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Hedgehog signaling is required for pituitary gland development

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SUMMARY

Pituitary gland development serves as an excellent model system in which to study the emergence of distinct cell types from a common primordium in mammalian organogenesis. We have investigated the role of the morphogen Sonic hedgehog (SHH) in outgrowth and differentiation of the pituitary gland using loss- and gain-of-function studies in transgenic mice. *Shh* is expressed throughout the ventral diencephalon and the oral ectoderm, but its expression is subsequently absent from the nascent Rathke's pouch as soon as it becomes morphologically visible, creating a *Shh* boundary within the oral epithelium. We used oral ectoderm/Rathke's pouch-specific 5′ regulatory sequences (*Pitx1*^{HS}) from the bicoid related pituitary homeobox gene (*Pitx1*) to target overexpression of the Hedgehog inhibitor Hip (Huntingtin interacting protein) to block Hedgehog signaling, finding that SHH is required for proliferation of the pituitary gland. In addition, we provide evidence that Hedgehog signaling, acting at the *Shh* boundary within the oral ectoderm, may exert a role in differentiation of ventral cell types (gonadotropes and thyrotropes) by inducing *Bmp2* expression in Rathke's pouch, which subsequently regulates expression of ventral transcription factors, particularly *Gata2*. Furthermore, our data suggest that Hedgehog signaling, together with FGF8/10 signaling, synergizes to regulate expression of the LIM homeobox gene *Lhx3*, which has been proved to be essential for initial pituitary gland formation. Thus, SHH appears to exert effects on both proliferation and cell-type determination in pituitary gland development.

Key words: Pituitary, Organogenesis, Boundary, Sonic hedgehog, Hip, Cell signaling, Mouse

INTRODUCTION

The pituitary gland originates through the interaction of two different ectodermal tissues, the neural and oral ectoderm. The neural ectoderm gives rise to the posterior pituitary, whereas part of the oral ectoderm will develop into the anterior and intermediate pituitary gland containing at least six distinct cell phenotypes (Treier and Rosenfeld, 1996; Sheng and Westphal, 1999; Burrows et al., 1999; Dasen and Rosenfeld, 1999).

Recent work has begun to unravel general mechanisms of pituitary organ induction based on defining the obligatory interactions between the neural and oral ectoderm as a prerequisite for pituitary gland formation. A dual induction from the diencephalon is required for formation of Rathke's pouch, the primordium of the pituitary gland (Ericson et al., 1998; Takuma et al., 1998; Treier et al., 1998). Both a BMP4 signal and FGF activity from the ventral diencephalon are required for the development of a definitive pouch. Two LIM homeobox factors *Lhx3/Lhx4* have been shown to be the earliest molecular pituitary markers expressed within the oral ectoderm and required for formation of a definitive pouch (Sheng et al., 1997). Subsequently a ventrodorsal BMP2 gradient, appearing at a *Shh* boundary (Treier et al., 1998), together with an opposing dorsoventral FGF8 gradient serve to determine ventral/intermediate cell phenotypes (gonadotropes, thyrotropes, somatotropes, lactotropes) and dorsal cell phenotypes (melanotropes and corticotropes), respectively, with attenuation of the BMP2 signal ultimately required for terminal differentiation.

A consequence of these signaling gradients is apparently to establish overlapping expression patterns of several transcription factors in Rathke's pouch, allowing positional determination of pituitary cell types by E10.5-E12.5. Several factors exhibit spatially restricted domains of expression, including *Isl1* (Ericson et al., 1998; Treier et al., 1998), *P-Frk* (Treier et al., 1998), *Brn4* (Sornson et al., 1996; also known as *Pou3F4*), and *Gata2* (Dasen et al., 1999). In the final stages, the pituitary cell types express a series of differentiation markers which appear in distinct temporal and spatial patterns between E14.5-E16.5, including corticotropes, secreting adrenocorticotropin hormone (ACTH); melanotropes, secreting melanocyte-stimulating hormone (MSH); thyrotropes, secreting thyroid-stimulating hormone (TSH); gonadotropes, secreting luteinizing hormone (LH) and follicle-stimulating hormone

(FSH); somatotropes, secreting growth hormone (GH); and lactotropes, secreting prolactin (Prl). In addition, an embryonic cell type produces TSH (rostral tip cells). LH, FSH and TSH are heterodimers sharing a common α-glycoprotein subunit (αGSU) and a specific β-subunit (reviewed by Treier and Rosenfeld, 1996). The well-defined nature of these cell types make the pituitary gland an excellent model system in which to investigate the molecular mechanisms that underlie the appearance of specific cell phenotypes during mammalian organogenesis (Treier and Rosenfeld, 1996; Dasen and Rosenfeld, 1999).

The expression pattern of *Shh* in cells adjacent to Rathke's pouch during its formation led us to speculate whether SHH could exert essential roles in the early phase of pituitary gland formation. Indeed, Hedgehog proteins have been proved to play important roles in the development of various vertebrate and invertebrate tissues (Basler and Struhl, 1994; Johnson and Scott, 1998; Ruiz i Altaba, 1999). Although SHH has been implicated in proliferative aspects of organ development (Bellusci et al 1997; Duprez et al 1998; Pepicelli et al., 1998; St-Jacques et al., 1998; Rowitch et al., 1999; Chiang et al., 1999; Drossopoulou et al., 2000), less is known about how *hedgehog* boundaries in epithelia may contribute to differentiation of cell types in mammalian organs in vivo (Dahmann and Basler, 1999). We have therefore used a transgenic approach utilizing the Hedgehog inhibitor Hip (Hedgehog interacting protein) (Chuang and McMahon, 1999) to study a potential role of Hedgehog signaling during pituitary gland formation. Here, we demonstrate a critical role for Hedgehog signaling in early phases of pituitary organogenesis, both in proliferation and differentiation of the gland.

MATERIALS AND METHODS

Generation of transgenic mice

Transgenic mice were produced as described (Crenshaw et al., 1989). Construction of transgenes using the 14 kb *αGSU* promoter or the 9 kb *Pitx1* promoter in combination with a vector cassette containing a 5′-globin intron and a 3′-polyadenylation signal from the human growth hormone has been described previously (Treier et al., 1998), by generation of transgenic mice expressing *lacZ* or CRE under control of this information, we have demonstrated the temporal and spatial specificity of its expression. The *Pitx1^{HS}-Hip* transgene contains a mouse *Hip* cDNA that encodes for a HIP protein lacking the last 22 amino acid residues involved in membrane anchoring. The *αGSU-Shh* transgene contains the full-length cDNA for *Shh*. The *αGSU-Fgf8* transgene has been described previously (Treier et al., 1998).

