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Direct and indirect requirements of Shh/Gli signaling in early pituitary development

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Abstract

Induction of early pituitary progenitors is achieved through combined activities of signals from adjacent embryonic tissues. Previous studies have identified a requirement for oral ectoderm derived Sonic Hedgehog (Shh) in specification and/or proliferation of early pituitary progenitors, however how different *Gli* genes mediate Shh signaling to control pituitary progenitor development has not yet been determined. Here we show that *Gli2*, which encodes a major Gli activator, is required for proliferation of specific groups of pituitary progenitors but not for initial dorsoventral patterning. We further show that the action of Gli2 occurs prior to the closure of Rathke's pouch. Lastly, we show that Shh/Gli2 signaling controls the diencephalic expression of *Bone morphogenetic protein 4 (Bmp4)* and *Fibroblast growth factor 8 (Fgf8)*, two genes that are known to play critical roles in patterning and growth of Rathke's pouch. Our results therefore suggest both cell-autonomous and non-cell autonomous requirements for *Gli2* in regulation of pituitary progenitor specification, proliferation and differentiation.

Keywords

Gli1; Gli2; Gli3; Shh; mouse; pituitary; patterning; proliferation

INTRODUCTION

The pituitary gland is a master endocrine organ that produces a number of hormones regulating many essential physiological functions in the body. It is comprised of the adenohypophysis (anterior and intermediate lobes) and neurohypophysis (posterior lobe). The anterior lobe contains five different hormone-producing cell types that secrete adrenocorticotrophic hormone (ACTH), growth hormone (GH), prolactin (PRL), thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) while the posterior lobe contains nerve endings that secrete oxytocin and vasopressin

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(Reviewed in Camper et al., 2002; Zhu et al., 2007). Both hypopituitarism and hyperpituitarism have been identified in a number of human congenital pituitary disorders (Dattani et al., 1998; Pfaffle et al., 1992; Roessler et al., 2003; Thomas et al., 2001; Wu et al., 1998).

Development of the pituitary gland requires extensive interactions between different embryonic tissues. Between ~E8.5 and E9.0, a portion of the oral ectoderm thickens and invaginates to form a structure called Rathke's pouch (RP). Several lines of evidence suggest that induction of RP requires signals derived from the diencephalon. First, mutation of *Tebp* (*Nkx2.1*), which is expressed in the ventral diencephalon but not in RP, causes apoptosis and degeneration of nascent RP (Takuma et al., 1998). Second, mutation of *Bmp4*, which is expressed in the ventral diencephalon, causes a loss of early RP (Takuma et al., 1998; Winnier et al., 1995). Similar phenotypes were also observed in embryos over-expressing *Noggin*, a BMP antagonist, in the oral ectoderm (Treier et al., 1998). On the other hand, mutation of *Noggin* results in expansion of pituitary tissues (Davis and Camper, 2007). Lastly, analysis of embryos mutant for *Fgfr2*, which encodes a receptor for FGF1/3/7/10 and is expressed in the diencephalon, revealed a requirement for FGF signaling in the diencephalon (De Moerlooze et al., 2000). In both *Fgfr2* and *Fgf10* mutants, increased apoptosis causes severe defects in infundibulum and RP development (De Moerlooze et al., 2000; Ohuchi et al., 2000). By ~E11, RP closes off from the oral ectoderm and by ~E12, RP detaches from the oral ectoderm. Interestingly, the critical period for pituitary progenitor specification appears to be between ~E10 and ~E12/13. Nascent RP isolated from E10.5 mouse embryos will express early markers of pituitary pouch *Isl1* but not *Lhx3* (*Lim1*) when cultured in vitro. When combined with ventral diencephalic tissues, nascent RP can be induced to express *Lhx3* and other pituitary markers (Ericson et al., 1998). The action of diencephalic tissues can be recapitulated by beads soaked with FGF8, suggesting at least that FGF8 secreted from the diencephalon is critical in specifying different pituitary cell types. However, by E11.5, the pituitary explants become refractory to FGF8 induction (Ericson et al., 1998). By E12/13 (E13/15 in rat embryos), isolated RP can differentiate into different pituitary cell types even when cultured alone, suggesting that pituitary progenitors have already been specified when RP detaches from the underlying oral ectoderm (Nemeskery et al., 1976).

In addition to dorsal diencephalic signals, *Shh* is expressed in the oral ectoderm immediately adjacent to RP (Treier et al., 1998). The requirement of *Shh* signaling in pituitary development is supported by the following experiments. Over-expression of *Shh* in the developing RP using an α *GSU* promoter, which drives ectopic expression of *Shh* in RP starting from ~E12.5, results in an expansion of ventral pituitary cell markers *TSH* and *LH* and causes pituitary hyperplasia (Treier et al., 2001). On the other hand, blocking *Shh* signaling through expression of a *Shh* antagonist, Hedgehog-interacting protein (*Hhip*), using the *Pitx1* promoter causes pituitary hypoplasia (Treier et al., 2001). However, interpretation of some of these results is complicated by the fact that manipulations of secreted signals can affect multiple tissues. In addition to the oral ectoderm, *Shh* is also abundantly expressed in the ventral diencephalon, immediately above the early RP. As a result, RP may be exposed to *Shh* derived from both oral ectoderm and ventral diencephalon, and the above experiments might have affected oral ectoderm and diencephalon development in addition to development of RP. Preliminary analysis of the pituitary phenotype resulting from a loss of *Gli2*, which encodes the major *Gli* activator, provides some initial insight into the requirement of *Gli* genes. Mutations in *Gli2* result in a variable loss of pituitary, demonstrating a requirement for *Shh*/*Gli2* signaling in pituitary development (Park et al., 2000). However, whether different cell types are generated in the anterior pituitary or whether the development of posterior pituitary is affected has not yet been determined. To address how *Gli* genes mediate *Shh* signaling to control pituitary

specification and proliferation, we analyzed pituitary phenotypes of embryos mutated for individual *Gli* genes, which are required for the transcription output of Shh signaling. We then conditionally disrupted *Gli* genes in RP and found a critical role for Shh signaling before RP detached from the oral ectoderm. Lastly, we found Shh signaling is also required in the diencephalon to control pituitary development through the regulation of two critical growth factors.

