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Pituitary Gland Development and Disease: From Stem Cell to Hormone Production

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Abstract

Many aspects of pituitary development have become better understood in the last two decades. The signaling pathways regulating pituitary growth and shape have emerged, and the balancing interactions between the pathways are now appreciated. Markers for multi-potent progenitor cells

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are being identified, and signature transcription factors have been discovered for most hormone producing cell types. We now realize that pulsatile hormone secretion involves a 3-D integration of cellular networks. About a dozen genes are known to cause pituitary hypoplasia when mutated due to their essential roles in pituitary development. Similarly, a few genes are known that predispose to familial endocrine neoplasia, and several genes mutated in sporadic pituitary adenomas are documented. In the next decade we anticipate gleaning a deeper appreciation of these processes at the molecular level, insight into the development of the hypophyseal portal blood system, and evolution of better therapeutics for congenital and acquired hormone deficiencies and for common craniopharyngiomas and pituitary adenomas.

Keywords

adenohypophysis; anterior pituitary; Rathke's pouch; stem cell; neural ectoderm; organizing center

Introduction

The pituitary gland is known as the "master gland" of the body, acting as central endocrine regulator of growth, reproduction, metabolism and response to stress. To exert its function, unique cell types in the anterior pituitary gland, including lactotrophs, somatotrophs, thyrotrophs, corticotrophs and gonadotrophs, secrete polypeptide hormones: prolactin (PRL), growth hormone (GH), thyroid stimulating hormone (TSH), adrenocorticotropic hormone (ACTH) and the gonadotropins - luteinizing (LH) and follicle stimulating (FSH) hormones, respectively.

The anatomical steps of pituitary development have been described in many species, beginning a century or more ago [1, 2]. Because the basic aspects of pituitary development and functions of the pituitary hormones are fairly well conserved across all vertebrates, lessons learned in bird (chick-quail), amphibian (bullfrog), fish (zebrafish), and mammal (mouse and rat) all provided important contributions to our current understanding of pituitary development and disease. Transplant studies laid the foundation for understanding the signaling that influences pituitary hormone production [3-6]. Electron microscopy ushered in the ability to distinguish hormone producing cell types based on their size, shape and secretory granules, and this was superseded by the availability of antibodies specific to individual hormones, permitting the emergence of differentiating cells to be tracked during embryogenesis [7-9]. The molecular biology era brought the discovery of signature transcription factors that are important for cell specification and lineage determination, and the discovery of the signaling molecules that were predicted in early transplant experiments (reviewed in: [10-12]).

A recent area of active investigation is aimed at understanding the nature of pituitary progenitors, including cells with stem-like characteristics during embryogenesis and in the adult organ (reviewed in [13, 14]). Much still needs to be learned about the recruitment of progenitors, differentiated cell hypertrophy and hyperplasia during puberty, pregnancy, wound healing, and cases of unusual physiological demand. For a deeper basic understanding of pituitary development we need to know how the hypophyseal portal

system develops, which is necessary for hypothalamic releasing hormones to reach the pituitary gland and for transporting hormones to their target tissues [15]. The mechanisms that regulate the formation of pituitary cell networks and the role of these networks in hormone secretion are also under investigation (reviewed in [16]).

Studies of pituitary development have given us the ability to carry out molecular diagnoses for many of the rare familial pituitary adenomas and congenital pituitary hormone deficiency disorders. This is important for predicting risk, disease progression and for assessing treatments. Despite this progress, at least half of the congenital disorders still do not have diagnoses, and the common pituitary adenomas are mostly still mysterious and can be extremely difficult to treat [17-19]. In this review we intend to focus on the areas where future investigation is needed and refer to recent reviews that cover the aspects of pituitary development and disease that are fairly well understood. We hope that future basic science studies will usher the way for improved detection, treatment and prevention.