The number of transgenic mice obtained at the reported stage and the number showing an abnormal phenotype (respectively in parentheses) are as follows: *Pitx1^{HS}-Hip* E11.5 (12:9), E13.5 (10:8), E17.5 (9:7); *αGSU-Shh*, E17.5 (7:7); *αGSU-Fgf8*, E17.5 (9:7).

In situ hybridization and immunohistochemistry

Tissues were fixed in either 10% formalin for analysis by in situ hybridization or in 10% formaldehyde, 60% ethanol for 2-3 hours for immunohistochemistry. Hybridization with ^{35}S-labeled antisense RNA probes and exposure were all done as previously described (Simmons et al., 1990). In brief, frozen sections (16 mm) were mounted onto Superfrost plus slides. The sections were prehybridized followed by hybridization with ^{35}S-labeled antisense cRNA probes for the respective cDNAs. After hybridization and washing the sections were incubated with RNase A (20 mg/ml) at 37°C for 20 minutes. RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion. Autoradiographic exposure was for 4-7 days. Immunohistochemistry was done on 5-7 mm thick paraffin sections stained by an indirect immunoperoxidase method. Peroxidase activity was visualized with the DAB/metal enhancer (Pierce; Rockford, Ill.).

Isolation and cultivation of Rathke's pouch

Rathke's pouches were dissected from E9.5 mouse embryos with electrolytically sharpened tungsten needles after collagenase (Sigma, type I) treatment. After 30 minutes recovery in Dulbecco's minimum essential medium (DMEM) which contained 10% fetal calf serum, Rathke's pouches were recombined in collagen gel either with control QT6 quail cells or with SHH-producing QT6 cells (a gift from Dr D. Duprez; see Duprez et al., 1998). For recombinant cultures, control QT6 cells and SHH-producing QT6 cells were grown in 35 mm petri dishes until a dense monolayer was formed. Small pieces (approx. 50 μm in diameter) of monolayer were dissected from these cultures with tungsten needles and placed in close proximity to Rathke's pouches in drops of collagen. Collagen gel from collagen type I (Collaborative Research) was prepared according to the manufacturer's protocol. Recombinants were cultivated for 48 hours in serum-free DMEM/F12 medium supplemented with penicillin-streptomycin and N2 (Gibco-BRL). They were fixed in 4% formaldehyde in PBS (30 minutes), washed with PBS overnight and stained with rabbit affinity-purified anti-LHX3 antibody (1:2500 dilution in PBS, 10% fetal calf serum, 0.1% Triton X-100, 16 hours at +4°C). Antibodies against LHX3 were a gift from Dr S. Pfaff. After washing in PBS (4 times, 6 hours) cultures were processed with anti-rabbit HRP-conjugated antibodies (dilution 1:500; Chemicon) for 16 hours at +4°C and washed 4 times with PBS (3 hours). Peroxidase was visualized with amino-ethyl carbasole (Calbiochem) according to the manufacturer's protocol.

RESULTS

A *Pitx1* enhancer with oral ectoderm and Rathke's pouch specificity

PITX1 (Lamonerie et al., 1996; Szeto et al., 1996) is a transcription factor expressed during very early stages in the oral ectoderm and prior to and during Rathke's pouch formation. A 9 kb *Hin*dIII-*Srf*I (*Pitx1^{HS}*) fragment of the upstream region of *Pitx1* directs expression of β-galactosidase (β-gal) to early Rathke's pouch and oral ectoderm. From seven founder transgenics, β-gal expression was detected in four of them. Expression was specific to the oral ectoderm and Rathke's pouch within the head region in all four of them. The other three founders showed no expression of the reporter transgene, presumably due to integration position effects. Fig. 1B shows whole-mount β-gal staining of 10.5 d.p.c. *Pitx1^{HS}-lacZ* founders with transgene expression only in Rathke's pouch and oral ectoderm and in the branchial arch, without evidence of expression elsewhere within the head region, including an absence of expression in the ventral diencephalon.

Expression of *Shh* in the ventral diencephalon and oral ectoderm

Shh is uniformly expressed throughout the oral ectoderm, but its expression is restricted from the nascent Rathke's pouch as soon as it becomes morphologically visible (Fig. 1A1,A2). This creates a *Shh* boundary within the oral epithelium whereby cells that are destined to form Rathke's pouch, the primordium of the anterior pituitary, no longer express *Shh*. In addition, *Shh* can be detected throughout the ventral

diencephalon adjacent to Rathke's pouch. *Shh* expression is subsequently lost within the oral epithelium after E12.0 and within the diencephalon after E14.0 (Fig. 1A3). Furthermore, *patched 1 (Ptc1*; also known as *Ptch)* (Fig. 1A2), the multipass transmembrane protein receptor for SHH and a direct transcriptional target of the Hedgehog pathway is expressed at high levels in all cells of Rathke's pouch suggesting that cells of the nascent pituitary gland are receiving a Hedgehog signal.

Pitx1^{HS}-Hip transgenic embryos display pituitary hypoplasia

To test whether SHH may play a role during pituitary organogenesis, we first analyzed *Shh*$^{-/-}$ mice (St.Jacques et al., 1998). However, in *Shh*$^{-/-}$ mice, we were never able to detect even a rudimentary Rathke's pouch (data not shown). This is not surprising because *Shh*$^{-/-}$ mice exhibit cyclopia and several midline structures of the brain are missing including the *T/Ebp/Nkx2.1* expression domain (Pabst et al., 2000). Therefore, the absence of the pituitary gland in *Shh*$^{-/-}$ embryos could be a direct or indirect effect of SHH action, as it has been shown that the dysmorphogenesis of the ventral diencephalon in *T/Ebp/Nkx2.1* null mice (Kimura et al., 1996) leads to a complete disruption of pituitary gland development. It has been suggested that the observed dysmorphogenesis in *T/Ebp/Nkx2.1* null mice results in a displacement of the *Fgf8* expression domain within the ventral diencephalon, such that it no longer contacts the oral ectoderm (Kimura et al., 1996). Furthermore, in vitro experiments have shown that FGF8 can maintain *Lhx3* expression (Ericson et al., 1998), a LIM homeobox protein essential for pituitary development (Sheng et al., 1996), leading to the suggestion that *Lhx3* may be a direct FGF8 target in vivo. Because of the dysmorphogenesis of the ventral diencephalon in *Shh*$^{-/-}$ mice, we wanted to investigate a possible direct role for Hedgehog signaling in pituitary organogenesis, which precludes any model disrupting ventral diencephalon morphogenesis.

