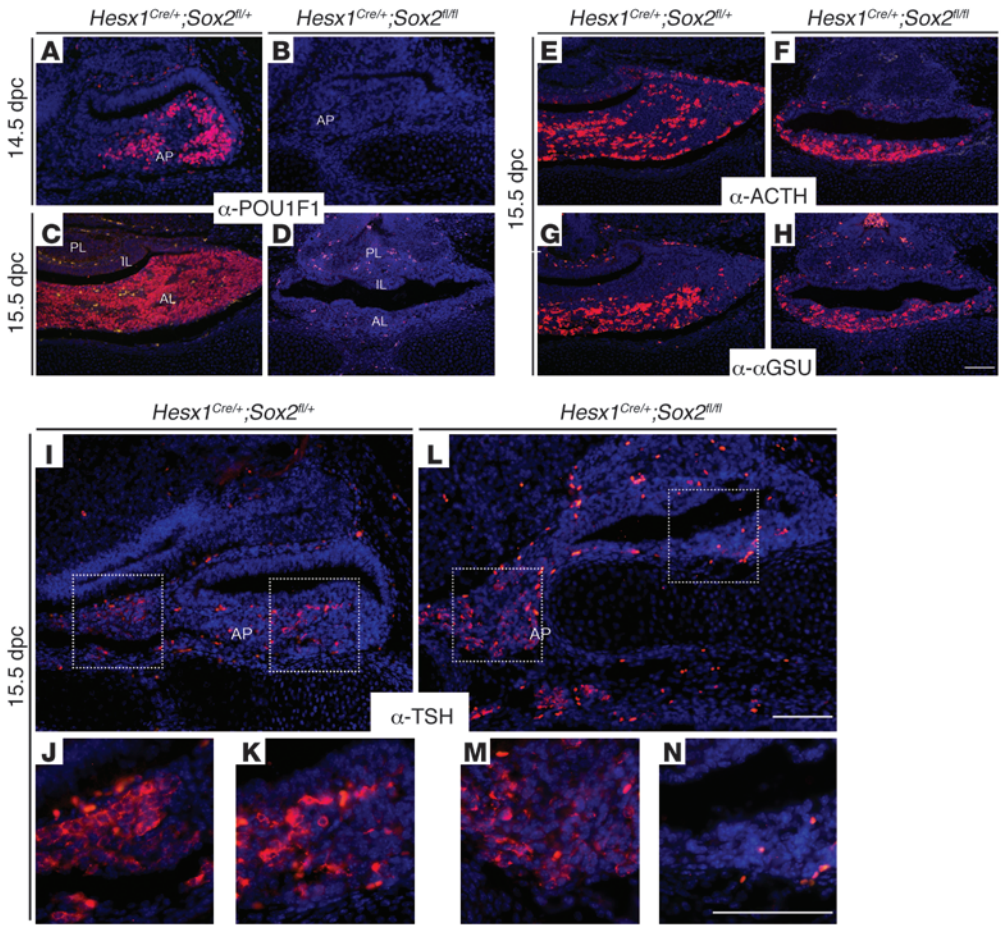

Figure 3

Corticotrophs and rostral-tip POU1F1-independent thyrotrophs are specified in *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ developing pituitaries, but POU1F1 expression is not initiated during embryonic development. Immunofluorescent detection of specific cell lineage marker expression on sagittal (**A**, **B**, and **I**–**N**) and transverse histological sections (**C**–**H**) of *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/+}$ control and *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ mutant embryos at 14.5 and 15.5 dpc. (**A** and **B**) POU1F1^{+} cells are abundant in the caudomedial region of the control (**A**), but are not detected in the mutant (**B**) AP at 14.5 dpc. (**C** and **D**) A day later, most of the cells in the anterior lobe express POU1F1 in the control (**C**). However, the mutant pituitary is devoid of POU1F1^{+} cells (red signal is autofluorescence by blood cells) (**D**). (**E**–**H**) Despite the evident hypoplasia, ACTH^{+} and αGSU^{+} cells are detected at large numbers in the mutant (**F** and **H**) pituitary. The red signal around the PP area is due to blood cells in **G** and **H**. (**I** and **L**) TSH^{+} cells corresponding with the rostral-tip POU1F1-independent thyrotrophs are identified in the control (**J**) and mutant (**M**) developing anterior lobe. However, the TSH^{+} cells marking the POU1F1-dependent thyrotrophs, located in caudomedial regions, can be recognized only in the control (**K**), but not in the mutant (**N**), pituitary. The autofluorescence signal in **N** is due to trapped blood cells. Scale bars: 100 μm.

Next, we analyzed proliferation of RP progenitors at 12.5 dpc, just as a morphological phenotype becomes noticeable, and 14.5 dpc, when anterior lobe hypoplasia is fully evident. Cyclin D2 controls G_1/S cell-cycle progression and is mainly expressed in RP progenitors in the periluminal epithelium (12). Quantification of cyclin D2–expressing cells out of total DAPI-stained nuclei in the periluminal epithelium revealed a significant decrease in this ratio in the *Hesx1*$^{Cre/+}$*;Sox2*$^{fl/fl}$ mutant pituitary compared with stage-matched controls (Figure 4, A–E). Actively dividing cells can also be detected by a short pulse (90 minutes) of BrdU, which is incorporated during DNA replication at the S-phase of the cell cycle. In the pituitary gland, it is well established that BrdU-labeled cells are mostly detected in RP progenitors lining the cleft when the pulse is administered from 12.5 dpc to 14.5 dpc (11, 12). Quantitative analysis showed a significant reduction of total numbers of BrdU^{+} cells relative to DAPI-stained nuclei in mutant relative to control pituitaries at both stages analyzed (Figure 4, F–J). Therefore, lack of SOX2 in RP progenitors leads to a decreased proliferation rate during the developmental window when progenitors of the majority of the anterior lobe hormone-producing cells exit the cell cycle (11).