Regulating the pituitary organizer and the growth and shape of Rathke's pouch

The pituitary gland is primarily derived from two ectodermal structures, the neural ectoderm, which gives rise to the posterior lobe, and the surface ectoderm, which produces Rathke's pouch, the precursor to the anterior and intermediate lobes (adenohypophysis or pars distalis and pars intermedia, respectively). The posterior lobe, or neurohypophysis, forms from the ventral diencephalon, and its formation and patterning is detailed in another article in this volume. The patterning of the ventral diencephalon is critical not only for establishing the pituitary posterior lobe, but also for producing an organizing center that establishes the proper size and shape of Rathke's pouch (Fig. 1). Analysis of genetically modified mice has greatly advanced our understanding of the roles of various signaling pathways in pituitary development (Table 1). The organizing center consists of an overlapping expression domain of bone morphogenetic protein and fibroblast growth factors (BMP4, FGF8, and FGF10) in the ventral diencephalon where it evaginates to form an infundibulum [20, 21]. Rostral to the organizing center and the infundibulum is a domain of sonic hedgehog (SHH) expression [21]. BMP4 is an essential inductive signal for Rathke's pouch formation because *Bmp4*$^{-/-}$ mice fail to form the pituitary placode or Rathke's pouch at e9.5 [5]. After placode formation and pouch induction, FGF signaling is necessary for cell proliferation in Rathke's pouch. The ligand and receptor mutants, *Fgf10*$^{-/-}$ and *Fgfr2IIIb*$^{-/-}$, form Rathke's pouch, but it fails to expand and is lost through apoptosis [22, 23]. *Nkx2.1*$^{-/-}$ mice do not express *Fgf8* in the ventral diencephalon and Rathke's pouch is hypoplastic, which phenocopies the *Fgf10*$^{-/-}$ pituitary [5]. Mice with a hypomorphic mutation in *Fgf8* have a variable phenotype, including reduction in the size of the pituitary anterior lobe (adenohypophysis), loss of the pituitary posterior lobe, and neural ectoderm midline defects, including holoprosencephaly [24]. Thus, both BMP and FGF are critical at early stages of pouch induction and growth, and there is evidence for dosage sensitivity.

The FGF family is large, and many of the genes are expressed in the pituitary gland. Unique roles of FGF8 and FGF10 in the pituitary organizing center are not completely clear. FGF8 plays a central role in establishing the neuroectoderm midline, while *Fgf10*$^{-/-}$ mice do not

display midline defects [23-25]. We hypothesize that FGF8 is more broadly required for patterning the neuroectoderm derived pituitary organizing center. FGF8 and 10 may work in concert for mouse infundibulum development in a manner similar to FGF3 and FGF10 in chick infundibular development. It would be useful to analyze the expression pattern of *Fgf10* in the *Nkx2.1*$^{-/-}$ and *Fgf8* hypomorphic mutants, and *Fgf8* expression in *Fgf10*$^{-/-}$ mice, to understand whether there are compensatory changes in expression. *Fgf18* is expressed the organizing center [26, 27], and FGFs 13, 14, and 17 are detected in the embryonic pituitary transcriptome [28]. Thus, the potential for functional redundancy amongst the FGF family is great.

There is an intricate interplay between the signaling pathways in the ventral diencephalon. Single gene disruptions in one pathway influence expression of genes in a different signaling pathway. Noggin is expressed in the pituitary organizer and inhibits BMP4 activity. *Nog*$^{-/-}$ mice have an expanded domain of BMP4, and a reduction in FGF10 expression, revealing interaction between these signaling pathways [29]. A larger domain of surface ectoderm is induced to become Rathke's pouch, and the pituitaries have highly variable dysmorphologies. The Wnt signaling pathway also affects BMP and FGF expression. *Wnt5a*$^{-/-}$ mice and *Tcf7l2*$^{-/-}$ (TCF4) mice have expanded expression domains of both BMP4 and FGF10 and enlargement of the gland. The effects are consistent, with *Wnt5a* mutants exhibiting a modest dysmorphology that resolves by birth and *Tcf7l2* mutants having a greatly enlarged pituitary gland that protrudes through the cartilage plate [30-33]. WNT5A is likely to act through the non-canonical Wnt signaling pathway, and little or no stabilized beta catenin is detectable in the nuclei at that stage. Thus, the overgrowth characteristic of *Tcf7l2* mutants is likely due to a loss of transcriptional repression.