To permit a transgenic approach, we linked the *Pitx1^{HS}* regulatory upstream sequences with the DNA sequence encoding *Hip*. HIP is a type I membrane protein that binds with high affinity to all mammalian Hedgehog family members, and like PTC1, is a general transcriptional target of the Hedgehog signaling pathway (Chuang and McMahon 1999). Ectopic expression studies indicate that HIP acts as an antagonist of Hedgehog signaling, a property it also shares with PTC1. Ectopic expression of HIP in the developing skeleton generates a limb phenotype closely resembling a loss of Indian hedgehog activity (Chuang and McMahon, 1999). Furthermore, ectopic expression of Hip in the developing zebrafish embryo produces an adaxial muscle phenotype that closely resembles that produced by zebrafish mutants lacking Hedgehog signals (M. Hammerschmidt and A. P. M., unpublished data). Indeed, despite the widespread expression of Hip during a period of fish development known to be regulated by many different classes of signals, the only phenotype detected appears to be a loss of Hedgehog signaling. Thus, Hip appears to be a direct and highly specific antagonist of Hedgehog proteins, exhibiting a functional effect on the Hedgehog proteins analogous to that which Noggin exerts against specific members of the BMP family. We utilized a truncated form of HIP, which lacks the transmembrane domain and has been shown to efficiently become secreted into the extracellular space (Chuang and McMahon 1999). This enabled us to use a transgenic founder analysis strategy to determine the consequences of blocking Hedgehog signaling within Rathke's pouch and the oral ectoderm for pituitary organogenesis.

Fig. 1C shows representative examples of the morphological consequences of targeted HIP overexpression within the oral ectoderm, analyzed at various time points during mouse embryonic development. Rathke's pouch has separated from the oral ectoderm by E11.5 during normal development. In contrast, in *Pitx1^{HS}-Hip* transgenic embryos only a rudimentary pouch is visible, resembling the developmental stage equivalent to E9.0. The nine transgenic founders analyzed showed a visible morphological phenotype with varing degrees of organ hypoplasia; a representation of the most severe form is shown (in Fig. 1C). At E17.5, normally the pituitary gland is fully developed containing an anterior and intermediate lobe, as well as the posterior pituitary (Fig. 1C). In contrast, only a cystic rudiment of the pituitary gland can be found in high copy number *Pitx1^{HS}-Hip* transgenic embryos.

These results provide strong evidence that Hedgehog signaling plays an essential role during pituitary organogenesis. In this analysis, we have not disturbed the development of the ventral diencephalon, as occurs in *Shh*$^{-/-}$ embryos. Not only is the ventral diencephalon morphologically intact, but it's normal development is further substantiated by the observation that the expression domain of *Fgf8*, one of the family of signaling factors from the diencephalon required for Rathke's pouch formation, is not altered in these mice (Fig. 2B), in contrast to alterations observed in *T/Ebp/Nkx2.1* null mice. Therefore, the data are most consistent with the conclusion that cells within Rathke's pouch directly receive a hedgehog signal that is pivotal for pituitary gland formation.

Ventral cell type markers in Rathke's pouch are absent in *Pitx1^{HS}-Hip* transgenics

To better understand the failure of pituitary organogenesis in *Pitx1^{HS}-Hip* transgenic embryos, we performed in situ hybridization on the nine transgenic founders obtained at E11.5 with probes for molecular markers previous shown to play essential roles in pituitary gland formation. Although at E11.5 development of Rathke's pouch is severely disturbed in *Pitx1^{HS}-Hip* transgenics, *Lhx3* expression can be readily detected even in the rudimentary pouch that is seen (Fig. 2A7). These data indicate that the induction of *Lhx3* expression through FGF factors is still occurring and further confirms that signals from the ventral diencephalon are not impaired, in contrast to the situation in *Shh*$^{-/-}$ or *T/Ebp/Nkx2.1*$^{-/-}$ mice (Takuma et al., 1998). However, *Prop1* (Sornson et al., 1996), a tissue-specific paired-like homeodomain factor, which is mutated in the Ames mouse and is required for later appearance of the PIT1 lineage, is only minimally expressed, albeit initially detected (Fig. 2A8). *Bmp2* is known to be induced at the *Shh* boundary in a ventral to dorsal gradient opposing the patterning effects of the dorsally expressed FGFs. Performing in situ analysis with a *Bmp2*-specific probe we could not detect *Bmp2* expression in the nascent pouch rudiment in the analyzed *Pitx1^{HS}-Hip* transgenic animals. However, *Bmp2* expression persists in the surrounding ventral mesenchyme, showing that the block of *Bmp2* expression is specific for ectodermal cells of the pouch (Fig. 2A6,A12).