We reasoned that this significant reduction in the proliferative capacity of early RP periluminal progenitors could account, at least partially, for the observed phenotype whereby only anterior lobe differentiated cells (i.e., corticotrophs, rostral-tip thyrotrophs, and gonadotrophs) whose precursors exit cell cycle early are present, while cells derived from late precursors (i.e., somatotrophs and mature thyrotrophs) are missing. To test this concept further, we carried out a pulse/chase BrdU experiment by injecting a single dose of BrdU into pregnant mice at 10.5 dpc and analyzing the embryos at 14.5 dpc. This temporal window was expected to allow actively proliferating 10.5-dpc RP progenitors to go through

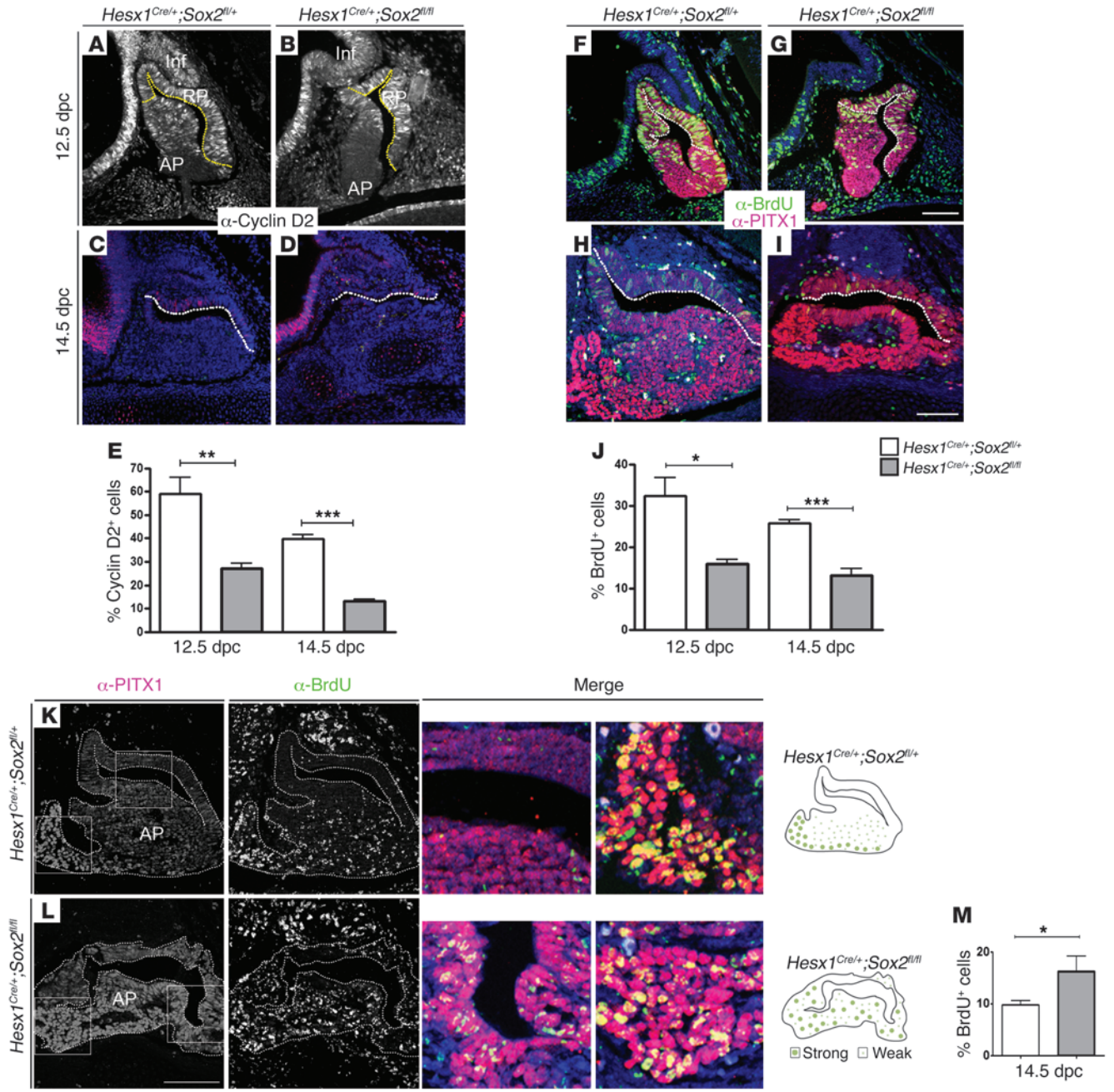

Figure 4

Reduced cell proliferation of RP periluminal progenitors in *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ embryos. The region of the periluminal epithelium used for quantitative analysis is delineated in **A**–**I**. (**A**–**D**) Cyclin D2 immunofluorescence identifies cycling cells at the G_1/S transition in RP, but not in the developing AP, at 12.5 and 14.5 dpc in both genotypes. (**E**) Quantitative analysis demonstrates a significant decrease in cycling cells in the mutant compared with the control pituitaries at both stages analyzed (12.5 dpc, $^{**}P = 0.0011$; 14.5 dpc, $^{***}P < 0.0001$; Student's *t* test). (**F**–**I**) BrdU immunodetection after a 90-minute pulse showing the presence of cycling cells in S phase mainly within the RP periluminal epithelium in both genotypes. PITX1 staining distinguishes pituitary tissue from surrounding mesenchyme. (**J**) There is a significant reduction in the percentage of BrdU$^+$ cells from the total PITX1-stained nuclei in the mutant relative to the control pituitaries (12.5 dpc, $^{*}P = 0.0252$; 14.5 dpc, $^{***}P = 0.0001$; Student's *t* test). (**K**–**L**) BrdU and PITX1 double immunostaining on control (**K**) and mutant (**L**) pituitaries. BrdU was injected at 10.5 dpc and labeled cells traced at 14.5 dpc. In the control pituitary, BrdU labeling is strong in the rostral-tip and ventromedial region of the AP, weak in the dorsomedial region, and almost absent in the periluminal epithelium. In the mutant pituitary, BrdU retention in the periluminal epithelium is evident and a dorsomedial region of weak BrdU signal is not clearly observed. (**M**) There is a significant increase in BrdU retention in the mutant relative to the control pituitaries ($^{*}P = 0.0158$; Student's *t* test). Scale bars: 100 μm.