MATERIAL AND METHODS

Mouse breeding

Mouse breeding was carried out according to protocols approved by Case Western Reserve University (CWRU) Institutional Animal Care and Use Committee. The *Rosa26-lacZ* Reporter (R) mouse (Soriano, 1999) was used to monitor Cre-mediated recombination. To generate *FoxG1Cre;Gli* conditional mutant embryos, *FoxG1Cre;Gli2^{zfd/+}* male mice were bred to *Gli2^{lox/lox};R/R* or *Gli2^{lox/lox};Gli3^{xt/+};R/R* female mice. To generate embryos with activated Hh signaling in RP, *Pitx2Cre* male mice were bred to *R26SmoM2* female mice. To generate *Gli2* mosaic embryos, *Gli2^{lacZ/+}* mice were bred with *Gli2^{zfd/+}; Rosa26 (R26)-EGFP/R26-EGFP* mice. The resulting morula stage embryos were co-cultured with *CD1* embryos. Embryos that had aggregated after overnight culture were then transferred into pseudo-pregnant female for further development. The genotyping of *Cre*, *Gli2^{lox}*, *Gli2^{lacZ}*, *Gli2^{zfd}* and *Gli3^{xt}* mice has been described (Bai et al., 2002; Bai and Joyner, 2001; Corrales et al., 2004; Jeong et al., 2004; Maynard et al., 2002; Mo et al., 1997).

Histology, immunostaining and RNA *in situ* hybridization

Embryos were dissected in cold PBS and fixed in 4% paraformaldehyde (PFA) for 1-3 hrs at 4°C, washed with PBS, sunk in 30% sucrose and embedded in OCT (Tissue-Tek). Sagittal tissue sections were collected at 12 µm on a Leica Cryostat. 3-6 embryos were analyzed for each embryonic stage. Methods of X-gal staining, antibody staining and RNA *in situ* hybridization have been described (Bai et al., 2002; Bai and Joyner, 2001). The following antibodies were used: Pax6 (1:500, Covance), Isl1 (1:100, Abcam), Lhx3 (1:100, Developmental Studies Hybridoma Bank), BrdU (1:20, Becton-Dickinson), Ki67 (1:500, Abcam), phospho-Histone H3 (1:100, Upstate), cleaved Caspase-3 (1:200, Cell Signaling), ACTH (1:1000), GH (1:1000), LH (1:500), PRL (1:2500) and TSH (1:500, all from National Hormone & Peptide Program) and β-galactosidase (1:500, Biogenesis). Hoechst 33258 (Molecular Probes) was used to visualize nuclear staining. All sections were examined with a Leica DMLB epi-fluorescence microscope fitted with a SPOT camera in the CWRU Genetics Imaging Facility (supported by NIH-NCRR, RR-021228).

BrdU labeling, cell cycle, cell death analysis and quantification

Pregnant mice were injected intraperitoneally with BrdU (Sigma) at 100 µg/g body weight 1 hr prior to dissection. The percentage of BrdU+, Ki67+, pHH3+, Caspase3+ cells was calculated by dividing the number of the positive cells by the total number of nuclei (Hoechst) inside RP or other specified region. To calculate the fraction of S-phase cells within cell cycle, we divided the number of BrdU+ cells by the total number of Ki67+ cells and converted the number to a percentage. For each time point, we used 2 mid-sagittal sections from each embryo and 4-6 embryos from at least two different litters for analysis. As the number of Caspase3+ cells is low in both WT and *Gli2* mutants, we counted 3-4 sections from each embryo (total 1-5 Caspase3+ cells per embryo, with n=3 for *Gli2* mutants and n=6 for WT). The number in charts was displayed as mean ± S.E.M. Student's *t*-test was used to calculate the *P* value and to determine whether the results were significantly different from each other (** indicates *P*<0.01; * indicates *P*<0.05).

Quantification of pituitary cell types at E17.5. To ensure consistent sampling of cell types among different embryos, we performed immunostaining analysis for each marker on two mid-sagittal sections from each embryo. A total of 6 WT embryos, 6 *Gli2*^{-/-} embryos and 4 *FoxG1Cre;Gli2*^{-/-} embryos from at least three litters were used in the analysis.

We used the following method to normalize the contribution of EGFP-labeled cells to RP of mosaic embryos. We first selected an area in the dorsal mid/hindbrain region, which does not have active Shh/Gli signaling, and calculated the percentage of EGFP⁺ cells over the total number of cells. This number was used as a baseline for the level of mosaicism in that embryo. We then calculated the percentage of EGFP⁺ cells within RP or in different regions of RP, and normalized the percentage against the baseline in that embryo. Results from three embryos were then used to determine whether there was a significant difference between the contribution of WT and *Gli2* mutant cells.

RESULTS

Loss of *Gli2* leads to a selective loss of specific pituitary cell types

Previous studies reported a variable loss of the pituitary (3/6) in *Gli2* mutants when examined at E12.5 (Park et al., 2000). To determine how *Gli2* mediates Shh signaling to influence pituitary development, we examined patterning of the pituitary gland and the production of different pituitary cell types in wild type (WT) and mutant embryos.

We first examined the expression of two markers that are normally expressed throughout the E12.5 pituitary pouch, *Lhx3* and *Prop1*. *Lhx3* encodes a LIM-homeodomain transcription factor that is required for specification of pituitary cell lineages (Sheng et al., 1996) and *Prop1* encodes a paired-like homeodomain transcription factor required for initial determination of the *Pit1* (Pou1f1)-expressing lineage of the pituitary (Sornson et al., 1996). In *Gli2* mutants, both *Lhx3* and *Prop1* were found throughout the pituitary gland, suggesting that early pituitary cells were specified normally (Fig. 1A-D). We then examined the expression of markers for different regions of the pituitary pouch. *Gata2* encodes for a ventrally restricted transcription factor that is normally expressed in pituitary cells located at the anterior tip of RP, most of which have just exited cell cycle at E12.5 (Dasen et al., 1999; Treier et al., 1998) (Fig. 1E). In *Gli2* mutants, *Gata2*⁺ cells were found in the anterior tip of the pouch, as was the case in WT embryos (Fig. 1F). We also examined the expression of two other pituitary markers, *Pax6* and *Isl1*. *Pax6* is normally expressed in a dorsal-to-ventral gradient and *Isl1* is restricted to the pituitary tip at E12.5 (Fig. 1G,I). The expression pattern of *Pax6* and *Isl1* in *Gli2* mutants is similar to the pattern seen in WT embryos (Fig. 1H,J). This demonstrates that loss of *Gli2* does not affect the overall patterning of the pituitary.