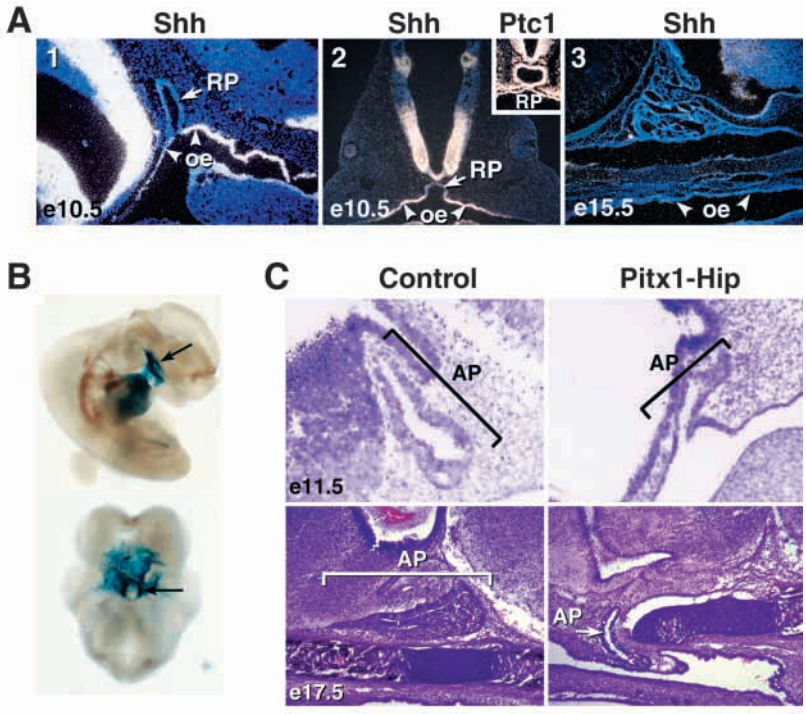

Fig. 1. Expression of *Sonic hedgehog* and morphological consequences of Hip overexpression during early stages of pituitary development. (A) In situ hybridization analysis for *Sonic hedgehog (Shh)* during early stages of pituitary development (embryonic day of mouse development is indicated in the left lower corner for each panel). In sagittal and transverse sections of E10.5 mouse embryos *Shh* expression is observed throughout the oral ectoderm, whereas the invaginating part of oral ectoderm that becomes Rathke's pouch is void of any detectable expression. This creates a SHH boundary within the oral epithelium between two ectodermal domains of *Shh*-expressing and -nonexpressing cells. By E13-E15.5, SHH expression is no longer observed throughout the oral ectoderm. A probe for *patched 1* (*Ptc1*) encoding a multipass membrane protein, which is a direct transcriptional target of Hh signaling is highly expressed throughout Rathke's pouch from E9.5 on. (B) Whole-mount *Pitx1^{HS}-lacZ* embryos at E10.5 stained for β-gal showing expression only in Rathke's pouch and the oral ectoderm within the head region (arrow indicates Rathke's pouch). (C) Hematoxylin-Eosin staining of cryosections from wild-type and *Pitx1^{HS}-Hip* transgenic mouse embryos, shown at the same scale of magnification. In *Pitx1^{HS}-Hip*-misexpressing embryos the gland has not progressed beyond a thin layer of epithelium still connected to the oral cavity at E11.5, long after the normal gland has closed, proliferated and separated from the oral ectoderm. By E17. 5, instead of the nascent gland, in which all component cell types have terminally differentiated, in wild-type embryos only a cyst rudiment is detectable in *Pitx1^{HS}-Hip* transgenic mice. INF, infundibulum; RP, Rathke's pouch; OE, oral ectoderm; AP, anterior pituitary; VD, ventral diencephalon.

Consistent with this result, we observe that *Gata2*, which encodes a zinc finger-containing transcription factor previously shown to be a direct transcriptional target of BMP2 in the pituitary gland (Treier et al., 1998; Dasen et al., 1999), fails to be expressed in Rathke's pouch of all E11.5 *Pitx1^{HS}-Hip* transgenic mouse embryos analyzed (Fig. 2A4,A10). In addition, all other ventral transcription factors, exhibiting expression domains in Rathke's pouch beginning at the ectodermal boundary of *Shh* exclusion, fail to become expressed, as further exemplified by *Brn4* (Sorenson et al., 1996) (Fig. 2A5,A11). In contrast, transcripts of *Isl1* (Ericson et al., 1998: Treier et al 1998), which encode a LIM homeobox transcription factor expressed in the oral ectoderm prior to and during Rathke's pouch formation are not altered (Fig. 2A3,A9). The observation that *Isl1* expression across the *Shh* boundary is unaffected in *Pitx1^{HS}-Hip* transgenics demonstrates that the block of Hedgehog signaling may affect only factors that become expressed de novo at the *hedgehog* boundary after the appearance of Rathke's pouch.

By E13.0, the arrest of pituitary development in *Pitx1^{HS}-Hip* transgenic embryos has become even more apparent (Fig. 3A). In wild-type mice, the developing pituitary gland has undergone a marked increase in organ volume with concomitant expression of *Lhx3* (Fig. 3A1). In contrast, in the most severe cases of the eight transgenic *Pitx1^{HS}-Hip* embryos obtained at E13.0, only a cystic pouch is present, which remains partly connected to the oral ectoderm (Fig. 3A8). Furthermore, *Lhx3* expression has now ceased in the ventral part of the cystic structure and only remains within the dorsal cells, where expression of *Prop1*, a dorsally expressed factor, is now clearly visible (Fig. 3A11). As was the case at E11.5, no expression of ventral factors including *Bmp2*, *Gata2* or *Brn4* is detectable in the rudimentary pouch of any of the transgenic embryos analyzed (Fig. 3A9,A10,A12). This is further underscored by the absence of the first two terminal differentiation markers, POMC and TSHβ, which are normally detectable after E12-E12.5 (Fig. 3A6,A13,A7,A14) during normal pituitary development (Japon et al., 1994). These results demonstrate that Hedgehog signaling is required for both proliferation and appearance of ventral cell type markers in pituitary organogenesis in vivo.

Pitx1^{HS}-Hip transgenic mice display a pituitary phenotype similar to that of mice with a targeted deletion of *Lhx3* (Sheng and Westphal 1996), and exhibit a delayed ventral exclusion of *Lhx3* expression. This prompted us to speculate that *Lhx3* expression may not only be regulated by FGF signaling, but may in addition be controlled by Hedgehog signaling. To test whether SHH is able to regulate *Lhx3* expression, we cocultured Rathke's pouch explants with SHH-expressing QT6 cells (Duprez et al., 1998). Only when the pouch explants were cocultured with SHH-expressing QT6 cells could we see a strong induction/maintenance of LHX3 immunoreactivity, whereas explants cocultured with control QT6 cells only showed barely detectable LHX3 levels (Fig. 3B). This result suggests that Hedgehog signaling, directly or indirectly, modulate, LHX3 expression levels. The discrete expression of *Lhx3* in only that part of the oral ectoderm that is destined to become Rathke's pouch and subsequent expression in the developing gland therefore appears to result from a combinatorial regulation by two signaling molecules, FGF8 and SHH.

