Pax6 is essential for establishing ventral-dorsal cell boundaries in pituitary gland development

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***Pax6*, a highly conserved member of the paired homeodomain transcription factor family that plays essential roles in ocular, neural, and pancreatic development and effects asymmetric transient dorsal expression during pituitary development, with its expression extinguished before the ventral → dorsal appearance of specific cell types. Analysis of pituitary development in the *Small eye* and *Pax6* −/− mouse mutants reveals that the dorsoventral axis of the pituitary gland becomes ventralized, with dorsal extension of the transcriptional determinants of ventral cell types, particularly *PFrk*. This ventralization is followed by a marked decrease in terminally differentiated dorsal somatotrope and lactotrope cell types and a marked increase in the expression of markers of the ventral thyrotrope cells and SF-1-expressing cells of gonadotrope lineage. We suggest that the transient dorsal expression of *Pax6* is essential for establishing a sharp boundary between dorsal and ventral cell types, based on the inhibition of *Shh* ventral signals.**

As in patterning events and the development of the imaginal disc in *Drosophila* (1), a spatially specific signaling code sets a pattern of transcription factors that establish specific cell types from common primordium. The pituitary gland has provided a particularly useful model for understanding the signaling transcriptional control of mammalian organogenesis. The mature gland contains six hormone-producing cell types that arise from a common primordium. They are defined by the peptide hormones they release including corticotropes secreting adrenotropin; melanotropes secreting melanocyte-stimulating hormone; thyrotropes secreting thyroid-stimulating hormone (TSH); gonadotropes secreting luteinizing hormone (LH) and follicle-stimulating hormone (FSH); somatotropes secreting growth hormone (GH); lactotropes secreting prolactin (PRL); and a transient embryonic population in the mouse arising rostral tip. LH, FSH, and TSH are heterodimers consisting of a common α-glycoprotein subunit (αGSU) and a distinct β-subunit (2).

The anterior pituitary gland is derived from the anterior neural ridge after the invagination of the oral ectoderm, Rathke's pouch, and the direct cell contact with the overlying neural epithelium of the ventral diencephalon in mouse embryonic day (e) 8.5 (3, 4). Commitment, determination, and cell differentiation of the pituitary gland are controlled by sequential series of gradients of signaling molecules and tissue-specific transcription factors (5–7). A number of extrinsic and intrinsic signaling molecules regulate initial events in pituitary organogenesis. *Shh* is uniformly expressed along the oral ectoderm but excluded from the invaginating Rathke's pouch, creating an ectodermal boundary from which a ventral to dorsal gradient of *BMP2* arises. Signaling molecules expressed in the ventral diencephalon, including *BMP4*, *Wnt5a*, and *Fgf8*, control organ commitment and proliferation events. By e10, there are opposing ventral → dorsal *BMP* and dorsal → ventral *Fgf* activity gradients in Rathke's pouch and determine ventral cell phenotypes (gonadotropes, thyrotropes, somatotropes, and lactotropes) and dorsal cell phenotypes (melanotropes and corticotropes; refs. 5–7). These signaling gradients can establish the patterns of transcrip-

tion factors along the pituitary–hypothalamic axis between e10–e12, including *Isl-1* (7), *Brn4* (8), *PFrk* (7), and *GATA2* (9) ventrally. *Nkx3.1* (10), *Six3* (11), *Prop-1* (8), and *Pax6* (12) were expressed dorsally. The induction of the POU domain transcription factor *Pit1* is required for the initial activation of the GH, PRL, and thyrotrope genes, before the appearance of the pituitary cell lineages (13, 14) and after the ventral → dorsal gradient of GATA2 that provides the molecular memory of the control ventral transient signaling events. The orphan nuclear receptor SF-1 is the most ventral in the pituitary gland for the activation of LHβ and FSHβ (15, 16).