Although patterning of the *Gli2* mutant pituitary is largely normal, most *Gli2* mutant pituitaries were found to be smaller than WT pituitaries (Fig.1), suggesting defects in cell proliferation and/or excessive cell death in mutant pituitary tissues. To distinguish between these two possibilities, we examined expression of Ki67, a marker for proliferating cells, BrdU incorporation, a marker for cells in S-phase, and phospho-Histone H3 (pHH3), a marker for cells in M-phase. At E12.5, we detected reductions in the percentage of cells expression Ki67 in *Gli2* mutant pituitaries (Fig. 1K,L,Q, $P=0.009$), BrdU⁺ cells (Fig. 1K,L,Q, $P=0.0006$) and cells in M-phase based on pHH3 levels (Fig. 1M,N, $P=0.0123$). To test if excessive cell death contributed, we examined the expression of cleaved-Caspase3 and found no significant difference between mutant and WT pouches (Fig. 1O,P). Together, these data suggest that loss of *Gli2* affects proliferation of pituitary progenitor cells at E12.5.

To determine whether loss of *Gli2* affects the production of different pituitary cell types, we analyzed the expression of cell-type specific markers at E17.5, a stage when all mature cell

types are normally present (Fig. 2A-J). Five hormone-secreting cell types we examined include corticotropes (C), which secrete ACTH; thyrotropes (T), which secrete TSH; gonadotropes (G), which secrete LH; somatotropes (S), which secrete GH; and lactotropes (L), which secrete PRL. We found that all five markers were detected in *Gli2* mutant pituitaries (Fig. 2 A-J). However, when the number of different cell types was counted in *Gli2* mutants, we found a reduction in the number of C, S and L cells (Fig. 2K), suggesting a requirement for *Gli2* for normal generation of these three cell types.

Progenitor proliferation in the nascent pituitary requires cell-autonomous expression of *Gli2*

Several possibilities could account for the proliferation defects in *Gli2* mutant pituitaries. First, *Gli2* is required cell-autonomously in RP to respond to Shh signal derived from the oral ectoderm, as suggested by previous studies (Treier et al., 1998). Second, *Gli2* could be required for cells in RP to respond to diencephalic derived Shh. Third, *Gli2* may be required in the diencephalon for the expression of other growth signals, as diencephalic defects were found in *Gli2* mutants in our previous study (Park et al., 2000). To distinguish these possibilities, we generated *Gli2* mosaic embryos and analyzed mutant cells in an otherwise WT background.

Two different *Gli2* alleles, *Gli2^{zfd}* and *Gli2^{lacZki}* (Bai and Joyner, 2001; Mo et al., 1997), were used to generate *Gli2* mutant embryos. EGFP⁺ embryos were derived by crossing *Gli2^{lacZki/+}* and *Gli2^{zfd/+}*; *EGFP/EGFP* mice. The EGFP-labeled embryos were then aggregated with morula stage, un-labeled *CD1* embryos *in vitro*. After overnight incubation, those embryos that had successfully aggregated were transferred into pseudo-pregnant females and allowed to develop. Embryos were then dissected and genotyped with primers for the two *Gli2* alleles. Those that contained *Gli2^{zfd}*, *Gli2^{lacZki}* and WT *Gli2* alleles were mosaic embryos containing WT and *Gli2* mutant cells while those that contained only *Gli2^{lacZki}* and WT *Gli2* alleles were used as controls. Both control and mosaic embryos appeared to have morphologically normal RP (Fig. 3A-F). To determine whether *Gli2* mutant cells can compete with WT cells and adopt a pituitary cell fate, we stained mosaic pituitary with Lhx3 antibody (Fig. 3A,D). We found that all EGFP⁺ *Gli2* mutant cells expressed Lhx3 in mosaic pituitary examined (Fig. 3B,E, n=3), suggesting that *Gli2* mutant cells can contribute to pituitary and adopt pituitary cell fate in mosaic embryos (Fig. 3C,C',F,F'). However, there were fewer EGFP positive cells in mosaic *Gli2* pouches (Fig. 3E,K,N) than in controls (Fig. 3B,H). To determine whether GFP⁺ mutant cells are less likely to be integrated into mosaic pituitaries, we divided RP into two regions, anterior wall (A) and posterior wall (P), along the pouch lumen (Fig. 3M). When we calculated the percentage of GFP⁺ cells in each division and normalized this number to the level of mosaicism in each embryo (see Materials and Methods), we found mutant cells contributed significantly less to the anterior wall of the pituitary (Fig. 3N, $P_A=0.002$). Interestingly, mutant cells contributed less to the posterior wall as well, although the reduction was not as dramatic (Fig. 3N, $P_P=0.031$). Lastly, mutant cells were also underrepresented in the infundibulum (Fig. 3N, $P_I=0.011$), suggesting a requirement of Shh/*Gli2* signaling in the development of posterior pituitary.

One potential reason why there are fewer mutant cells in the anterior wall of the pouch is that mutant cells do not proliferate as well as WT cells. To determine whether this was the case, we counted the number of EGFP⁺ BrdU⁺ mutant cells and the total number of unlabeled, BrdU⁺ S-phase cells (Fig. 3G-L), and calculated the percentage of mutant cells over the total number of S-phase cells in each region (Fig. 3O). Indeed, we found EGFP⁺ cells were proliferating less in the anterior wall of the pouch (Fig. 3O). There were also fewer proliferating EGFP⁺ cells in the posterior wall, although the reduction was not as drastic as in the anterior wall of the pouch, suggesting that the reason there were fewer *Gli2*

mutant cells inside RP is that these is a cell-autonomous requirement of *Gli2* for proliferation.

Removal of *Gli2* function before the closure of RP causes proliferation defects in some pituitary progenitors

Previous studies have shown that pituitary development is controlled by *Shh* expressed from the oral ectoderm (Treier et al., 1998; Treier et al., 2001). To determine the critical period of Shh/Gli signaling for pituitary proliferation, we conditionally removed *Gli2* function in the pituitary by using a conditional *Gli2* allele and two *Cre* lines, *FoxG1Cre* (Hebert and McConnell, 2000) and *Pitx2Cre* (Liu et al., 2003).