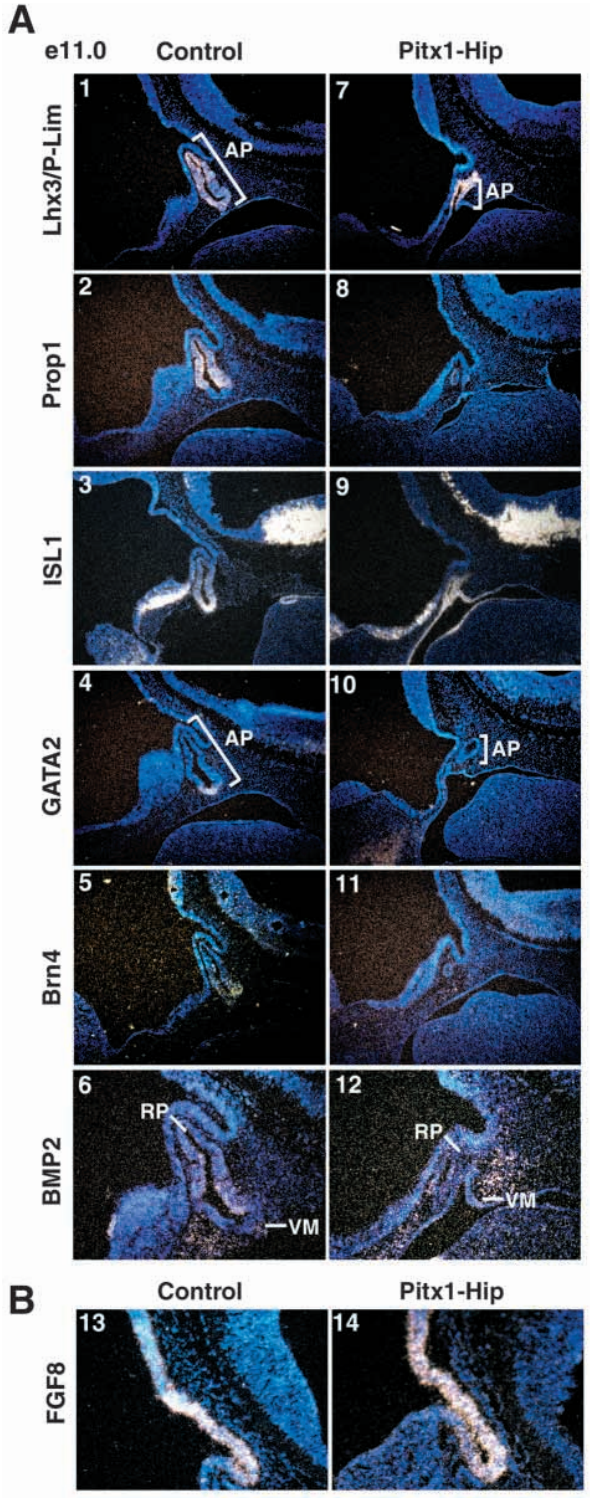

Fig. 2. Loss of ventral anterior pituitary gland patterning in *Pitx1*^{HS}*-Hip* transgenic embryos. (A) Pituitary phenotypes of E11.5 mice expressing the *Pitx1*^{HS}*-Hip* transgene. Pituitaries of E11.5 *Pitx1*^{HS}*-Hip* transgenic embryos (A7-12) and their wild-type littermate (A1-6) are shown. In situ analysis for *Lhx3/P-LIM*, *Prop-1*, *Isl1*, *Gata2* and *Brn4*, *Bmp2*, are shown, with failure to detect expression of *Bmp2* (A12), *Gata2* (A10) or *Brn4* (A11) in the hypomorphic *Pitx1*^{HS}*-Hip* pituitary pouch. In contrast, expression of *Isl1* can be still detected (A9). (B) FGF8 expression within the ventral diencephalon is not affected in *Pitx1*^{HS}*-Hip* embryos.

Ventral cell types are expanded in *αGSU-Shh* transgenics

To complement the in vivo Hedgehog loss-of-function studies with gain-of-function studies, we again utilized an in vivo transgenic approach to express the full-length coding region of *Shh* linked to the regulatory sequences of *αGSU*, which targets expression into Rathke's pouch (Fig. 4A and Kendall et al., 1994; Treier et al., 1998). Comparison of pituitary glands from wild-type and *αGSU-Shh* transgenic mouse embryos at E17.5 revealed a striking threefold increase in organ volume and cell number within the pituitary gland of *αGSU-Shh* transgenic embryos (Fig. 4B). *Lhx3* expression seems slightly increased in the ventral part of the gland, further supporting the model that this domain of *Lhx3* expression is under SHH control. Most strikingly, *Bmp2* transcripts, which are normally no longer detectable in the wild-type gland at this stage, are clearly present in lateral parts of the gland where *αGSU* regulatory sequences drive the highest expression of the transgene (Fig. 4B2,B9). These data suggest that pituitary cells can respond to SHH activity with upregulated or sustained *Bmp2* transcription. The increase in organ size is mainly due to an increase of the αGSU-expressing cell population, consisting of thyrotropes, producing TSHβ, and gonadotropes, producing LHβ (Fig. 4B3,B10,B6,B13,B7,B14). The field of both terminal differentiation markers is broadly expanded, with premature onset of leutinising hormone β gene expression. Whereas corticotrope cell number and distribution appears unchanged (Fig. 4B5,B12), the diminished field of growth hormone-expressing cells is pushed more dorsally (Fig. 4B15,B16). This result demonstrates that SHH has, at least, a positive effect on gonadotrope and thyrotrope cell type proliferation and determination.

FGF8 is sufficient to induce *Lhx3* expression in the oral ectoderm

Recent experiments have shown that *Lhx3* expression can be maintained in Rathke's pouch explants by addition of FGF8 (Ericson et al., 1998). Furthermore, mice lacking the IIIb form of fibroblast growth factor receptor 2 have severe defects in pituitary organogenesis, demonstrating that a FGF activity is required for outgrowth of the pituitary gland (De Moerlooze et al., 2000). We and others (Crossley and Martin, 1995) have shown earlier that *FGF8* is expressed in a correct spatial and temporal pattern in the infundibulum to be a good candidate for this activity. Nevertheless, FGF8 does not bind with high affinity to the IIIb form of fibroblast growth factor receptor 2, although it has been suggested that accessory molecules might differentially modify the relative activity. In contrast, FGF10 does bind to FGFR2b, and has been reported to be expressed at later stages (E14) in the rat infundibulum (Yamasaki et al., 1996). Therefore, we compared directly the spatial and temporal expression profiles of three FGF family members in the infundibulum: *Fgf8*, *Fgf10* and *Fgf18*. All three genes are already expressed by approx. E9.0 in the ventral diencephalon, opposite the region of the oral ectoderm from which Rathke's pouch will morphologically appear and in which expression of *Lhx3* is first observed (Fig. 5A). Therefore, *Fgf10* most likely constitutes the most critical endogenous mitogenic activity for pituitary progenitor cells signaling through FGFR2b.