The WNT gene family is large and many members are expressed in the developing pituitary and surrounding areas. *Wnt11* and *Wnt16* are expressed in the ventral diencephalon [33], and generally act as non-canonical and canonical WNTs, respectively. Thus, *Wnt16* is one candidate for regulating the pituitary organizer through TCF7L2. Recently, the ROR1 and ROR2 receptors have been identified as mediating non-canonical WNT5A signaling. The features of *Ror1*$^{-/-}$; *Ror2*$^{-/-}$ mice such as limb truncation phenocopy many aspects of the *Wnt5a*$^{-/-}$ mice, although the pituitary phenotype was not reported [34]. More work is needed to define the important players and to understand the roles of canonical and non-canonical WNT signaling in development of the pituitary organizer.

The SHH signaling pathway is important for regulating pituitary growth, and transcription factors from the SOX, T-box and GLI families are involved in SHH expression and activity. Sonic hedgehog is expressed in both the oral ectoderm and the pituitary organizer within the ventral diencephalon. The pituitary placode arises from a patch of oral ectoderm that is negative for SHH expression, which is similar to the emergence of the pancreatic bud from the gut tube in a SHH negative zone. SHH expression in the ventral diencephalon is necessary to restrict the growth of the pituitary gland. Conditional loss-of-function of *Shh* in the ventral diencephalon is associated with expansion of the organizing center and pituitary enlargement [35]. *Shh* transcription in the ventral diencephalon is regulated by SOX2 and SOX3, which bind the *Shh* enhancer, SBE2, and activate expression. A dose dependent reduction in *Sox2* and *Sox3* leads reduced *Shh* expression in the ventral diencephalon and an

expansion of the pituitary organizer [35]. The action of SOX2 and SOX3 are blocked by the T-box transcription factors TBX2 and TBX3. They bind SOX2 and SOX3, preventing activation of *Shh* expression through the SBE2 enhancer. *Tbx3*$^{-/-}$ mice exhibit expanded SHH expression and reduced expression of BMP4 and FGF10, leading to a hypomorphic pituitary [36]. SHH signals are transduced through the Gli transcription factors, *Gli2* and *Gli3*. GLI2 primarily activates and GLI3 primarily represses SHH transcriptional targets [37]. *Gli2*$^{-/-}$ embryos have reduced *Bmp4* and *Fgf8* expression in the pituitary organizer and hypomorphic pituitaries, while *Gli2*$^{-/-}$; *Gli3*$^{-/-}$ embryos have no pituitary at all [38]. Given the active and repressive roles of GLI proteins, it is difficult to determine if early, active SHH signaling is necessary to induce the expression of *Bmp4* and *Fgf8* in the pituitary organizer, or if the repressive activity of GLI2 and GLI3 are necessary in the pituitary organizer to ensure the expression of *Bmp4* and *Fgf8*.

The homeobox transcription factors LHX2 and RX, which contain LIM and paired type homeodomains, respectively, are important regulators of the pituitary-organizing center. Both *Lhx2*$^{-/-}$ and *Rx*$^{-/-}$ embryos have enlarged pituitaries that are typical of genetic mutants that have expanded expression of both BMP4 and FGF8 and reduced SHH expression in the ventral diencephalon. *Lhx2* mutants have expanded FGF8 expression, but BMP4 expression appeared unchanged [39]. The *Rx*$^{-/-}$ mice have reduced FGF10 expression, and while neither BMP4 nor FGF8 expression were examined, we predict that their expression domains are expanded [40]. Additional characterization of these mouse models may reveal additional transcriptional regulation of the pituitary organizer and the subsequent induction of Rathke's pouch.