FoxG1 (Forkhead box G1) is initially expressed in the anterior neural ridge and in the early telencephalon (Shimamura and Rubenstein, 1997). To determine whether *FoxG1Cre* can be used to remove *Gli2* function before RP closes, we used *FoxG1Cre* to recombine a *Rosa26-lacZ* reporter (*R*) and monitored the activity of β -galactosidase expressed from the *Rosa26-lacZ* reporter. We found that at E9.5, all cells encompassing the invaginating RP were positive for β -galactosidase (Fig. 4A). The staining of β -galactosidase within all RP cells was confirmed at E10.5 and E12.5 (Fig. 4B,C), suggesting that *FoxG1Cre* can be used to disrupt *Gli2* function before the closure of RP. As *FoxG1* is not expressed in the diencephalon, the manipulation should not affect the normal role of the diencephalon in regulating the growth and patterning of the pituitary.

At E12.5, the pituitaries of conditional *Gli2* mutants (*FoxG1Cre; Gli2^{flox/zfd}*) appeared morphologically normal. Analysis of early pituitary markers, *Lhx3*, *Porpl*, *Gata2*, *Pax6* and *Isl1*, also revealed a pattern of expression resembling that of the WT embryos (Fig. S1A-K), consistent with our results that *Gli2* does not control pituitary patterning (Fig. 1). Nevertheless, we reasoned that if *Gli2* is required to mediate Shh signaling to control progenitor proliferation before the closure of RP, then we would expect proliferation defects in the *FoxG1Cre; Gli2^{flox/zfd}* conditional mutants. To this end, we examined the proliferation of conditional mutant cells at E12.5 by analyzing BrdU incorporation and expression of Ki67 and pHH3. Compared with WT embryos, we found no significant differences in the percentage of cells incorporating of BrdU, or cells expressing Ki67 and pHH3 in conditional *Gli2* mutants (Fig. 4D,G). Furthermore, removal of *Gli2* did not change the pattern of cell death, as there was no significant change in the number of cells expressing Caspase3 (Fig. 4F,I). To determine whether there are regional differences in cell proliferation, we divided the pouch into four regions: anterior (dorsal and ventral, A^d and A^v respectively), rostral tip (rT) and posterior (P) (Fig. 4J). At this stage, most of the rT cells had already exited the cell cycle while the rest of the cells in RP were still proliferating. Interestingly we found a decrease in the percentage of BrdU incorporation in the A^v region of the *FoxG1Cre; Gli2* mutants (Fig. 4K). The reduction in BrdU incorporation appeared to reflect a reduction in proliferation, rather than changes in cell cycle length, as the number of proliferating cells (Ki67+) was also decreased and the ratio of BrdU/Ki67 remained largely unchanged (Fig. 4L).

If proliferation of progenitors in the A^v region was affected, then we would expect to see a reduction in the number of mature pituitary cells generated from this region. We therefore quantified the number of each mature pituitary cell type in *FoxG1Cre; Gli2* mutants at E17.5 (Fig. 5A-J). Consistent with this expectation, we found a smaller number of corticotropes (C) and lactotropes (L) (Fig. 5K), suggesting a requirement of *Gli2* in the proliferation and differentiation of their precursors.

The reduction in the number of pituitary cells could impede normal growth during the early postnatal period. To test whether this is the case, we monitored the growth of

FoxG1Cre;Gli2 mutant pups. Compared with WT pups, mutant pups had a lower body weight (Fig. 5L), and even though mutant pups were born alive, most died within 30 days after birth. The growth defects in *FoxG1Cre;Gli2* mutants likely reflect disruption of pituitary function, although the possibility that it might also result from loss of *Gli2* function in endodermal tissues was not examined.

We also used *Pitx2Cre*, which is active in RP (Liu et al., 2003) slightly later than *FoxG1Cre* to disrupt *Gli2* function. *Pitx2Cre*-mediated recombination of *Rosa26-lacZ* can be detected in a few cells at E9.5 and more cells at E10.5 and E12.5 (Fig. 6A-C). However, when *Pitx2Cre* was used to remove *Gli2* function, we found no obvious defects in patterning or proliferation of mutant pituitaries at E12.5 (data not shown), suggesting that *Gli2* is required before *Pitx2Cre* is active in RP.

Overlapping roles of *Gli* genes in pituitary development

Previous studies have shown that the three *Gli* genes share overlapping and redundant functions (Bai et al., 2004). To address whether *Gli2* is the primary *Gli* transcription factor to mediate *Shh* signaling during pituitary development, we analyzed the pituitary phenotypes of *Gli1* and *Gli3* mutants at E12.5. Both mutants had normal pituitary patterning and proliferation at E12.5 (data not shown), consistent with the notion that *Gli2* is the primary *Gli* transcription factor in the pituitary. We next examined pituitary development in *Gli2/Gli3* double mutants, which do not express *Gli1* and therefore lose all *Gli* function (Bai et al., 2004), and found no pituitary in all double mutants examined (n=4). One potential reason for the complete loss of pituitary in *Gli2/Gli3* double mutants is that *Gli* mediated *Shh* signaling is required in the diencephalon for the initial formation of RP. To address this issue, we generated conditional mutant embryos that lost all *Gli* function specifically in the pituitary using *FoxG1Cre* (*FoxG1Cre; Gli2^{flox/zfd}; Gli3^{xt/xt}*). At E12.5, the pituitary gland in these conditional mutant embryos was smaller. However, when pituitary specific cell markers were used to examine the initial patterning, we found that *Lhx3* is expressed normally throughout the pituitary gland (data not shown). In addition, *Isl1* showed typical staining in the rostral tip and *Pax6* appeared to express in a dorsal-to-ventral pattern, similar to WT embryos at this stage (Fig. S1K,L). These results confirm that early pituitary patterning is not affected by loss of *Gli* function, and that excessive production of *Gli* repressor in *pitx1-Hhip* transgenic embryos, as in *Shh* mutants, may be responsible for the severe pituitary phenotypes.