This prompted us to investigate whether FGFs, including FGF8, indeed do regulate *Lhx3* in vivo expression as suggested

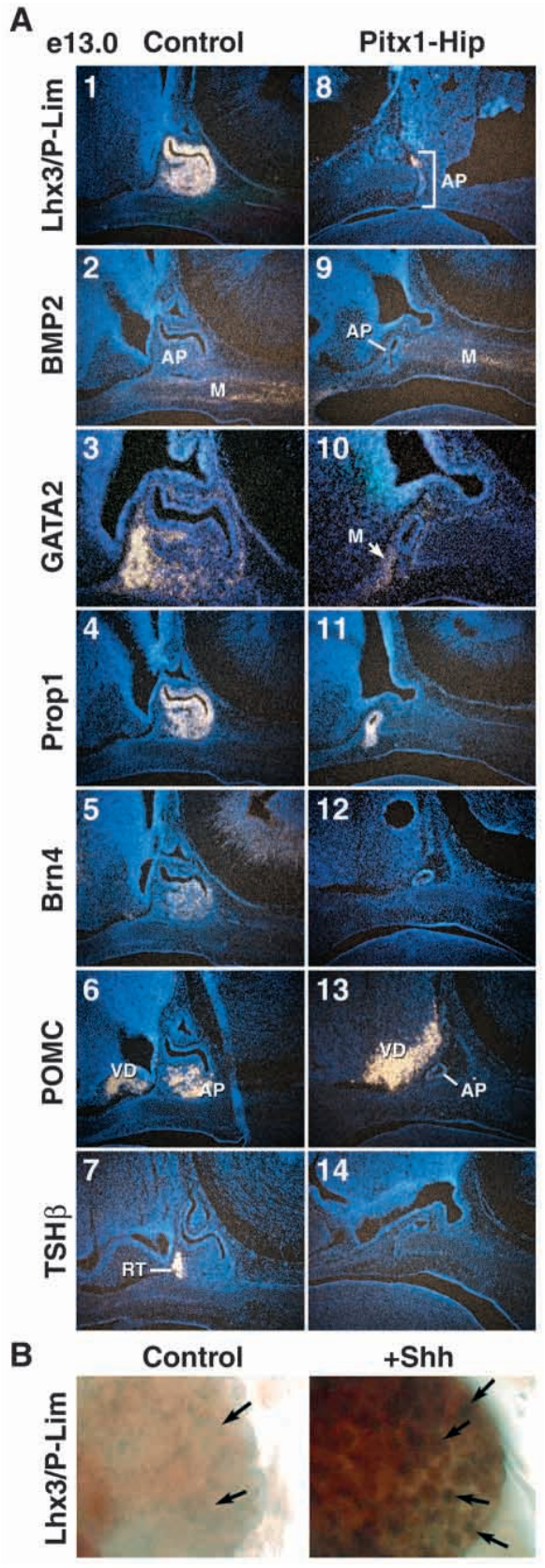

Fig. 3. Pituitary phenotype of E13.0 mice expressing the *Pitx1^{HS}-Hip* transgene. (A) Analysis of pituitaries in wild-type (A1-7) and of *Pitx1^{HS}-Hip* (A8-14) littermates. In situ analysis for *Lhx3/P-LIM*, *Bmp2*, *Gata2*, *Prop1*, *Brn4*, *Pomc* and *Tshβ*.
(B) Induction/maintenance of LHX3 expression (arrow) by SHH signaling. E9.5 Rathke's pouch explants cultured in the presence of QT6 cells showed negligible levels of LHX3 expression (left) as revealed by immunohistochemistry, while E9.5 Rathke's pouch explants cocultured with QT6 cells secreting SHH exhibited strong LHX3 expression. Results are from four independent experiments.

through in vitro experiments. Using a transgenic approach to express *Fgf8* under the αGSU promoter reveals that Lhx3 expression is ectopically activated in the oral ectoderm, driving multiple invaginations within the oral ectoderm. Fig. 5B,C shows in situ hybridization with a *Lhx3*-specific probe. *Lhx3* expression in the wild-type gland is restricted from initial invagination to Rathke's pouch, with no expression visible within the oral ectoderm (Fig. 5B). Overexpression of *FGF8* within Rathke's pouch however leads to a dysmorphogenic gland (Treier et al., 1998). When we analyze the invaginated structures using a *Lhx3*-specific hybridization probe, we find that the multiple invaginations that have formed small pouch cavities or long open cysts are still expressing high levels of *Lhx3* transcripts at a time when *Lhx3* levels are becoming undetectable in the wild-type gland. More surprisingly, when we analyze more lateral sections within the same embryo, where the oral ectoderm exhibits no morphological alteration, we also find strong *Lhx3* expression within the oral ectoderm (Fig. 5C). These results indicate that FGF8 is indeed sufficient to activate *Lhx3* expression in the mouse oral ectoderm in vivo.

DISCUSSION

Mutations in the human *sonic hedgehog* gene (Odent et al., 1999), the mouse *Gli1* and *Gli2* genes (Park et al., 2000) as well as the *you-too* mutation in zebrafish affecting a Gli2 related transcription factor (Karlstrom et al., 1999), together with our described expression profile of *Shh* during the appearance of Rathke's pouch (Treier et al., 1998), suggested that Hedgehog signaling may play an instrumental role in pituitary organogenesis. We analyzed the significance of Hedgehog signaling during pituitary gland development by using a *Pitx1^{HS}* enhancer to overexpress Hip, a hedgehog inhibitor within the oral ectoderm and Rathke's pouch. This approach allowed us to avoid disturbing the development of the ventral diencephalon as shown for *Shh$^{-/-}$* embryos (Pabst et al., 2000) and *Gli1/Gli2* double mutant embryos (Park et al., 2000). This is crucial as the ventral diencephalon expresses *Fgf8*, *Fgf10*, *Fgf18*, as well as *Bmp4* in addition to *Shh*, which have been shown to provide crucial instructive signals for pituitary organogenesis. *Pitx1^{HS}-Hip* transgenic embryos display pituitary hypoplasia and show a loss of induction of ventral transcription factors expressed at the *Shh* boundary.