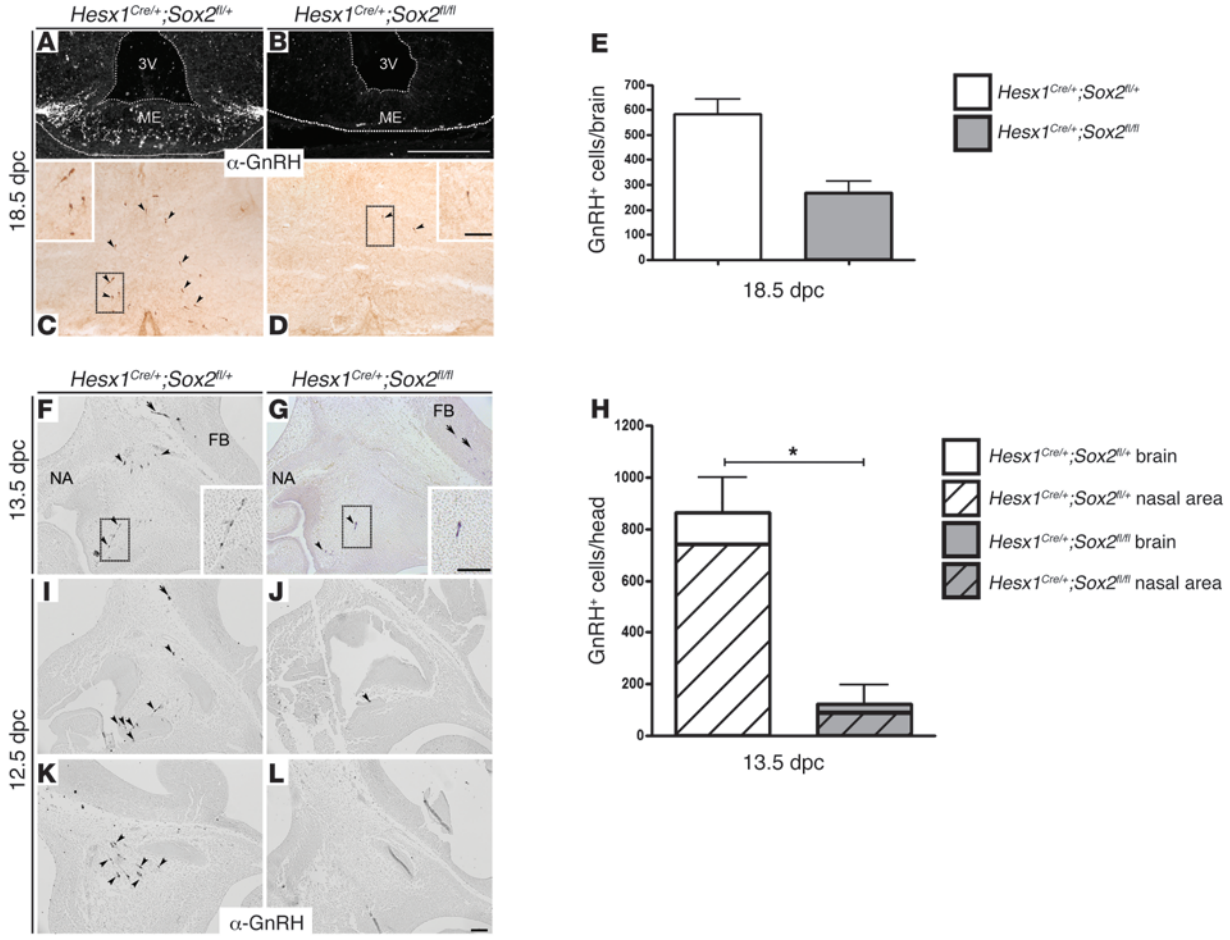

Figure 5

Significant reduction of GnRH neurons in the brain and nasal regions of *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant embryos. (**A** and **B**) Specific immunostaining shows the accumulation of GnRH in the axon terminals of GnRH neurons in the median eminence (ME) of the control (**A**), but not the mutant hypothalamus, at late gestation (**B**). (**C** and **D**) Representative pictures of GnRH neurons (arrowheads) in the brain at the level of the medial preoptic area. (**E**) Quantitative analysis of total numbers of GnRH neurons in the brain of both genotypes at 18.5 dpc (n = 2 per genotype). (**F** and **G**) Representative pictures of migrating GnRH neurons in the nasal area (NA; arrowheads) and forebrain (FB; arrows) at 13.5 dpc. (**H**) Quantitative analysis of total numbers of GnRH neurons in the heads of embryos of both genotypes (n = 3 per genotype; *P = 0.011; Student's t test). (**I**–**L**) At 12.5 dpc, GnRH neurons are detected around the OE (arrowheads) and are migrating toward the forebrain (arrows) in the control embryo (**I** and **K**) (n = 2). There is an evident reduction in the mutant embryo (**J** and **L**) (n = 2). Scale bars: 100 μm; 10 μm (insets).

several cell cycles, thereby diluting the marker through subsequent S-phases in half with each division. In contrast, early born cells exiting the cell cycle around the time of BrdU injection, and not further dividing, would retain a steady level of marker. In control 14.5-dpc embryos, as expected, most of the strongly stained BrdU$^+$ cells were located in the rostral tip of the gland, corresponding to the rostral-tip POU1F1-independent thyrotrophs and corticotrophs (Figure 4K and refs. 11, 30). In these embryos, BrdU staining was not detected in the periluminal epithelium containing RP progenitors, as subsequent rounds of divisions would have diluted the initially incorporated label. An intermediate zone of the anterior lobe immediately ventral to the periluminal space, where POU1F1$^+$ cells normally reside, showed weaker BrdU incorporation, as these cells exit the cell cycle later in development (Figure 4K). This BrdU staining pattern created a distinct visual gradient in the control pituitaries that was not as well defined in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant littermates. Rather, there was more uniform BrdU staining throughout the hypoplastic anterior lobe of the mutant pituitary containing the rostral-tip thyrotrophs and corticotrophs, as previously shown (Figure 4L). Consistently, BrdU$^+$ cells remained in the ventral part of the periluminal epithelium, indicating that these progenitors had retained the label for longer. Quantification of the ratios between BrdU$^+$ cells/total DAPI-stained nuclei revealed a significant increment in the mutant pituitaries compared with controls (Figure 4M). The pattern of BrdU staining in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant developing pituitary is consistent with the marked defects previously described in POU1F1 cell lineage specification and less affected corticotroph and rostral-tip thyrotroph differentiation. In addition, the persistence of BrdU-labeled cells in RP periluminal progenitors is compatible with the lower proliferation capacity observed in the mutant pituitary at 12.5 and 14.5 dpc. In summary, these studies demonstrate an essential requirement for *Sox2* during proliferation of RP progenitors and expansion of the AP.