Activities of signaling pathways intrinsic to Rathke's pouch

BMPs and FGFs are expressed in and around Rathke's pouch, and the roles of these signaling factors in cell specification within the anterior lobe are controversial. Loss of function models support the role of these signaling molecules in growth and shape, but not cell specification, while gain of function models suggest excess signaling can influence cell specification and/or affect the size of specific cell populations. At e10.5 of mouse development FGF8 and FGF10 are expressed in the infundibulum, dorsal to Rathke's pouch, and BMP is expressed on the ventral side and adjacent mesoderm [20, 21]. Counteracting gradients of FGF and BMP signaling have been proposed to regulate specification of anterior lobe cell types depending on where the progenitor cells are located relative to the gradient [20, 21]. Gonadotropes are enriched on the ventral side of the developing anterior lobe, and somatotropes are initially located more dorsally, which could mean that progenitor cells closer to the source of BMP2 become gonadotropes, while progenitor cells closer to the source of FGF become somatotropes. No experiments, such as diI or genetic labeling, have been performed to follow progenitor cells and their descendants from a specific starting position near the lumen of Rathke's pouch to a final location and specific cell type in the anterior lobe. The discovery of cell type specific networks within the anterior lobe and the movement of hormone secreting cells to form those networks suggest that the final position of cells in the anterior lobe cannot be directly correlated with a starting position in Rathke's pouch [41]. In fact, a birth dating study showed that progenitor cells that exit the cell cycle concurrently are scattered throughout the anterior lobe, implying the active movement of

cells throughout the anterior lobe [42]. Therefore, we do not know if all progenitor cells near the lumen are equivalent or if the progenitor cells are patterned dependent on location within Rathke's pouch prior to cell cycle exit.

Anterior lobe cell types begin to exit the cell cycle and start to differentiate between e11.5 and e13.5 [42, 43]. As progenitor cells exit the cell cycle they enter a non-cycling, undifferentiated state that is characterized by the expression of p57Kip2 (*Cdkn1c*) [44]. These cells are visible on the ventral side of the lumen as they leave the epithelia of the lumenal area and enter the anterior lobe. The timing of cell cycle exit beginning at e11.5 correlates with a period when Rathke's pouch explants become refractory to exogenous signals including FGF and BMP [20, 21], suggesting that signals intrinsic to Rathke's pouch are likely to drive cell specification. Altering the expression of BMP and FGF in the pituitary organizer does not significantly alter anterior lobe cell specification [29, 31-33, 39]. Embryos homozygous for an FGF8 hypomorphic mutation have fewer gonadotrope cells, indicating that extrinsic FGF may be influence anterior lobe cell specification and/or population size at birth [24, 39].

More work is necessary to elucidate the intrinsic roles of BMP and WNT within Rathke's pouch. BMP2, WNT4, WNT6, WNT11, and WNT16 are all expressed in the pouch and could have roles [21, 29, 33]. Expression of a dominant negative *Bmpr2* receptor in Rathke's pouch effectively reduces BMP signaling, and the consequences are loss of the POU1F1 (*Pit1*) lineage, which is comprised of thyrotropes, somatotropes and lactotropes, and a concomitant expansion of corticotropes [21]. Stimulating BMP signaling by driving BMP4 expression in Rathke's pouch promotes the differentiation of intermediate cell types, especially those expressing *Gata2* and *Isl1* expressing cells, but it prevents the terminal differentiation of all hormone cell types except corticotropes [21]. It is difficult to be certain whether the consequences of non-physiological gain of function experiments are truly reflective of intrinsic signaling pathway functions.

The expression of multiple WNTs in Rathke's pouch raises the possibility of functional redundancy and the use of both canonical and non-canonical pathways. *Wnt4* deficiency leads to a reduction in somatotropes and thyrotropes, whereas a loss of *Wnt6* has no obvious effect on pituitary cell specification [21, 33]. Canonical Wnt signaling appears critical for cell specification because the conditional inactivation of β-catenin in the early pouch leads to a loss or reduction in all cell types except corticotropes [45]. Expression of an activated form of β-catenin in the early Rathke's pouch causes variable phenotypes depending on the cre driver used to activate β-catenin. With *Pitx1-cre*, the pituitary is arrested early in organogenesis [45]. With *Hesx1-cre*, an increase in pituitary stem cells is observed leading to the formation of craniopharyngiomas and cell specification is altered, reducing all cell types, except corticotropes [46]. Despite the presence of a gene encoding a degradation resistant form of β-catenin in all anterior lobe cells, nuclear localized β-catenin is not observed in the cells outside of the stem cell niche that have begun to differentiate. The anterior pituitary appears to have mechanisms to suppress activation of β-catenin. While both *Pitx1* and *Hesx1* are expressed very early in Rathke's pouch formation, they are also expressed in other anterior structures prior to pouch formation [6]. Spatial and temporal differences in expression of these two cre drivers likely contribute to the disparate phenotypes that are observed. In addition,