The observed proliferation defect may be due to the combined block of Hedgehog signaling from both the ventral diencephalon and the oral ectoderm. Further, we provide evidence that *Lhx3* is under dual control of FGF and SHH signaling. *Lhx3$^{-/-}$* knock out mice display a phenotype similar to *Pitx1^{HS}-Hip* transgenics suggesting that the maintenance of high *Lhx3* expression levels is crucial for proliferation of pituitary progenitor cells, and ultimately requires a synergistic signaling input from both FGF and SHH, respectively.

Thus, in the pituitary gland, as in other organs, proliferation

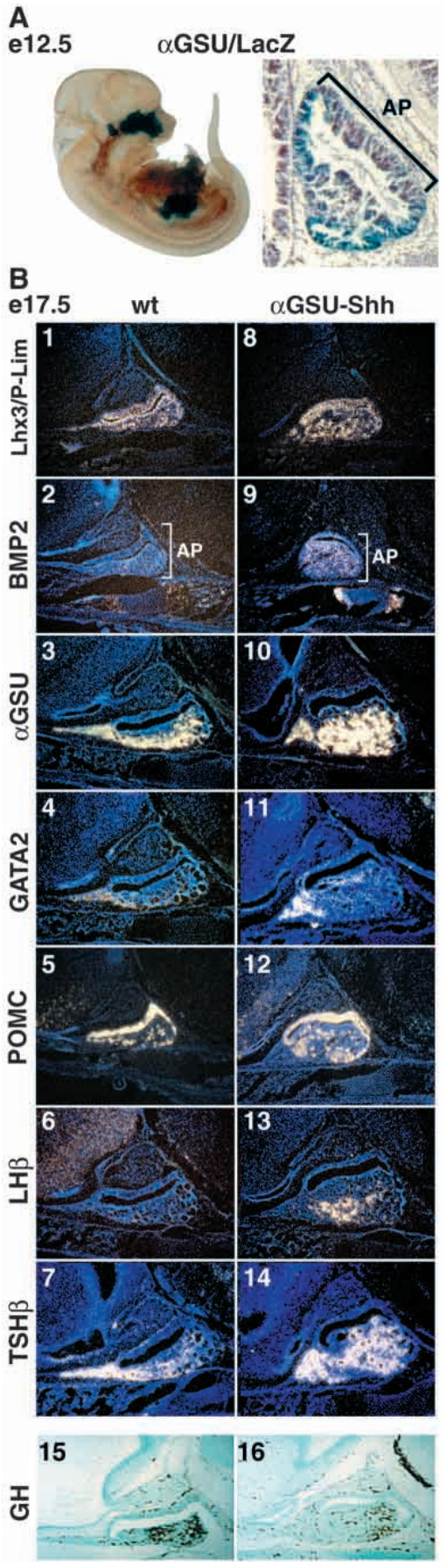

Fig. 4. Overexpression of SHH leads to ventral pituitary cell type expansion. (A) Whole-mount stained embryos at E12.5 carrying a *αGSU-CRE* transgene and the ROSA26 conditional reporter showing only expression of β-gal throughout Rathke's pouch and within part of the oral mesenchyme in the head region. (B) Phenotypic appearance of the pituitary gland in *αGSU-Shh* transgenic embryos at E17.5, with expression profile in an E17.5 transgenic embryo (B8-B14) and its wild-type littermate (B1-B7). In situ analysis was performed with the following markers: *Lhx3, Bmp2, αGSU, Gata2, Pomc, Lhβ* and *Tshβ.* Immunohistochemical analysis for the trophic hormone GH is also shown (B15,B16). Seven transgenic embryos were analyzed, all showing the same phenotype.

and terminal differentiation are closely linked. We have, however, been able to show that factors with expression domains initiated at the *hedgehog* boundary and that determine ventral cell types in Rathke's pouch are selectively affected by the *Pitx1*^{HS}*-Hip* transgene. We postulate that this is most likely due to a failure of *Bmp2* induction within Rathke's pouch, indicating that the *Shh* boundary within the oral epithelium is instrumental in the induction of ventral factors expressed in Rathke's pouch. This scenario is reminiscent of the compartment boundaries organizing anteroposterior patterning in the *Drosophila* wing (Zecca et al., 1995; Lawrence and Struhl, 1996). In that case, posterior cells organize growth and patterning in both compartments by secreting Hedgehog protein that then induces a stripe of neighboring anterior cells across the compartment boundary to secrete Decapentaplegic. In the case of pituitary development, we would suggest that SHH induces *Bmp2* expression in ventral Rathke's pouch cells, with BMP2 serving as the critical signal for at least *Gata2* gene expression. We favor this interpretation of our results, because a FGF factor expressed within the anterior pituitary that is required for pituitary development has not yet been identified, although in other organs and in the vertebrate limb (Sun et al., 2000; Drossopoulou et al., 2000) both SHH/BMP and SHH/FGF signaling relays exist.

The suggestion that SHH plays a role both in proliferation and in cell-type determination of the pituitary gland is further supported by *αGSU-Shh* overexpression studies that result in an induction of *Bmp2* and an expansion of ventral cell types producing LHβ and TSHβ. This phenotype mimics the pituitary phenotype we have previously reported for mice harboring a mutation in the coding sequence of the transcription factor *Pax6* (Kioussi et al., 1999). Together, this situation is reminiscent of results obtained in the ventral mouse neural tube (Ericson et al., 1997; Briscoe et al., 1999). In the ventral tube of *Pax6* mutant mice, progenitor cells missing PAX6 generate neurons characteristic of cells normally exposed to greater SHH activity. This raises the possibility that SHH at the boundary may even have polarizing activity and specific pituitary cell types may likewise be determined from a progenitor cell population by responding to graded SHH/BMP signaling leading to a ventral to dorsal cell fate transformation in *αGSU-Shh* transgenics. Two additional observations support this view. First, we do not see growth hormone-positive cells intermingled with thyrotropes and gonadotropes in the most rostral part of the gland as in the wild-type gland, where *Shh* expression is highest. Second, the overall number of growth-hormone-positive cells is relatively reduced, suggesting that more pituitary progenitor cells may have adopted a ventral fate.