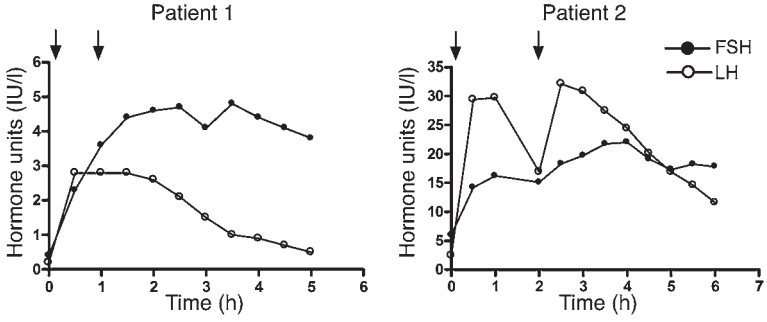

Figure 6
Gonadotrophin responses to GnRH stimulation in 2 female patients harboring heterozygous *SOX2* mutations. A bolus of 100 mg of GnRH was administered i.v. (first arrow) and LH and FSH measured at 0, 30, and 60 minutes. This was followed by a second injection of 500 mg of GnRH (i.v. bolus; second arrow) and further half-hourly measurements for 4 hours. Note the elevation of both LH and FSH circulating concentrations in both patients. Patient 1 received the second bolus 1 hour after the first one, while patient 2 had a lag of 2 hours between injections.

Lack of GnRH neurons in the hypothalamus of Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$ mutant embryos. Given that gonadotrophs were present at considerable numbers in the pituitaries of the *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutant embryos, we sought to explore whether defects in the neuroendocrine hypothalamic nuclei may account for the HH observed in patients carrying *SOX2* mutations.

In situ hybridization with specific *Avp* and *Oxt* antisense riboprobes revealed the presence of these neurons within the paraventricular and supraoptic nuclei of *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutant and control 18.5-dpc embryos (Supplemental Figure 7, A–H, and refs. 13, 14). Likewise, similar *Ghrh* and *Nr5a1* expression domains in the arcuate and ventromedial nuclei, respectively, were observed in *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutants and controls at 18.5 dpc (Supplemental Figure 7, I–L). Finally, immunostaining against GnRH at this stage revealed an expected pattern of expression in the median eminence of the hypothalamus in control embryos (Figure 5A). In contrast, an evident lack of GnRH immunoreactivity was apparent in the median eminence of the *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutants (Figure 5B). In keeping with this finding, quantitative analysis revealed a substantial reduction of GnRH neurons in the *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* mutant brain when compared with controls at 18.5 dpc (control 583.5 ± 60.50, mutant 269.0 ± 44.00; n = 2; Figure 5, C–E). This reduction was also observed at 12.5 dpc and 13.5 dpc, when GnRH neurons migrate from the OE to the brain (Figure 5, F–L). Of note, numbers of migrating GnRH neurons within the nasal area were significantly reduced, suggesting that the initial defect may be in the neurogenesis of this cell type (Figure 5H). Collectively, these data suggest that the neuroendocrine hypothalamus of the *Hesx1$^{Cre/+}$;Sox2$^{fl/fl}$* embryos is not overtly affected, but rather, generation of GnRH neurons is significantly impaired and projections to the median eminence completely absent.

Human patients harboring SOX2 mutations respond to GnRH stimulation. The data derived from the mouse model imply that reduced GnRH neurogenesis may account for the HH observed in patients harboring *SOX2* heterozygous mutations. We sought to explore our findings further by assessing the responses to a GnRH stimulation test of 2 patients carrying mutations in *SOX2*.

Patient 1 harbored a missense mutation in *SOX2* (p.L97P) that leads to loss of DNA binding with impaired transactivation (20) and was previously reported (42). She had bilateral microphthalmia with left sclerocornea and aphakia. At the age of 12 years, a GnRH stimulation test showed poor response, with a peak LH of 3.1 IU/l and peak FSH of 4.0 IU/l 60 minutes after GnRH stimulation. Two years later, she had a height of 138.4 cm (–2.65 SD score) with a weight of 32.5 kg (–1.6 SD score). She was completely prepubertal; the uterus was prepubertal and neither ovary was identified on ultrasound imaging. MRI revealed right microphthalmia with a prosthesis in situ, a colobomatous left globe, small optic nerves, and chiasm, a normal AP, and a thickened upper third of the pituitary stalk. Investigations revealed a normal free thyroxine, cortisol, IGF1, IGFBP3, and peak GH to glucagon stimulation. The PRL concentration was low (Supplemental Table 3). GnRH testing was repeated using 2 different i.v. doses (100 and 500 μg); the initial injection of GnRH caused an increase in circulating concentrations of FSH (from 0.4 to 3.6 IU/l) and LH (from <0.2 to 2.8 IU/l) after 1 hour (Figure 6). FSH concentrations were further increased after a second higher dose of GnRH, reaching a peak of 4.8 IU/l. In contrast LH concentrations were no further elevated after a second injection; indeed, there was a decrease.

Patient 2, with a haploinsufficient c.60insG *SOX2* mutation, was previously described with bilateral anophthalmia, learning difficulties, and oesophageal atresia (20). She was noted to have isolated HH, with a low response to GnRH stimulation (peak LH of 6.1 IU/l and FSH of 5.7 IU/l, both at 60 minutes after GnRH stimulation). Measurement of overnight spontaneous LH and FSH concentrations revealed impaired FSH and LH secretion (FSH 3.2-4.7 IU/l and LH 1-2 IU/l) with no pulsatility. Peak GH to glucagon stimulation was normal. Pubertal induction was performed using ethinylestradiol. More recently, the ethinylestradiol was stopped, and GnRH testing was repeated using a dose of 100 μg followed by 500 μg as performed with patient 1. There was a dramatic increase in LH concentration, from 2.5 to 29.7 IU/l, with an increase in FSH from 6.0 to 16.2 IU/l following the administration of 100 μg GnRH, with a further increase in LH to 32.1 IU/l and FSH to 22.0 IU/l following the administration of the higher dose of GnRH. Both IGF1 and IGFBP3 are low at the time of writing (Supplemental Table 3), suggesting the possibility of adult GH deficiency, although cortisol and thyroid function tests as well as the PRL concentrations are normal.

The responses in both patients are in keeping with former studies (43–46), and in particular, that of the second patient suggests that functional gonadotrophs that are able to receive the GnRH signal and secrete gonadotrophins are present in the pituitary gland, even if this is hypoplastic on MRI evaluation.

Discussion

Human patients carrying heterozygous *SOX2* mutations commonly display a phenotype characterized by AP hypoplasia on imaging (1, 4, 20, 47, 48). However, most pituitary hormones are detected at normal concentrations, with the exception of occasional GH deficiency; on the other hand, the gonadotrophins FSH and LH are present at abnormally low concentrations, causing typical HH (1, 2, 10, 45, 46). HH may be present even in patients without pituitary hypoplasia, suggesting that there may be a specific requirement of *SOX2* for normal development of the hypothalamic-gonadotroph axis (49). Since the pathogenesis

of these abnormalities in the patients is poorly understood, the analysis of the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mouse model presented here has proven helpful in the investigation of the mechanisms underlying the phenotype of these patients.