Activation of *Hh* signaling in RP increases pituitary proliferation without affecting patterning

If disruption of *Gli* function affects pituitary cell proliferation, then we expect that activation of *Shh* signaling increase pituitary proliferation. To test this, we activated Hedgehog (*Hh*) signaling using *Pitx2Cre* and a conditional allele encoding an active form of *Smo* inserted in the *Rosa26* locus (*R26SmoM2*) (Jeong et al., 2004). At E12.5, *Pitx2Cre; R26SmoM2* mutant pouch appeared similar to wild type pouches, as *Lhx3*, *Prop1*, *Gata2* and *Isl1* were expressed normally (Fig. 6D-K). The expression pattern of these markers remained unchanged even when examined at E14.5 (Fig. 6O-V), suggesting that activation of *Hh* signaling using *Pitx2Cre* does not alter the general patterning of the pituitary.

We next examined markers for cell proliferation in mutant pituitary pouches. Although there were no significant differences in the number of *Ki67+* and *pHH3+* cells in the whole pituitary pouch (data not shown), the number of proliferating cells (*BrdU+* and *BrdU/Ki67*) was significantly increased in the *A^d* region of the pouch (Fig. 6L,M,N). The observation that cells in the dorsal anterior region over proliferate in response to *Shh* signaling suggests that these cells normally do not receive saturating amounts of *Shh*. The phenotype became

more prominent at E14.5, as *Pitx2Cre;SmoM2* mutant pituitaries were much bigger in size than WT controls (Fig. 6O-V). Together, these data support our observation that the primary role of Shh/Gli2 signaling is to control the proliferation, rather than patterning, of pituitary progenitors.

Patterning of pituitary requires diencephalic function of *Gli2*

In addition to the partial loss of anterior pituitary as reported previously (Park et al., 2000), we found that all *Gli2* mutants had no posterior pituitary (n=43). The complete loss of the posterior pituitary suggests a requirement of *Gli2* in ventral diencephalon. As diencephalon-derived signals such as BMP4 and FGF8 are required for induction and proliferation of the anterior pituitary (Ericson et al., 1998; Takuma et al., 1998; Treier et al., 1998), we were curious whether the expression of these two signals was perturbed in *Gli2* mutants. We therefore examined the expression of *Shh*, *Bmp4* and *Fgf8* in *Gli2* mutants using in situ hybridization on E9.5 embryos and on embryo sections (Fig. 7). We found a normal expression of *Shh* in the ventral diencephalon of *Gli2* mutants (Fig. 7A,B). However, while *Bmp4* was detected strongly in the medial ventral diencephalon in WT embryos (Fig. 7E), no *Bmp4* expression was detected in the ventral diencephalon of *Gli2* mutants (Fig. 7F, n=3 embryos). Similarly, while *Fgf8* is strongly expressed in the diencephalon of WT embryos (Fig. 7I), the expression in *Gli2* mutants was greatly reduced (Fig. 7J, n=3 embryos). Similar results were obtained when *in situ* hybridization was performed on E9.5 embryo sections (Fig. 7C,D,G,H,K,L, n=7 embryos). Together, these results suggest that *Gli2* is required for diencephalic expression of *Bmp4* and *Fgf8* and that the loss of expression of these two genes in diencephalon may be responsible for the variable pituitary phenotypes in *Gli2* mutants.

DISCUSSION

Shh signaling is primarily required for proliferation, rather than specification, of early pituitary progenitors

Shh signaling has been shown to be involved in controlling cell fate specification and proliferation in different tissues. In the spinal cord, Shh can induce different types of spinal cord neurons based on the concentration of Shh or the length of action (Dessaud et al., 2008). In the cerebellum, however, the major function of Shh appears to control proliferation of granular precursors (Kenney and Rowitch, 2000). Analysis of *Gli* mutants and gain-of-function embryos provided further support that Gli transcription factors, in particular *Gli2*, mediate different functions of the Shh signaling in different tissues. For example, *Gli2* mutants do not generate floor plate cells and have only residual V3 interneurons in the spinal cord (Ding et al., 1998; Matise et al., 1998). Similarly, *Gli2* mutants have defects in the generation of ventral telencephalic neurons (Yu et al., 2009). On the other hand, *Gli2* mutants show reduced proliferation of granular progenitors but no loss of cell types in the cerebellum (Corrales et al., 2004).

Because *Shh* mutants have severe patterning defects during early embryonic development, the role of Shh signaling in pituitary development has previously only been examined in gain- or loss-of-function transgenic embryos (Treier et al., 2001). Blocking Shh signaling by expressing Shh antagonist Hhip in the oral ectoderm caused loss of anterior pituitary markers *Gata2* and *brn4* (*Pou3f4*), and a smaller pituitary. On the other hand, ectopic expression of *Shh* in the anterior pituitary resulted in the expansion of anterior marker *Gata2*, increased production of pituitary cells and a bigger pituitary. In both situations, changes in the expression of anterior markers were accompanied by a corresponding change in cell proliferation. Our current study provides several lines of evidence that Shh/Gli signaling functions primarily to promote pituitary cell proliferation. First, the patterning of *Gli2* mutant pituitaries is largely normal but the pituitaries have a clear proliferation defect.

Second, *Gli2* mutant cells are less likely to contribute to the pituitary, in particular to anterior pituitary, because these mutant cells do not proliferate as well as WT cells in mosaic embryos. Lastly, patterning of early pituitary is normal in conditional *Gli2* mutants when *Gli2* function is disrupted by using *FoxG1Cre* or *Pitx2Cre*. Even when both *Gli2* and *Gli3* are disrupted in the pituitary, cells in RP still express appropriate pituitary markers at E12.5.

Our analysis also revealed a critical window of Shh/Gli signaling in controlling proliferation and differentiation of pituitary progenitors. Removal of *Gli2* using *FoxG1Cre* affects proliferation and differentiation of several pituitary progenitors. These effects are direct results of loss of *Gli2* function in RP, as diencephalic expression of *Bmp4* and *Fgf8* is not altered in these embryos (Supplementary Figure S2). Additional removal of all *Gli* function in the pituitary have a more drastic effect, as *FoxG1Cre;Gli2;Gli3* mutant pituitary glands are much smaller than *FoxG1Cre;Gli2* pituitary glands. However, removal of *Gli2* function using *Pitx2Cre*, which becomes active slightly later than *FoxG1Cre*, does not appear to have obvious effect on patterning or proliferation of the pituitary progenitors. Nevertheless, ectopic activation of Shh signaling using *Pitx2Cre* causes massive over-proliferation of all pituitary progenitors of the pituitary, suggesting that although Shh/Gli signaling is not required at this stage, pituitary progenitors are still responsive to ectopic Shh signaling. It further suggests that Shh signaling must be tightly regulated even at this later phase.