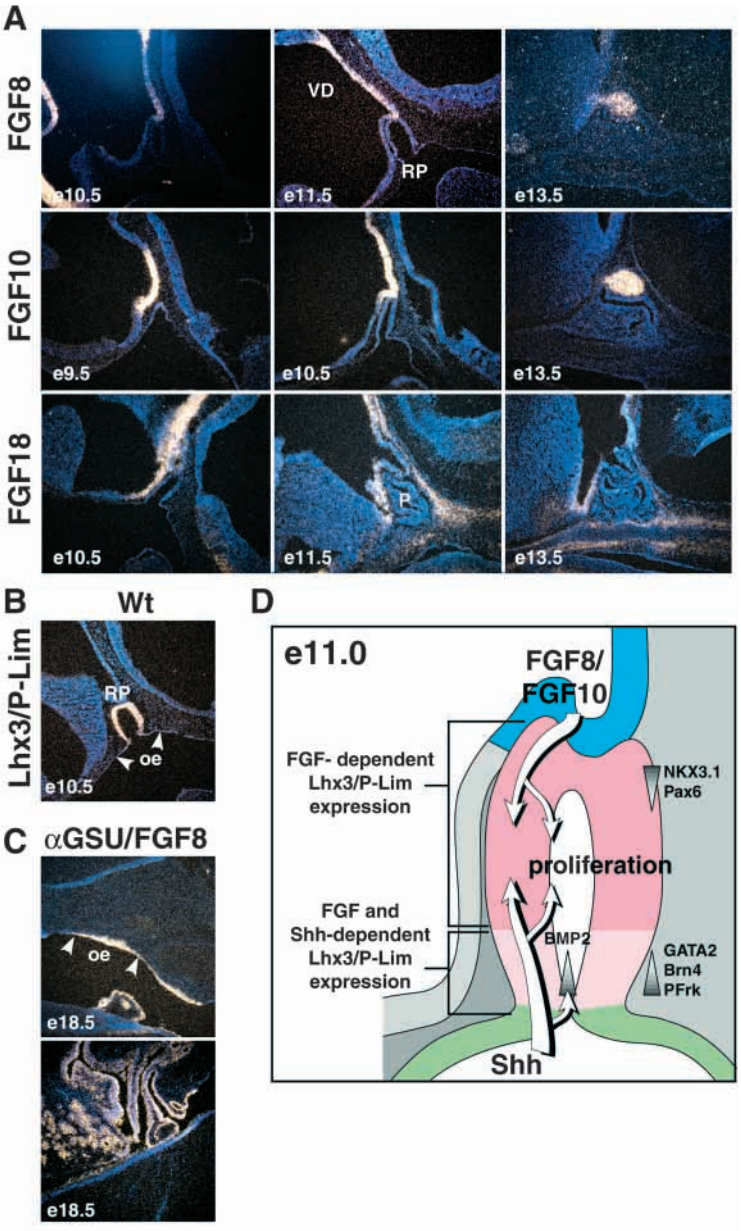

Fig. 5. Role of FGFs and SHH in the regulation of *Lhx3*. (A) In situ hybridization analysis for *Fgf8, Fgf10* and *Fgf18* expression within the diencephalon during mouse embryonic development. A, anterior lobe; I, intermediate lobe; RP, Rathke's pouch; P, pituitary; VD, ventral diencephalon. (B,C) Modulation of *Lhx3* expression by FGF8. (B) In wild-type embryos *Lhx3* is only expressed from early on in cells destined to become Rathke's pouch but is never seen within the remaining oral ectoderm. (C) Ectopic expression of FGF8 in *αGSU-Fgf8* transgenic embryos is sufficient to induce *Lhx3* in the surrounding oral ectoderm, which drives the invagination of the oral ectoderm, resulting in striking dysmorphogenesis and new clusters of lumenal-like cells that bud off as seen in the midsagittal section of an E18.5 *αGSU-Fgf8* embryo analyzed with a *Lhx3* in situ probe (lower panel); the upper panel is a more lateral section of the same embryo. oe, oral ectoderm. (D) Model of Rathke's pouch patterning at E11.0. The boundary between the oral ectoderm expressing *Shh* and the nonexpressing region forming Rathke's pouch functions as an organizing center for ventral gene induction, including *Bmp2* directing patterning and proliferation of the gland. FGF8/FGF10 expressed in the infundibulum functions antagonistically to SHH/BMP2. These ventrodorsal SHH/BMP and dorsoventral FGF activity gradients within Rathke's pouch lead to the induction of several temporally and spatially restricted transcription factors, several of which are listed on the right, that are postulated to combinatorially divide Rathke's pouch into zones with different identities. These zones are proposed to impose the determination of cell lineages at this developmental stage.

While there seems to be no striking upregulation of *Gata2* in *αGSU-Shh* transgenic pituitaries, which may be due to the fact that *Bmp2* levels are much less elevated compared to those in transgenic mice overexpressing *Bmp2* (Dasen et al., 1999), we still observe a similar phenotypic consequence – an expansion of ventral cell types (gonadotropes and thyrotropes).

Thus, exploring the role of SHH in pituitary organogenesis has further elucidated the early complementary and reciprocal signaling events involved in pituitary development, suggesting a model (Fig. 5D) where the interplay of several classes of signaling molecules including SHH, BMP4 and FGF8/FGF10, is responsible for the induction and patterning of a mammalian organ. It appears that the exclusion of *Shh* from the primordium of organs that arise by budding morphogenesis may be a common, but clearly not universal, strategy (Hebrok et al., 1998; Pepicelli et al., 1998) through which organ size later becomes determined; in addition, creating a *Shh* boundary can direct initial organ patterning (Hogan, 1999; Kim and Melton, 1998). It is tempting to speculate that one critical role of BMP4, expressed in the ventral diencephalon, is to suppress *Shh* expression in the adjacent region of the oral ectoderm destined to become Rathke's pouch, analogous to the role of BMP4 on *Shh* gene regulation recently described in tooth development (Zhang et al., 2000). The cells in the invaginating oral ectoderm, which no longer express *Shh*, are able to respond appropriately to FGF8, FGF10, and SHH signals by expressing the LIM homeobox gene *Lhx3*, which we have shown is under dual control of FGF8 and SHH signaling. Therefore, *Lhx3* may provide the positional cue that governs the fate of oral ectodermal cells to become Rathke's pouch and, in combination with other transcription factors, may direct proliferation and the expression of terminal markers within the pituitary gland.

In summary, we have shown that SHH exerts effects on both proliferation and cell type determination during pituitary gland development analogous to the role of IHH in bone

development. Whether SHH, in addition, displays polarizing activity in the patterning of pituitary progenitor cells in Rathke's pouch remains to be determined.

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