Providing a possible explanation for the anterior lobe hypoplasia frequently observed in human patients, our data demonstrate that SOX2 plays a critical role in the regulation of cell proliferation in RP periluminal progenitors during early development (Figure 4). The consequence of this proliferation defect is the generation of a small pool of undifferentiated cell lineage precursors, which is insufficient to allow the subsequent differentiation of enough numbers of hormone-producing cells at late gestation, resulting in severe pituitary hypoplasia. The prediction of this phenotype is that precursors of those cell lineages that exit cell cycle and begin differentiation earlier should be less affected than precursors of lineages that stop cycling later in development. In agreement with this notion, our data show that rostral-tip thyrotrophs, corticotrophs, and gonadotrophs, whose precursors become postmitotic from 11.5 dpc are present in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant pituitary at late gestation, but somatotrophs and POU1F1-dependent thyrotrophs, which represent late-born cell types, are drastically reduced in numbers (Figure 2, Supplemental Figure 1, and refs. 10, 11). Therefore, it seems reasonable to suggest that similar cellular defects may underlie the typical pituitary hypoplasia observed in patients carrying *SOX2* mutations, although the severity may be attenuated due to the presence of a wild-type *SOX2* allele in these patients. Supporting this notion, some patients with *SOX2* mutations manifest GH deficiency (in addition to the typical HH), suggesting a specific defect in somatotrophs (50, 51). In mouse, somatotrophs are the last hormone-producing cell type of the anterior lobe to be born and therefore, are possibly more susceptible if not enough precursors are generated at early stages of pituitary development, as our data from the mouse model demonstrate. Therefore, the propensity of human patients carrying *SOX2* mutations to develop GH deficiency may be caused by a reduced pool of progenitors in the developing pituitary.

The analysis of our mouse model gives relevant clues on the molecular alteration causing this phenotype. We show an almost complete absence of POU1F1 and a drastic downregulation of *Prop1* expression in *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ developing pituitaries (Figures 2 and 3 and Supplemental Figure 2). Reduction in *Prop1* expression could be the consequence of fewer cells present to commit to this fate, due to a reduced precursor pool (27, 52–54), or alternatively, impaired *Prop1* gene activation in the absence of SOX2 (55). In *Prop1*$^{-/-}$ mutants, there is a significant reduction in proliferation of RP progenitors, which upon cell-cycle exit fail to migrate into the developing anterior lobe (56). The similarities of these phenotypes suggest that the proliferation defects observed in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ developing pituitaries may be the additive result of reduced *Sox2* and *Prop1* functions. Furthermore, conditional removal of the Notch intracellular mediator recombination signal-binding protein for immunoglobulin κ J region (RBPJ), leading to the complete genetic ablation of Notch signaling, results in severe AP hypoplasia, with the concomitant reduction of *Prop1* expression and the POU1F1 cell lineage (57). Similarly, absence of the Notch targets *Hes1* and *Hes5* also results in AP hypoplasia due to reduced proliferative capacity of RP progenitors (41, 58, 59). Of interest, Notch signaling has been found to function downstream of *Sox2* within neural progenitor cell populations, such as the developing neural tube and retina (5, 60). Thus, it seems likely that SOX2 may mediate part of its function through the control of Notch signaling and *Prop1* expression within the pituitary.

It is not obvious why the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mice die perinatally, but we may assume that the disruption of the basisphenoid cartilage could compromise breathing and suckling of these neonates. Alternatively, loss of mature thyrotrophs required for lung maturation may further contribute to this phenotype, as previously shown in the *Prop1*$^{-/-}$ mutants (52). An unexpected finding is the presence of lactotrophs in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant pituitary at late gestation, despite the severe reduction of POU1F1 expression from the early onset (Figure 2 and Supplemental Figure 1). Do they represent a POU1F1-independent lactotroph lineage? Analysis of the Snell mouse model, which harbors a mutation in the *Pou1f1* gene, led to the identification of an independent thyrotroph population, not reliant on the former expression of *Pou1f1*, but lactotrophs were not analyzed (30). *Prl* transcriptional activation in the absence of *Pou1f1* has previously been reported in a cell line (61), and our data provide support to the concept that there may be a POU1F1-independent lactotroph cell population in the pituitary at late gestation. Previously, it has been shown that lactotrophs, identified by PRL expression, are detectable as early as 14.0 dpc in the developing mouse pituitary, concurrently with caudomedial POU1F1-dependent thyrotrophs and followed by somatotrophs at 16.0 dpc (10). Therefore, it is also possible that the sparse POU1F1$^+$ cells detected in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ pituitaries may be biased to a lactotroph fate, as they appear to be the first cells to be born in this lineage. However, the observation that lactotrophs are more abundant than POU1F1$^+$ cells in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant pituitary argues against this possibility. Whether POU1F1 may be required in vivo only for the initiation of *Prl* expression and not for its maintenance is a question that requires further analyses of PRL expression in other mouse models deficient for either *Prop1* or *Pou1f1*.

Our studies raise an important question: if gonadotrophs are present in humans carrying *SOX2* mutations, what is the cause of the HH commonly manifested in these patients? Terminal differentiation of gonadotrophs, as assessed by expression of FSH and LH, occurs in *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ mutant embryos (Figure 2 and Supplemental Figure 1), suggesting that in the patients, the HH is not of pituitary origin. As it is well established that gonadotroph function is reliant upon the inducing actions of hypothalamic GnRH neurons, either a loss of these neurons or their misdirected migration from their origin of the olfactory placode could underlie this condition in humans. Our data demonstrate a substantial reduction of GnRH neurons in the *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ embryos at all stages analyzed (Figure 5). This defect is specific to GnRH neurons, as other neurons within the neuroendocrine hypothalamus (AVP$^+$, OT$^+$, and GHRH$^+$) are not affected (Supplemental Figure 7). In addition to reduced GnRH numbers, axonal projections may be misdirected, as evidenced by the almost complete lack of GnRH immunostaining in the median eminence of *Hesx1*$^{Cre/+}$;*Sox2*$^{fl/fl}$ embryos (Figure 5). While patients with *SOX2* haploinsufficiency, and therefore 1 normal *SOX2* allele, may be expected to have higher numbers of GnRH neurons than our mouse model, our findings suggest that these neurons may not fully project their axons to the median eminence, resulting in a failure of gonadotroph stimulation and gonadotrophin production. Supporting this hypothesis, we show that 2 patients carrying inactivating heterozygous mutations in *SOX2* and manifesting HH can respond to GnRH stimulation (Figure 6). This implies