the dosage sensitivity demonstrated for HESX1 and other pituitary transcription factors could be contributors in cases where cre expression occurs at the expense of an endogenous allele [47-49].

The role of SHH signaling within the pouch is suggested by the results of gain and loss of function experiments in mice. *Shh* is initially expressed throughout the oral ectoderm, and it is excluded from the placode that forms Rathke's pouch. Despite this, Rathke's pouch cells are apparently receiving SHH signals because the downstream target gene patched (*Ptc1*) is expressed [50]. Receipt of these signals must be important because blocking SHH signaling by driving expression of the *Shh* inhibitor, *Hip*, in the pouch reduces proliferation of progenitor cells, *Bmp2* and *Lhx3* are not expressed, and the pituitary is very hypoplastic [50]. Similarly, overexpression of *Shh* in Rathke's pouch causes an increase in *Bmp2* expression and an increase in thyrotropes and gonadotropes [50]. Embryos with a conditional inactivation of *Gli2* in the pituitary, however, exhibit a reduction in progenitor proliferation, but the pituitary is well formed and other than a reduction in corticotropes, the hormone producing cells are unaffected [38]. Compensatory changes in gene expression may provide a partial rescue in loss of function mutants, but gain of function may exceed the ability make adjustments. Activating *Shh* signaling in the pituitary with ectopic expression of SmoM2 increases proliferation without altering cell specification [38]. The differences between the HIP transgenic and conditional *Gli2* loss of function studies may be indicative a broader range of action for the secreted inhibitor, HIP, such as inhibiting SHH signaling in the ventral diencephalon as well as Rathke's pouch, or the differences may implicate non-canonical SHH signaling in the pituitary, such as the activation of RAC1 or RHO in a Gli-independent manner [51]. The differing results for the ectopic stimulation of the SHH pathway in Rathke's pouch may be explained by non-canonical SHH signaling because *Ptc1* has *Smo* and *Gli* independent functions [51].

The Notch signaling pathway has a proven to be a prime candidate for driving anterior lobe cell specification. *Hes1* is a Notch responsive transcription factor, and *Hes1*$^{-/-}$ embryos have a cell fate switch from melanotropes to somatotropes [52]. The conditional loss of an intracellular mediator of Notch signaling, *Rbpjk*, in Rathke's pouch promotes the differentiation of corticotropes and the loss of the POU1F1 lineage [53]. Stimulation of Notch signaling in the corticotropes and melanotropes prevents their differentiation [54], while ectopic Notch signaling in the POU1F1 lineage prevents terminal differentiation [53]. The Notch ligand *Dll3* is expressed in corticotropes; although it is not required for corticotrope differentiation [55]. The *Dlk1* Notch ligand is expressed in all hormonal cell types, and loss of *Dlk1* leads to a decrease in all cell types, with the most significant reduction occurring in somatotropes [56, 57]. More studies are necessary to define the role of notch family receptors, ligands and target genes in regulating pituitary progenitor transitions to differentiation and cell specification.

In sum, BMP, FGF, WNT and Notch each have important roles in and around the pituitary gland. The multiplicity of ligands and receptors and interactions between pathways confer a degree of complexity that is difficult to unravel completely. Future research is needed to clarify the roles of individual signaling pathways in cell specification, and to understand the

compensatory changes that are possible to ensure the proper distribution of cell types within the pituitary anterior lobe.