Although Hedgehog signaling is largely conserved across species, clear differences exist as to how Hh/Gli signaling regulates pituitary development in mouse and fish. In mouse, *Gli2* functions as the major activator and is required for pituitary development (Park et al., 2000), while *Gli1* and *Gli3* are dispensable, although *Gli1* is redundant in function with *Gli2*. Furthermore, conditional removal of *Gli2* or all *Gli* function in pituitary reveals that Hh/Gli signaling controls proliferation, rather than patterning, of early pituitary progenitors. Our analysis also reveals that Hh/Gli signaling controls diencephalic expression of *Bmp4* and *Fgf8*, which have been shown to control early pituitary patterning and proliferation. On the other hand, zebrafish *dtr/gli1* is required for normal development as *dtr/gli1* null mutants have a pituitary phenotype including a reduced expression of pituitary markers *nk2.2* and *Lim3*, and reduction in the number of somatotropes, lactotropes and anterior corticotropes (Devine et al., 2009; Karlstrom et al., 2003). Blocking the function of *yot/gli2* results in minor pituitary phenotypes as the number of corticotropes is reduced. However, blocking the function of both *dtr/gli1* and *yot/gli2ab* function results in a significant reduction in almost all anterior pituitary cell types (Devine et al., 2009), a phenotype that is more severe than any single or compound mouse *Gli* mutants. It is possible that the differences observed are species specific, as the morphogenetic process of pituitary formation in zebrafish is distinct from that of the mouse or chick in at least two aspects. First, the zebrafish pituitary primordium is formed as a solid structure at the anterior edge of the head, which then moves caudally into the head in a process that is different from that in chick or mouse. Second, the formation of zebrafish pituitary gland does not involve a structure equivalent to Rathke's pouch and the invagination of oral/placodal ectoderm (Glasgow et al., 1997; Herzog et al., 2003). In addition, there are differences in the transcriptional regulation of *Gli* genes in mouse and fish, as mouse *Gli2* is not regulated by Hh signaling (Bai et al., 2002) while the zebrafish *dtr/gli1* appears to be regulated by Hh signaling (Devine et al., 2009). Alternatively, the more severe pituitary phenotype in zebrafish could also result from knockdown of gene function in both pituitary and diencephalon.

***Shh/Gli2* mediated diencephalic signal is required for pituitary development**

Our analysis of pituitary-specific *Gli* mutants revealed that blocking of Shh signaling within pituitary progenitors reduces proliferation and differentiation of pituitary progenitors but does not significantly affect patterning of the pituitary gland. But if that is the case, why would half of the *Gli2* mutants lack a pituitary? The answer may be in the ventral

diencephalon. Starting from E8.0, *Shh* is strongly expressed in the ventral diencephalon, immediately above the forming RP. Our previous analysis identified loss of ventral diencephalic tissues and reduced expression of *Nkx2.1* in some *Gli2* mutants. As ventral diencephalon is an important tissue in the induction of RP, we examined the expression of diencephalic marker genes between E8.5 and E9.5. We found that *Gli2* mutants had reduced expression of *Bmp4* and *Fgf8*, which are growth factors shown to be critical for the development of a definitive pouch (Takuma et al., 1998; Treier et al., 1998). Our study therefore identified two requirements for Shh/Gli signaling that are critical for pituitary progenitor development: one within RP to promote proliferation of pituitary progenitors and a second role to control expression of *Bmp4/Fgf8* in the ventral diencephalon to control early patterning of RP. Future work will be needed to determine whether the Gli2 transcription factor can bind directly to *Bmp4/Fgf8* regulatory elements to control their expression in the ventral diencephalon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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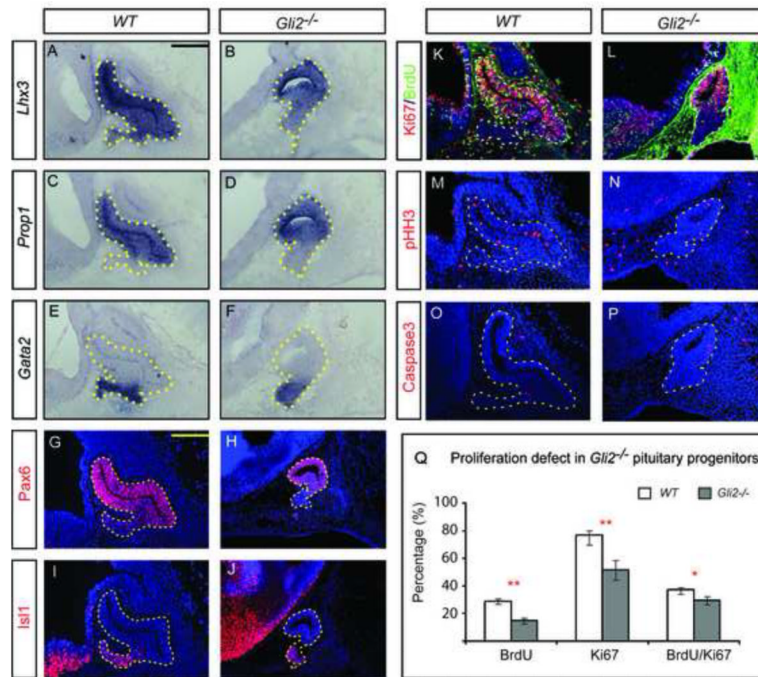


Figure 1.