Because *Pax6* is expressed in such a temporally and spatially restricted fashion in pituitary development, it was of particular interest to investigate its potential role in establishing the dorsal → ventral patterns of cell-type development. *Pax6* encodes a transcription factor containing both paired and homeodomain DNA binding motifs and is expressed in the developing eye and nervous system of a wide range of species from nematodes to vertebrates (17), as well as in the olfactory epithelium, pancreas, and pituitary gland in mammals. Studies on *Small eye (Sey)*, a semidominant mouse mutation, and on *Pax6* gene-deleted mice (18) have shown that *Pax6* is essential for the formation of the eye placode (17), certain structures of the forebrain and diencephalon (19–23), and differentiation of glucagon-producing α-cells in the pancreas (17, 24). In the spinal cord and hindbrain, *Pax6* establishes distinct ventral progenitor cell populations and controls the identity of motor neurons and ventral interneurons by mediating graded *Shh* signaling (5). In the absence of Pax6 functional protein, the gonadotropin-releasing hormone diencephalic neurons do not develop (25). These findings raise the possibility that *Pax6* is involved in pituitary gland development by controlling the dorsal → ventral appearance of cell-type specification.

In this article, we show that *Pax6* is an early dorsal marker of Rathke's pouch and early anterior pituitary gland, and its expression controls the established boundaries of somatotrope, lactotrope, and thyrotrope cell types. The absence of *Pax6* leads to a marked increase of the thyrotrope cell lineage, whereas the somatotrope and lactotrope cell lineage changes are very much diminished. These data support the hypothesis that *Pax6* is an essential component involved in forming the dorsal/ventral boundary in cell-type determination.
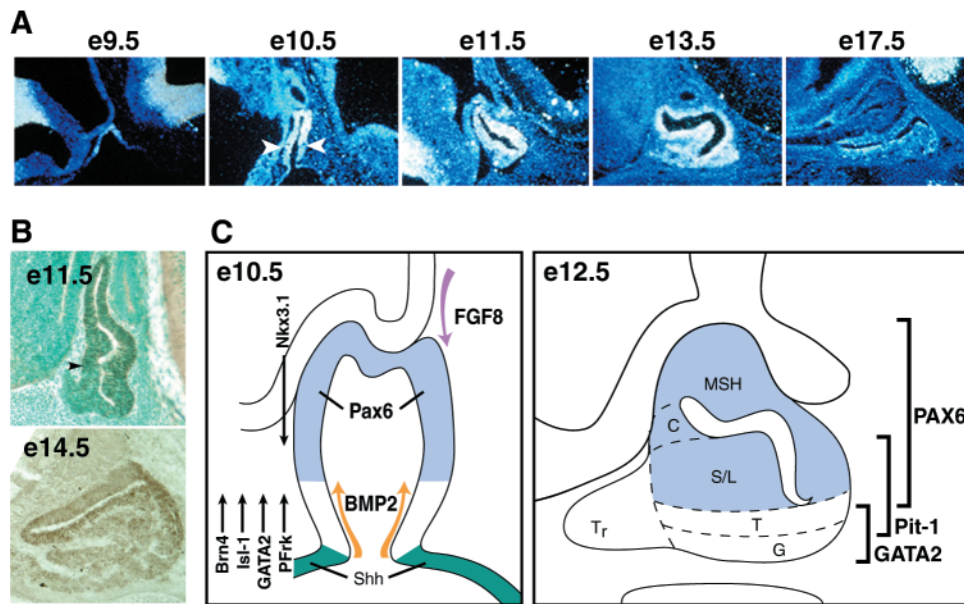
Materials and Methods

***Pax6* −/− Mice and *Sey/Sey* Mice.** Embryos were obtained from natural matings of *Pax6* +/− (18) and *Sey* +/− mice, generously provided by M. Goulding (Salk Institute, San Diego). The time point of vaginal plug observation was designated at 0.5 days