that functional gonadotrophs, able to bind injected GnRH and secrete FSH and LH, are present in these patients, but normal hypothalamic control is disrupted. All the patients from previous studies showed poor response to GnRH stimulation, similar to that of patient 1 in this study (4, 20, 49, 51). What we find very interesting and revealing is that when patient 2 was initially tested several years ago at a prepubertal stage, she showed a blunted response like that of patient 1 (20). However, when the test was repeated after estrogen treatment, the response to this stimulation test was essentially normal, possibly indicating a priming effect by estrogen (Figure 6). This finding reinforces our conclusion that gonadotrophs are present and functional in this patient and, in addition, it suggests that administration of GnRH may be useful in inducing puberty in patients with *SOX2* mutations.

Although this finding provides an explanation for the human phenotype observed, the mechanism underlying this defect remains unclear. A reduced dose of SOX2, as in $Sox2^{hyp/-}$ embryos (carrying a lower dose of *Sox2*), results in diminished neurogenesis, but the specification of all OE neuronal cell types, including olfactory, vomeronasal, and GnRH neurons, occurs as normal, albeit resulting in reduced numbers (5, 62). Moreover, these neurons are detected within the OE, nasal cavity, and forebrain of the hypomorphic mutants, implying that migration is not completely impaired. It is possible that the deletion of SOX2 within the OE of the $Hesx1^{Cre/+};Sox2^{fl/fl}$ mouse results in a partial reduction of neurogenesis, which ultimately accounts for the reduced GnRH neurons seen at late gestation. The detection of Cre-mediated reporter activity within the OE of $Hesx1^{Cre/+};R26^{YFP/+}$ mice supports this possibility (Supplemental Figure 4 and ref. 21).

There are some similarities as well as differences between the phenotypic features of patients with *SOX2* mutations and those in our mouse model. In humans, SOX2 haploinsufficiency disrupts the hypothalamic-gonadotrophic axis and leads to HH in all patients analyzed, and only occasionally, patients show GH deficiency, suggesting an additional impairment of the hypothalamic-somatotrophic axis. The mouse and human data presented here demonstrate that the HH is of hypothalamic and not pituitary origin. Unfortunately, we could not assess the nature of the GH deficiency in the patients for ethical reasons, but the mouse data strongly suggest that the underlying cause is likely to be an impairment of somatotroph differentiation rather than a deficit of GHRH neurons. Adrenal function and PRL concentrations are mostly normal in patients with *SOX2* mutations, and in the mouse, we show that ACTH- and PRL-expressing cells are present in abundant numbers. A major difference is that thyrotrophs are severely affected in the mouse model, but in humans, thyrotroph function is normal. We believe differences in the penetrance and severity of the phenotype are partially due to a *SOX2* dosage effect, as previously demonstrated (5), as humans have a wild-type *SOX2* allele, whereas there is no expression of SOX2 in the mouse model. In conclusion, the data presented here have revealed important insights into the pathogenic mechanisms underlying the endocrine phenotype observed in patients with *SOX2* mutations.

Methods

Mice. The $Hesx1^{Cre/+}$ and $Sox2^{fl/+}$ mouse lines have previously been described (5, 21). $Sox2^{fl/+}$ were crossed with $Hesx1^{Cre/+}$ animals in order to generate $Hesx1^{Cre/+};Sox2^{fl/+}$ mice. These mice were used for mating with either $Sox2^{fl/fl}$ or $Sox2^{fl/+}$animals. Genotyping of mice and embryos was carried out by PCR on tail-tip biopsies digested in DNAreleasy (Anachem) from mice and embryos using specific primers. The data presented here are representative examples of at least 3 individual embryos per genotype.

H&E and section in situ hybridization. Histological processing of embryos, H&E, and in situ hybridization on paraffin sections were performed as previously described (63, 64). The antisense riboprobes used in this study have previously been described (14, 22, 63, 64), with the exception of *Fgf10*, which was generated from a construct containing the *Fgf10* cDNA.

BrdU labeling and immunostaining. For BrdU labeling, pregnant mice were injected intraperitoneally with BrdU (100 mg/kg body mass), sacrificed as required thereafter (see text for details), and embryos fixed in 4% PFA and processed for immunodetection as follows: 6- to 8-μm paraffin sections were blocked in 5% heat-inactivated sheep serum in TBS/0.1% Tween-20 after antigen retrieval and incubated with primary antibodies overnight at 4°C and with secondary antibodies (either HRP- or fluorochrome-coupled) for 1 hour at room temperature. For immunostaining of GnRH neurons at E18.5, 16-μm cryosections were serially cut from the entire brain and immunostained as previously described (65). The anti-POU1F1 antibody was a gift from Simon Rhodes (Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana, USA), and Jacques Drouin (Molecular Genetics Research Unit, Institut de Recherches Cliniques de Montréal, Montréal, Quebec, Canada) provided us with the anti-TBX19 and anti-PITX1 antibodies. Working dilutions and sources of other antibodies used in this study have previously been detailed (22, 31, 65–67). For quantification of hormone-producing cells at 18.5-dpc, 2 control and 2 mutant pituitaries were analyzed using specific antibodies as described (22). Depending on the cell type, 2–7 histological sections from $Hesx1^{Cre/+};Sox2^{fl/fl}$ mutant and control pituitaries were counted, and proportions were calculated as the percentage of cell numbers in the mutant versus the control pituitary. Numbers of counted cells were as follows: (a) 5010 GH^+, 2478 TSH^+, 3533 $ACTH^+$, 2155 αGSU^+, 414 LH^+, 239 FSH^+, and 6177 $POU1F1^+$ in control pituitaries; (b) 43 GH^+, 90 TSH^+, 1483 $ACTH^+$, 780 αGSU^+, 107 LH^+, 123 FSH^+, and 88 $POU1F1^+$ in $Hesx1^{Cre/+};Sox2^{fl/fl}$ mutants.