Gli2 mutant pituitaries have normal patterning but show defects in proliferation. (A-F) RNA *in situ* hybridization of wild type (WT) and *Gli2* mutants using probes of *Lhx3*, *Prop1* and *Gata2*. (G-J) Immunostaining of Pax6 and Is11. (K-P) Analysis of proliferation and cell death by immunostaining of Ki67, BrdU, pHH3 and Caspase3 in Rathke's pouch. Note: The strong background staining in (L) is likely caused by leaky vasculature and elevated levels of mouse endogenous IgG in *Gli2* mutants. (Q) Quantification BrdU incorporation, the expression of Ki67 and Caspase3. The percentage of BrdU+, Ki67+, pHH3+, Caspase3+ cells was calculated by dividing the number of the positive cells by the total number of nuclei (Hoechst). All embryos (n=6) were at E12.5. * indicates $P<0.05$, ** indicates $P<0.01$. Scale bars: 100 μ m.

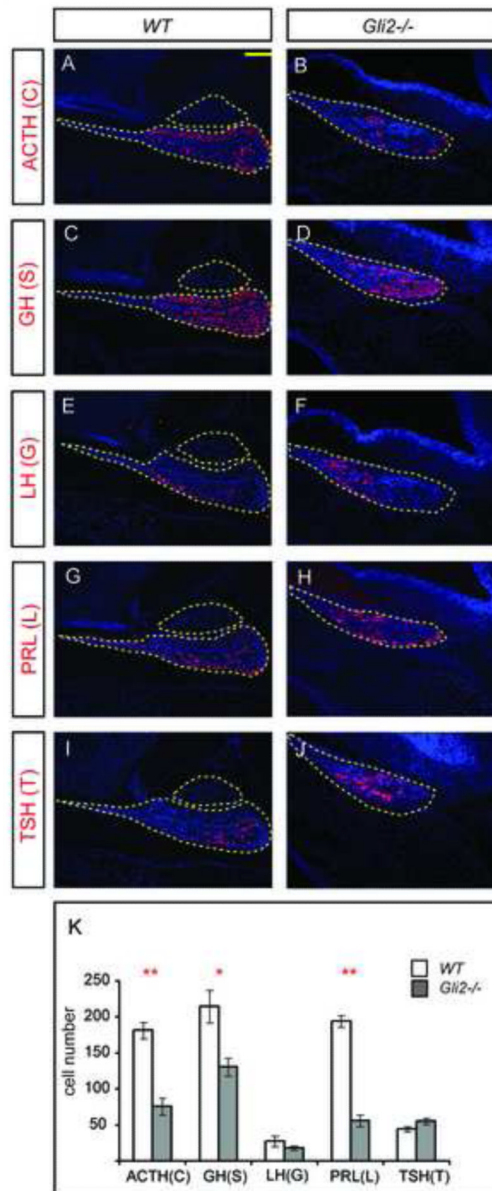


Figure 2. The number of corticotropes, somatotropes and lactotropes is reduced in *Gli2* mutants at E17.5. (A-J) Immunostaining of different pituitary cell type markers ACTH, GH, LH, PRL and TSH at in WT and *Gli2* mutant pituitaries. Outlined areas in WT represent anterior and posterior pituitaries. In *Gli2* mutants, only anterior pituitary is present. (K) Quantification of each pituitary cell type in WT and *Gli2* mutants (* indicates $P < 0.05$, ** indicates $P < 0.01$, $n = 6$ embryos). Two mid-sagittal sections from each embryo were used to quantify the number of different cell type.

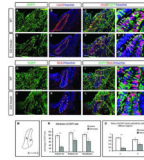


Figure 3.

In mosaics, *Gli2* mutant cells do not contribute as well in the anterior edge of the pituitary due to reduced proliferation there. (A-F) Immunostaining of Lhx3 in WT and *Gli2* mosaic embryos. (C',F',I',L') are higher magnification images of corresponding boxes indicated. Note: EGFP is expressed in cytoplasm while Lhx3 and BrdU are present in nuclei. (G-L) Immunostaining of BrdU and EGFP in WT and *Gli2* mosaic embryos. EGFP⁺ cells in WT embryos (A-C, G-I) were *Gli2*^{+/+} while EGFP⁺ cells in *Gli2* mosaic (D-F, J-L) were *Gli2*^{fl/fl}*lacki*. (M) Schematic representation of the subdivisions of the pituitary. R, rostral; C, caudal. (N) Distribution of EGFP⁺ cells in anterior wall (A) and posterior wall (P) of the anterior pituitary, and in infundibulum. (O) Distribution of BrdU⁺;EGFP⁺/BrdU⁺ cells in different regions of the anterior pituitary ($P_A < 0.01$; $P_P = 0.105$). See Material and Methods for normalization of the number of EGFP⁺ cells in each embryo. The anterior pituitary was outlined by dotted lines. White arrowheads indicate cells double-labeled with both green and red. Scale bars: 100 μ m. * represents $P < 0.05$, ** represents $P < 0.01$.

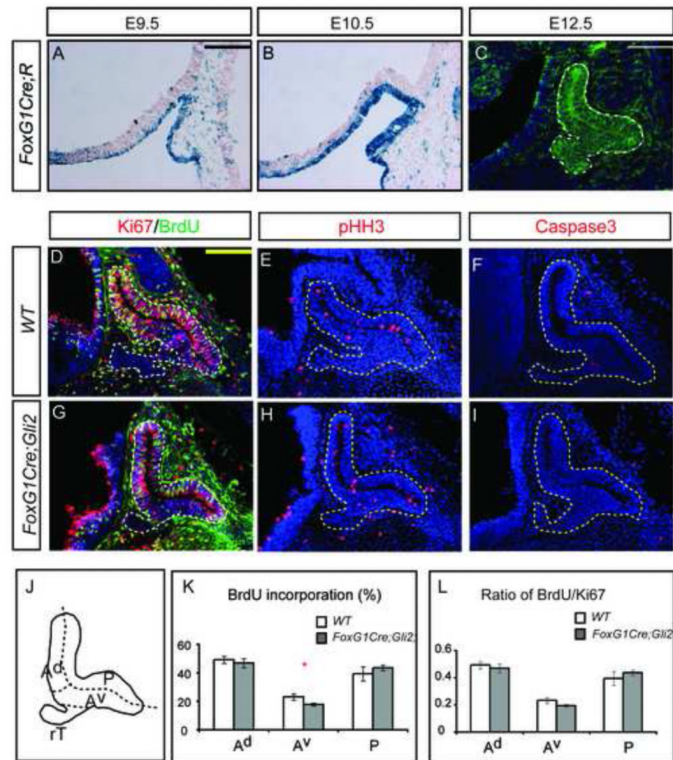
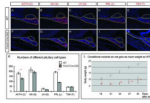


Figure 4.