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Fig. 1. *Pax6* expression pattern during pituitary gland development. (*A*) *Pax6* transcripts are detected very early in oral ectoderm at e9.5 as they occupy the *Shh*-nonexpressing area. Later, at e10–e12, *Pax6* is characterized by a dorsal[high] to ventral[low] expression pattern along the Rathke's pouch. *Pax6* transcripts are diminished during embryonic development, and no signal is detected before birth. (*B*) Pax6 protein is detected at the dorsal pituitary gland at e11.5. Pax6-positive cells are located only in the lumen at e14.5. (*C*) Schematic representation of Pax6 expression in combination with the ventral and dorsal signaling molecules and transcription factors. By e12.5, *Pax6* expression is excluded from the most ventral cell types, the gonadotropes and thyrotropes. MSH, melanocyte-stimulating hormone; C, corticotropes; S/L, somatotropes/lactotropes; T, thyrotropes; G, gonadotropes; T$_r$, rostral tip thyrotropes.

postcoitus, and embryonic days are listed as days postcoitus. Embryo heads were isolated and fixed in 4% (vol/vol) paraformaldehyde for 3–24 h until analyzed further. *Sey/Sey* mice were characterized by the absence of the optic vesicle, which made them easy to distinguished from the wild-type litter mates. Both *Sey/Sey* and *Pax6* −/− mice, which we will refer to as mutant, produced identical phenotypes.

***In Situ* Hybridization and Immunohistochemistry.** Hybridization with ^{35}S-labeled antisense RNA probes was performed as described (13) on 20-μm cryosections of mouse heads. Immunohistochemistry was done on 10-μm paraffin sections. Sections were incubated with primary antibodies in different dilutions in PBS/0.3% Triton/5% (vol/vol) goat serum overnight at 4°C. Detection antibodies were horseradish peroxidase coupled, and staining was visualized by using a diaminobenzidine metal conjugate (Pierce). Sections were counterstained with methyl green. Primary antibodies used were obtained as follows: Pax6 was generously provided by R. Reed (John Hopkins University, Baltimore); αGSU was obtained from the National Hormone and Pituitary Program, (National Institute of Diabetes and Digestive and Kidney Diseases, Rockville, MD); TSH, GH, and PRL were purchased from Dako.

Results

***Pax6* Is an Early Dorsal Marker of Pituitary Gland.** The onset of the pituitary gland is the result of signals from the ventral diencephalon and the oral ectoderm, under *Shh* exclusion occurring in the invaginating oral ectoderm on budding of Rathke's pouch at e9 (6, 7). *Pax6* transcripts are first detected during this early phase of pituitary gland commitment and remain expressed throughout the onset of positional commitment. *Pax6* is expressed in the nascent Rathke's pouch at e9.0 (Fig. 1) but is excluded from the ventral part of the *Shh* pouch, creating a ventral zone between the *Shh*-expressing cells and the *Pax6*-expressing cells. By e10–e12, as exogenous and endogenous signals have been established, *Pax6* expression is monitored at the dorsal Rathke's pouch, with

an apparent dorsal → ventral gradient. *Pax6* expression is in a certain zone of exclusion in the rostral part of the pituitary; the ventral gland is separated from the ectoderm, occupying the ventral lip of the pouch (7, 12). When pituitary cell lineage determination is initiated with the appearance of POMC-expressing cells on e12.5, followed by *Pit-1* on e13, *Pax6* expression is rapidly decreased. By the phase of terminal differentiation of the *Pit-1* lineages and gonadotropes (e16–17.5), *Pax6* transcripts are no longer detected (Fig. 1).

Thus, both the early *Pax6* expression in the pouch with exclusion from the most rostral and the caudal cells adjacent to the *Shh*-expressing cells at the ectodermal boundary suggest that it might be the component of the transcriptional apparatus that establishes a sharp boundary, modulating the transcriptional response required for positional determination of the cell types of the three *Pit-1* lineages, although they will not appear until 4–5 days later in pituitary organogenesis.