Cell proliferation studies. Cyclin D2–expressing and $BrdU^+$ cells, after a short pulse (90 minutes) within the periluminal epithelium, which contains rapidly dividing embryonic progenitors, were quantified in $Hesx1^{Cre/+};Sox2^{fl/fl}$ mutant and staged-matched controls. Three and 6 nonconsecutive histological sections were analyzed per embryo at 12.5 dpc and 14.5 dpc, respectively. Figures are represented as the percentage of positive cells from the total DAPI-stained nuclei (cyclin D2 labeling) or PITX1-expressing cells (BrdU labeling). This equated to approximately 400 nuclei per pituitary at 12.5 dpc and 350 nuclei at 14.5 dpc, and 3 control and mutant pituitaries were analyzed. For the BrdU label retention experiments, entire pituitaries were counted in both control (~700 nuclei) and mutant (~550 nuclei) embryos.

Statistics. For Mendelian ratio analyses, χ^2 tests were used. For all other analyses, 2-tailed Student's *t* tests were used to assess the differences between genotypes. SEM and *P* values were derived from a minimum of 3 pituitaries per condition. A *P* value of less than 0.05 was considered statistically significant. All quantitative data are presented as the mean ± SEM.

Study approval. All the experiments performed in mice were reviewed and approved by an internal ethical review committee at University College London and ratified by the UK government.

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1. Kelberman D, Rizzoti K, Lovell-Badge R, Robinson IC, Dattani MT. Genetic regulation of pituitary gland development in human and mouse. *Endocr Rev*. 2009;30(7):790–829.
2. Leiden University Medical Center. Leiden Open Variation Database. MRC Human Genetics Unit LOVD at MRC IGMM. Leiden University Medical Center Web site. http://lsdb.hgu.mrc.ac.uk/home.php. Acessed July 23, 2012.
3. Pevny L, Placzek M. SOX genes and neural progenitor identity. *Curr Opin Neurobiol*. 2005;15(1):7–13.
4. Kelberman D, et al. SOX2 plays a critical role in the pituitary, forebrain, and eye during human embryonic development. *J Clin Endocrinol Metab*. 2008; 93(5):1865–1873.
5. Taranova OV, et al. SOX2 is a dose-dependent regulator of retinal neural progenitor competence. *Genes Dev*. 2006;20(9):1187–1202.
6. Dabdoub A, et al. Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc Natl Acad Sci U S A*. 2008;105(47):18396–18401.
7. Que J, et al. Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development*. 2007; 134(13):2521–2531.
8. Fauquier T, Rizzoti K, Dattani M, Lovell-Badge R, Robinson IC. SOX2-expressing progenitor cells generate all of the major cell types in the adult mouse pituitary gland. *Proc Natl Acad Sci U S A*. 2008; 105(8):2907–2912.
9. Prince KL, Walvoord EC, Rhodes SJ. The role of homeodomain transcription factors in heritable pituitary disease. *Nat Rev Endocrinol*. 2011; 7(12):727–737.
10. Seuntjens E, Denef C. Progenitor cells in the embryonic anterior pituitary abruptly and concurrently depress mitotic rate before progressing to terminal differentiation. *Mol Cell Endocrinol*. 1999; 150(1–2):57–63.
11. Davis SW, Mortensen AH, Camper SA. Birthdating studies reshape models for pituitary gland cell specification. *Dev Biol*. 2011;352(2):215–227.
12. Bilodeau S, Roussel-Gervais A, Drouin J. Distinct developmental roles of cell cycle inhibitors p57Kip2 and p27Kip1 distinguish pituitary progenitor cell cycle exit from cell cycle reentry of differentiated cells. *Mol Cell Biol*. 2009;29(7):1895–1908.
13. Szarek E, Cheah PS, Schwartz J, Thomas P. Molecular genetics of the developing neuroendocrine hypothalamus. *Mol Cell Endocrinol*. 2010; 323(1):115–123.
14. Acampora D, et al. Progressive impairment of developing neuroendocrine cell lineages in the hypothalamus of mice lacking the Orthopedia gene. *Genes Dev*. 1999;13(21):2787–2800.
15. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev*. 2003;17(1):126–140.
16. Cavallaro M, et al. Impaired generation of mature neurons by neural stem cells from hypomorphic Sox2 mutants. *Development*. 2008;135(3):541–557.
17. Favaro R, et al. Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh. *Nat NeuroSci*. 2009; 12(10):1248–1256.
18. Ferri AL, et al. Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. *Development*. 2004;131(15):3805–3819.
19. Miyagi S, et al. Consequence of the loss of Sox2 in the developing brain of the mouse. *FEBS Lett*. 2008;582(18):2811–2815.
20. Kelberman D, et al. Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. *J Clin Invest*. 2006;116(9):2442–2455.
21. Andoniadou CL, et al. Lack of the murine homeobox gene Hesx1 leads to a posterior transformation of the anterior forebrain. *Development*. 2007; 134(8):1499–1508.
22. Gaston-Massuet C, et al. Increased Wingless (Wnt) signaling in pituitary progenitor/stem cells gives rise to pituitary tumors in mice and humans. *Proc Natl Acad Sci U S A*. 2011;108(28):11482–11487.
23. Shinoda K, et al. Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the Ftz-F1 disrupted mice. *Dev Dyn*. 1995;204(1):22–29.
24. Zhao L, et al. Steroidogenic factor 1 (SF1) is essential for pituitary gonadotrope function. *Development*. 2001;128(2):147–154.
25. Dolle P, Castrillo JL, Theill LE, Deerinck T, Ellisman M, Karin M. Expression of GHF-1 protein in mouse pituitaries correlates both temporally and spatially with the onset of growth hormone gene activity. *Cell*. 1990;60(5):809–820.
26. Li S, Crenshaw EB 3rd, Rawson EJ, Simmons DM, Swanson LW, Rosenfeld MG. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. *Nature*. 1990; 347(6293):528–533.
27. Gage PJ, Brinkmeier ML, Scarlett LM, Knapp LT, Camper SA, Mahon KA. The Ames dwarf gene, df, is required early in pituitary ontogeny for the extinction of Rpx transcription and initiation of lineage-specific cell proliferation. *Mol Endocrinol*. 1996;10(12):1570–1581.
28. Sornson MW, et al. Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. *Nature*. 1996; 384(6607):327–333.
29. Olson LE, et al. Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination. *Cell*. 2006;125(3):593–605.
30. Lin SC, Li S, Drolet DW, Rosenfeld MG. Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development*. 1994;120(3):515–522.
31. Lamolet B, et al. A pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. *Cell*. 2001; 104(6):849–859.
32. Pulichino AM, Vallette-Kasic S, Tsai JP, Couture C, Gauthier Y, Drouin J. Tpit determines alternate fates during pituitary cell differentiation. *Genes Dev*. 2003;17(6):738–747.
33. Poulin G, Turgeon B, Drouin J. NeuroD1/beta2 contributes to cell-specific transcription of the proopiomelanocortin gene. *Mol Cell Biol*. 1997; 17(11):6673–6682.
34. Takuma N, et al. Formation of Rathke's pouch requires dual induction from the diencephalon. *Development*. 1998;125(23):4835–4840.
35. Treier M, et al. Multistep signaling requirements for pituitary organogenesis in vivo. *Genes Dev*. 1998; 12(11):1691–1704.
36. Norlin S, Nordstrom U, Edlund T. Fibroblast growth factor signaling is required for the proliferation and patterning of progenitor cells in the developing anterior pituitary. *Mech Dev*. 2000; 96(2):175–182.
37. Ericson J, Norlin S, Jessell TM, Edlund T. Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. *Development*. 1998;125(6):1005–1015.
38. Ohuchi H, et al. FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. *Biochem Biophys Res Commun*. 2000; 277(3):643–649.
39. Sheng HZ, et al. Multistep control of pituitary organogenesis. *Science*. 1997;278(5344):1809–1812.
40. Charles MA, Suh H, Hjalt TA, Drouin J, Camper SA, Gage PJ. PITX genes are required for cell survival and Lhx3 activation. *Mol Endocrinol*. 2005; 19(7):1893–1903.
41. Monahan P, Rybak S, Raetzman LT. The notch target gene HES1 regulates cell cycle inhibitor expression in the developing pituitary. *Endocrinology*. 2009;150(9):4386–4394.
42. Ragge NK, et al. SOX2 anophthalmia syndrome. *Am J Med Genet A*. 2005;135(1):1–7.
43. Habiby RL, Boepple P, Nachtigall L, Sluss PM, Crowley WF, JrJameson JL. Adrenal hypoplasia congenita with hypogonadotropic hypogonadism: evidence that DAX-1 mutations lead to combined hypothalmic and pituitary defects in gonadotropin production. *J Clin Invest*. 1996;98(4):1055–1062.
44. Beranova M, et al. Prevalence, phenotypic spectrum, and modes of inheritance of gonadotropin-releasing hormone receptor mutations in idiopathic hypogonadotropic hypogonadism. *J Clin Endocrinol Metab*. 2001;86(4):1580–1588.
45. Bouligand J, et al. Isolated familial hypogonadotropic hypogonadism and a GNRH1 mutation. *N Engl J Med*. 2009;360(26):2742–2748.
46. Pitteloud N, et al. Role of seminiferous tubular development in determining the FSH versus LH responsiveness to GnRH in early sexual maturation. *Neuroendocrinology*. 2009;90(3):260–268.
47. Fantes J, et al. Mutations in SOX2 cause anophthalmia. *Nat Genet*. 2003;33(4):461–463.
48. Williamson KA, et al. Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. *Hum Mol Genet*. 2006;15(9):1413–1422.
49. Sato N, et al. Hypogonadotropic hypogonadism in an adult female with a heterozygous hypomorphic mutation of SOX2. *Eur J Endocrinol*. 2007; 156(2):167–171.
50. Tziaferi V, Kelberman D, Dattani MT. The role of SOX2 in hypogonadotropic hypogonadism. *Sex Dev*. 2008;2(4–5):194–199.
51. Alatzoglou KS, et al. SOX2 haploinsufficiency is associated with slow progressing hypothalamo-pituitary tumours. *Hum Mutat*. 2011;32(12):1376–1380.
52. Nasonkin IO, et al. Pituitary hypoplasia and respiratory distress syndrome in Prop1 knockout mice. *Hum Mol Genet*. 2004;13(22):2727–2735.
53. Raetzman LT, Ward R, Camper SA. Lhx4 and Prop1 are required for cell survival and expansion of the pituitary primordia. *Development*. 2002; 129(18):4229–4239.
54. Andersen B, et al. The Ames dwarf gene is required for