Early loss of *Gli2* function results in proliferation defects in progenitors in the anterior-ventral pituitary. (A-C) *FoxG1Cre* activity can be detected before the closure of RP, as detected by X-gal staining at E9.5 (A) and E10.5 (B), and by immunostaining at E12.5 (D-I). Analysis of proliferation and cell death in E12.5 WT and *FoxG1Cre;Gli2* conditional mutants. (J) Schematics of sub-divisions of the pituitary. (K,L) Quantification of BrdU incorporation and the ratio of BrdU/Ki67 in different regions of the pituitary. Scale bars: 100 μ m.

**Figure 5.**

Early loss of *Gli2* function results in the reduction of corticotropes and lactotropes at E17.5. (A-J) Immunostaining of different pituitary cell type markers. (K) Quantification of different pituitary cell types in WT and *FoxG1Cre;Gli2* mutants (* denotes $P < 0.05$, ** denotes $P < 0.01$, $n = 6$ embryos). (L) *FoxG1Cre;Gli2* mutants did not grow as well as WT pups after birth. Seven pups from four litters were tracked for each genotype. The number on each dot indicates surviving pups at that time. Scale bars: 100 μm .

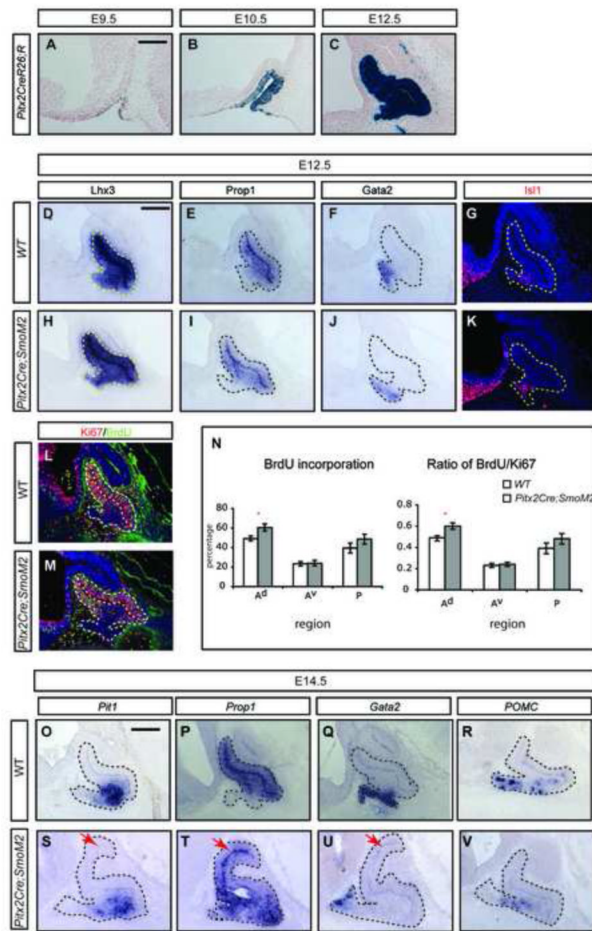


Figure 6.

Activation of Hh signaling after the closure of RP resulted in increased proliferation in the anterior-dorsal pituitary. (A-C) X-gal staining revealed the activity of *Pitx2Cre* from E9.5 to E12.5. (D-I) Analysis of patterning markers in E12.5 WT embryos (D,F,H) and in embryos with activated Hh signaling (*Pitx2Cre;SmoM2*) using RNA in situ hybridization (D-F,H-J) and immunostaining (G, K). (L, M) Immunostaining of BrdU incorporation and the expression of Ki67. (N) Quantification of BrdU incorporation and Ki67 expression. (* indicates $P < 0.05$, $n = 6$ embryos). (O-V) Activation of Hh signaling did not affect pituitary patterning, as revealed by RNA *in situ* hybridization of markers at E14.5. Scale bars: 100 μm .

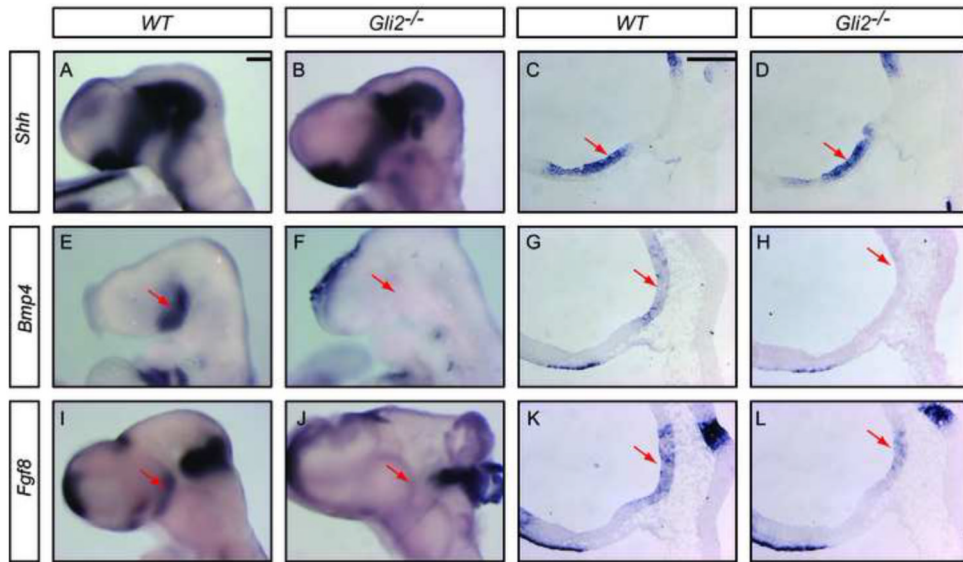


Figure 7. *Gli2* is required for the expression of *Bmp4* and *Fgf8* in the ventral diencephalon. (A-L) RNA *in situ* hybridization of *Shh*, *Bmp4* and *Fgf8* in E9.5 embryos (A,B,E,F,I,J) and embryo sections (C,D,G,H,K,L). Red arrows indicate diencephalic expression. Scale bars: 100 μ m.