***Pax6* Is Essential in Patterning the Developing Pituitary.** To begin to test this model of *Pax6* function in pituitary development, we examined pituitary development in mice by controlling these types by disruption of the *Pax6* gene. The first is the *Sey/Sey* mutation (26); the second is the targeted deletion of the *Pax6* locus (18). First, we examined the appearance of transcripts of signaling molecules and transcription factors as modulators of early pituitary cell-type commitment (7, 9), including the ventral expression of *Brn4*, *PFrk*, *Isl-1*, and *GATA2*. Their differential critical expression has been proposed to be mechanistically linked to ventral cell development programs (9). Therefore, we examined the expression patterns in the *Sey/Sey* embryos and *Pax6* gene-deleted mice to identify potential alteration patterns in their temporal and spatial expression. Indeed, by e12.5, the αGSU normally expressed in a rostral/ventral pattern and became widely expressed throughout the ventral anterior part of the gland (Fig. 2), and this expanded expression pattern remained until birth (see Fig. 4). In contrast, there was no difference of the dorsal → ventral gradient with the Nkx-related
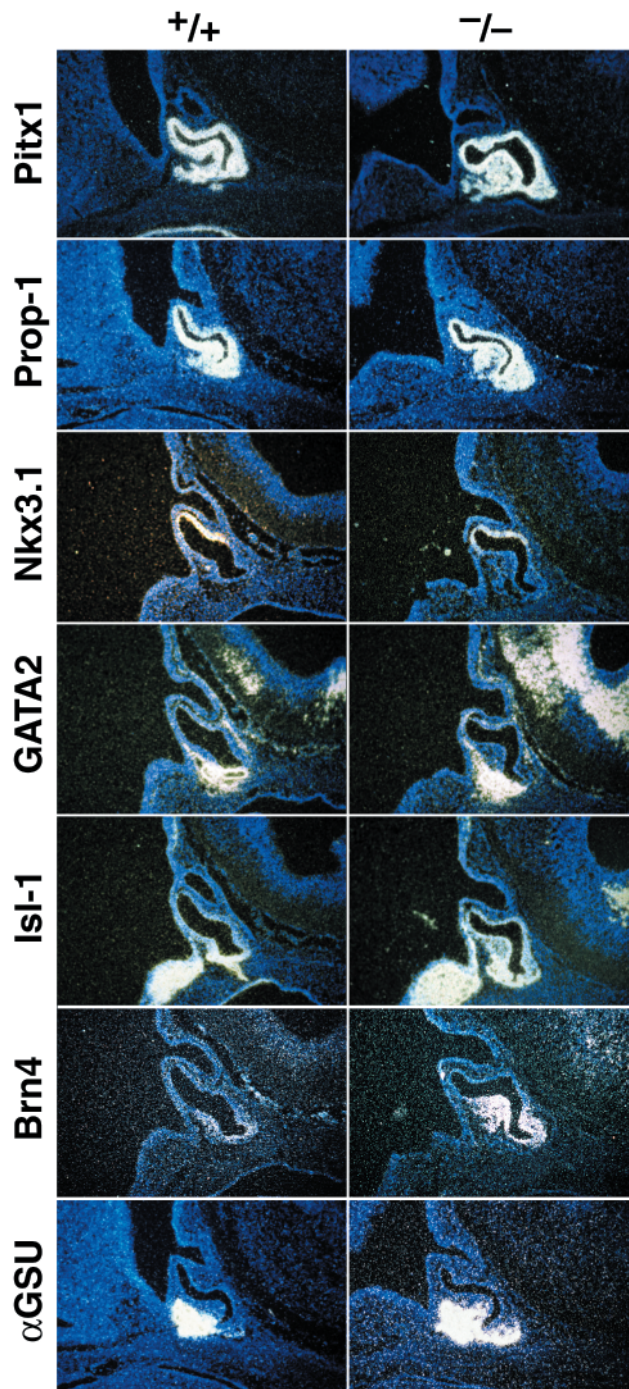
Fig. 2. Ventral markers are overexpressed in the absence of *Pax6* in the early stage of pituitary development. At e12 in Rathke's pouch, the ventral markers *αGSU*, *GATA2*, *Brn4*, *Isl-1*, and *Prop-1* are overexpressed in the *Pax6* mutant mice. No difference has been observed for the *POMC* and *Nkx3.1* levels. *Pitx1*, a uniformly expressed pituitary marker, showed an up-regulation at the ventral site of the pouch.