Pit-1 gene activation. *Dev Biol*. 1995;172(2):495–503.
55. Yoshida S, Kato T, Susa T, Cai LY, Nakayama M, Kato Y. PROP1 coexists with SOX2 and induces PIT1-commitment cells. *Biochem Biophys Res Commun*. 2009;385(1):11–15.
56. Ward RD, Raetzman LT, Suh H, Stone BM, Nasonkin IO, Camper SA. Role of PROP1 in pituitary gland growth. *Mol Endocrinol*. 2005;19(3):698–710.
57. Zhu X, et al. Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis. *Genes Dev*. 2006; 20(19):2739–2753.
58. Kita A, et al. Hes1 and Hes5 control the progenitor pool, intermediate lobe specification, and posterior lobe formation in the pituitary development. *Mol Endocrinol*. 2007;21(6):1458–1466.
59. Raetzman LT, Cai JX, Camper SA. Hes1 is required for pituitary growth and melanotrope specification. *Dev Biol*. 2007;304(2):455–466.
60. Bani-Yaghoub M, et al. Role of Sox2 in the development of the mouse neocortex. *Dev Biol*. 2006; 295(1):52–66.
61. Gellersen B, Kempf R, Telgmann R, DiMattia GE. Pituitary-type transcription of the human prolactin gene in the absence of Pit-1. *Mol Endocrinol*. 1995; 9(7):887–901.
62. Tucker ES, et al. Proliferative and transcriptional identity of distinct classes of neural precursors in the mammalian olfactory epithelium. *Development*. 2010;137(15):2471–2481.
63. Gaston-Massuet C, et al. Genetic interaction between the homeobox transcription factors HESX1 and SIX3 is required for normal pituitary development. *Dev Biol*. 2008;324(2):322–333.
64. Sajedi E, et al. Analysis of mouse models carrying the I26T and R160C substitutions in the transcriptional repressor HESX1 as models for septo-optic dysplasia and hypopituitarism. *Dis Model Mech*. 2008;1(4–5):241–254.
65. Cariboni A, Davidson K, Rakic S, Maggi R, Parnavelas JG, Ruhrberg C. Defective gonadotropin-releasing hormone neuron migration in mice lacking SEMA3A signalling through NRP1 and NRP2: implications for the aetiology of hypogonadotropic hypogonadism. *Hum Mol Genet*. 2011; 20(2):336–344.
66. Andoniadou CL, et al. HESX1- and TCF3-mediated repression of Wnt/beta-catenin targets is required for normal development of the anterior forebrain. *Development*. 2011;138(22):4931–4942.
67. Lanctot C, Gauthier Y, Drouin J. Pituitary homeobox 1 (Ptx1) is differentially expressed during pituitary development. *Endocrinology*. 1999;140(3):1416–1422.