homeobox gene *Nkx3.1*, the most dorsally expressed marker (Fig. 2; ref. 7). The expression pattern of the ventral markers was next assayed, including the POU domain factor *Brn4*; the wind-helix transcription factor *PFrk*; the *GATA2*, a zinc finger protein that has ventral induction in the pituitary gland in respond to *BMP2* (8); and *Isl-1*, encoding a LIM-homeodomain gene, which becomes restricted to a ventral expression pattern. We found a uniform dorsalization in the expression pattern of these four
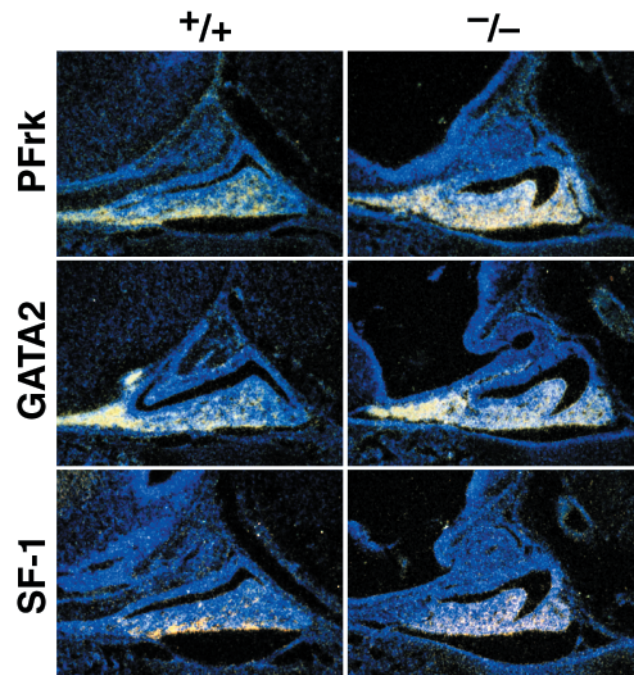
Fig. 3. Ventral markers are overexpressed in the absence of *Pax6* in the determined pituitary gland. At e15, the *PFrk*, *GATA2*, and SF-1 (a gonadotrope-specific marker) expression levels are increased in the absence of *Pax6*.

factors exemplified by the expression of *PFrk* and *GATA2* at e15.5 (Fig. 3). There was also an increase in the *Pit-1* expressing cells, which led to the prediction that there would be an increase in the thyrotrope population, because it requires both *Pitx1* and *GATA2* for determination (9).

Absence of *Pax6* Leads to an Increase in Ventral Cell Types and Decrease in Somatotropes/Lactotropes. Examination of the appearance of fully differentiated cell types revealed an increase in cells with the most ventral phenotypes. Thus, there was an increase rostrally in expression of both *αGSU* and *TSHβ*, (Fig. 4*A*). Ultimately, GH was ventrally diminished (Fig. 4 *A* and *B*). However, *POMC*-expressing cells, the most dorsal cell type, remained equivalent in wild-type and *Pax6* mutant mice (Fig. 4 *A* and *B*). The same results were obtained when expressing protein levels of αGSU and TSHβ, both of which were expanded in the mutant pituitary gland at postnatal day 0 and which were accompanied by the harbored down-regulation of GH and PRL (Fig. 4*B*). Similar observations for GH and PRL immunoreactivity have been described in another *Pax6* point mutation, *Pax6*neu (27). LHβ-expressing cells are not detected, consistent with the published effects of *Pax6* on gonadotropin-releasing hormone neurons (25), but the *SF-1* expression presumptive gonadotropes also increase expression in *Pax6* gene-deleted mice, suggesting an expansion of determined gonadotropes incapable of terminal differentiation based on the absence of the gonadotropin-releasing hormone (25). Together, these results show that the absence of *Pax6* results in an increased thyrotrope population but decreased numbers of lactotropes and somatotropes derived from the *Pit-1* lineage. Thus, there may be a dorsal extension of ventral cell type determination of *Pax6* expression in response to *Shh* ventrally. There is no evidence that *Pax6* can affect the BMP signaling response directly (7). Thus, we hypothesize that *Pax6* serves as a transcriptional regulator that opposes the effects of ventral signaling molecules, critical in causing determination of gonadotrope and thyrotrope cell determina-
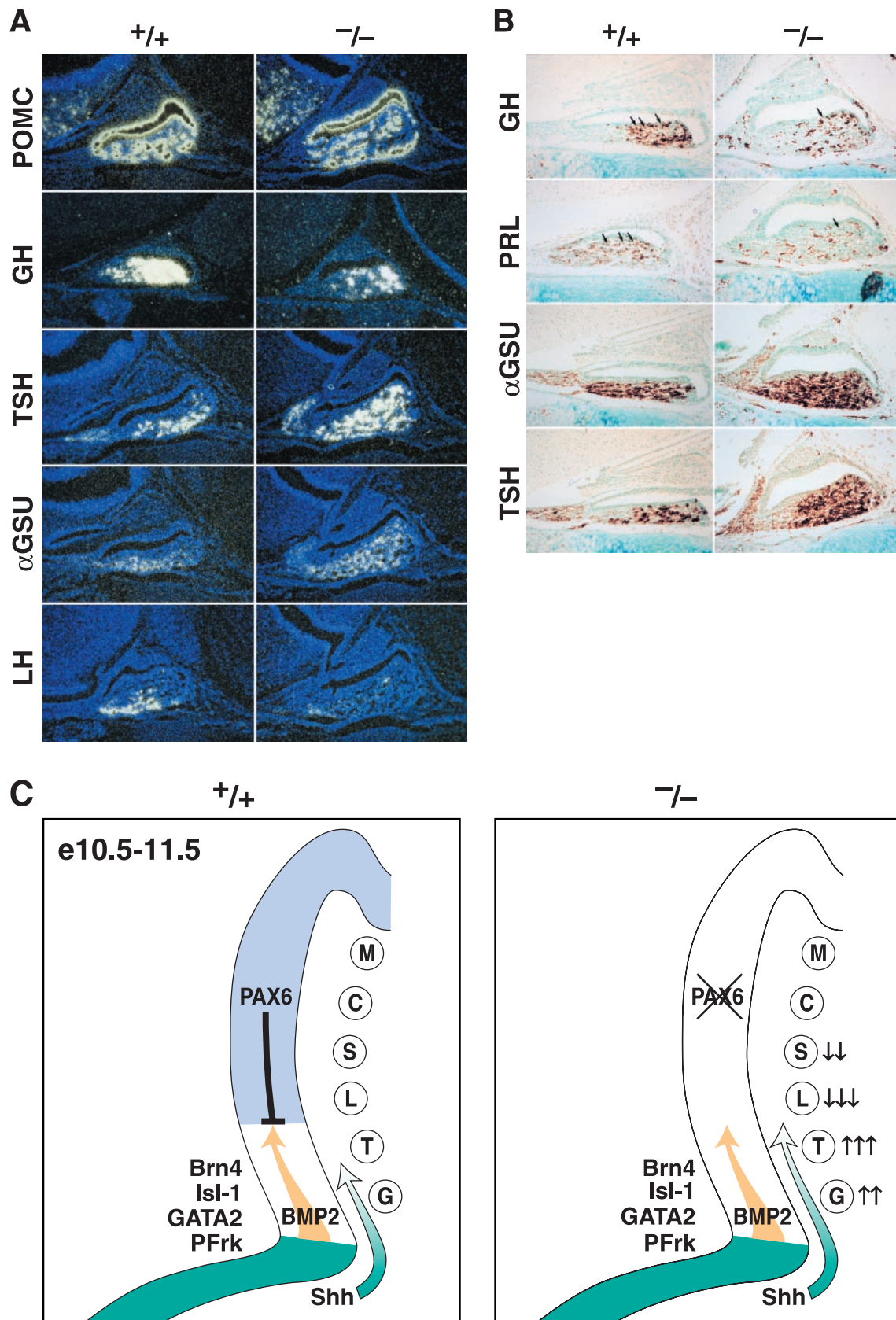
Fig. 4. Ventral pituitary cell types are increased in the absence of *Pax6*. (*A*) Newborn *Pax6* mutant mice are characterized by increased GH, TSH, and αGSU as well as by down-regulation of LH but not by a difference in POMC levels. (*B*) Very few dorsal GH- and PRL-positive cells are detected in mutant mice. (*C*) *Pax6* establishes the boundary along the dorsoventral pituitary axis as early as e10 in response to the inhibitory *Shh* gradient and maybe the *BMP2* from the ventral Rathke's pouch and oral ectoderm. In the absence of *Pax6*, there is a dorsal shift of the boundary, with up-regulation of the transcription factors determining the most ventral cell types, the gonadotropes and thyrotropes. M, melanotropes; other abbreviations are defined in the legend to Fig. 1.

tion events, thereby sharpening the delineation of dorsal cells in this differentiation phase of pituitary development.

Discussion

The critical events in the appearance of specific cell phenotypes from a common primordium seem to be the establishment of opposing dorsal → ventral *Fgf* and ventral → dorsal *Shh* and *BMP2* gradients. In turn, these events cause induction of a series of critical transcription factors expressed dorsally or ventrally. In this article, we have provided evidence that there are additional mechanisms to focus the boundaries at which specific cell types will arise. Thus, in early pituitary organogenesis, there is a dorsal expression of *Pax6* possibly induced by dorsal signals, and this expression remains throughout the period of determination of pituitary cell types and is extinguished by e14.5–e16.

Based on analysis of two different *Pax6* gene-deleted or mutant mice (18, 26), the role of *Pax6* seems to be to impose a sharp boundary of attenuation of the ventral signals that dictate thyrotrope and gonadotrope cell lineages. In the absence of *Pax6*, the ventral lineages, particularly thyrotropes, become dorsally extended at the expression of somatotropes and lactotrope cell types.

In the chicken and mouse neural tube, *Pax6* has similar spatial and temporal expression patterns. *Pax6* is expressed by cells at all dorsal → ventral positions, with an extended ventral^{low} → dorsal^{high} gradient, and its expression is extinguished soon after the differentiation of postnitotic motor neurons. *Pax6* controls the ventral progenitor cells, because no *Pax6* can be detected in *Sey/Sey* cellular populations. These ventral progenitor cells respond to *Shh* signal in a graded manner. The ventral^{low} → dorsal^{high} *Pax6* gradient is reconstructed *in vitro* by exposure of neural cells to differential Shh concentrations. Repression of

Pax6 requires high *Shh* concentrations, and *Pax6* expression remains sensitive to ambient *Shh* concentration during neurogenesis (28). In the pituitary gland, the ventral^{high} → dorsal^{low} gradient of *Pax6* might be a combinatorial effect of the graded Shh signal from the oral ectoderm and the hypothalamus. *Sey/Sey* mice also fail to establish boundaries that specify longitudinal domains in the forebrain, such as the hypothalamus–telencephalic transition zone, ventral thalamus, and a number of forebrain nuclei (19).

A defect in the hypothalamus of the *Sey/Sey* mice, which have an abnormally large third ventricle, affected formation of the transition zone between the telencephalic vesicle and hypothalamus (20). In the neural tube, *Pax6* (28) and *Nkx2.2* (29) control the ventral progenitor cell identity with respect in motor neurons or interneuron patterning in response to graded *Shh* signals (28). There is no delay or failure of Rathke's pouch formation in the *Pax6* mutants, because *Pitx1* (Fig. 2) and *Lxh3* (data not shown) are normally expressed in the e12.5 gland. Therefore, the most likely hypothesis is that the transient dorsal expression of *Pax6* in a cell-autonomous fashion permits lower *Shh* concentrations from inducing transcription factor expression to the ventral cell-type programs, thereby exerting a physiological role in creating a sharp boundary for somatotrope/lactotrope vs. thyrotrope/gonadotrope cell-type determination.

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