The role of signature transcription factors in cell specification

A collection of transcription factors have been identified that play important roles in the specification and/or expansion of pituitary hormone producing cells, and many are relevant in human disease (Table 2). The first of these was POU1F1, which was identified based on its role in trans-activating the growth hormone and prolactin genes [58-61]. Mice and humans with inactivating mutations in this gene generally have recessive hypopituitarism, characterized by a congenital lack of GH, PRL and TSH. POU1F1 is the signature transcription factor for the lineage that gives rise to the somatotropes, lactotropes and thyrotropes [62]. These cells fail to develop in POU1F1 mutants. Similar approaches identified other critical transcription factors like PITX1, the orphan nuclear hormone receptor, NR5A1 or Steroidogenic Factor 1, and the helix-loop-helix factor NeuroD1, and the T-box factor TPIT (Table 2) [63-67]. In some cases the transcription factor deficiency does not result in complete absence of the cell type, but the differentiation is incomplete. For example, NR5A1 deficient mice do not produce gonadotropins, but hyperstimulation with GnRH is an effective inducer, suggesting that gonadotrope differentiation does not require NR5A1 [68]. Similarly, corticotrope development does not depend on either NeuroD1 or TPIT, but POMC expression is delayed and/or reduced if they are deficient [65, 66]. The failure to promote differentiation along one particular path can be permissive for alternative pathways. In the absence of TPIT, intermediate lobe cell types differentiate into gonadotropes and POU1F1 independent thyrotropes, implying an important role of TPIT in repressing anterior lobe cell fates, in part by antagonizing NR5A1 [69, 70]. HES1 deficiency also can cause ectopic differentiation in the intermediate lobe: instead of melanotropes, the hypoplastic intermediate lobe contains POU1F1 dependent somatotropes. Premature cell cycle exit appears to be the underlying permissive factor [71].

The idea that single signature transcription factors direct cell specification is an overly simplistic one. Many transcription factors are required to produce the characteristic features of specialized hormone producing cell types. For example, additional factors, both positive and negative, are implicated in driving POU1F1 expressing cells towards the specialization in production of GH, PRL or TSH [72]. The glucocorticoid and estrogen receptors and ETS factors are examples of factors that promote specialization [73-75]. In addition, components of the combinatorial code of factors can change during development in order to achieve the fully differentiated hormone-producing cell or to maintain it (reviewed in [76]).

The current state of the art requires an understanding of the epigenetic regulation that makes chromatin accessible for transcription factor binding and the mechanisms that initiate this state. Genome-wide analysis of DNase sensitive open chromatin and transcription factor binding sites are powerful tools for dissecting the differentiation steps for hormone-producing cells. A recent example comes from the study of PAX7, which is a pioneer transcription factor that binds enhancers from many genes, opening the chromatin to permit binding of TPIT or suppressing binding [77]. In the absence of the PAX7 selector, intermediate lobe cells differentiate into corticotropes instead of melanotropes. An important

future challenge is to understand these initiating, selector steps for other hormone-producing cell types. These types of experiments are particularly challenging unless there are cell culture systems that can recapitulate the differentiation process because embryonic pituitary tissue availability is limited.

Early acting transcription factors

PROP1 is the earliest pituitary-specific transcription factor expressed in development. This paired homeodomain protein is required for both activation and silencing of several genes that individually have important roles in organogenesis. It is critical for initial activation of POU1F1 and NOTCH2 and for temporally appropriate silencing HESX1 and OTX2 [55, 78-81]. PROP1's switch from repressor to activator may be controlled by beta-catenin, but other evidence suggests that beta-catenin must be strongly suppressed for normal development [31, 45, 46]. In humans PROP1 deficiency can affect all hormone producing cell types of the anterior lobe [82], and in mice it causes a congenital deficiency of GH, TSH, PRL and reduced levels of gonadotropins [83, 84]. In the absence of PROP1 the proliferating cells located along Rathke's cleft fail to delaminate, mimicking failed epithelial to mesenchymal transition [85, 86]. This results in a highly dysmorphic and hypoplastic organ in mice and a variety of organ sizes in humans [76, 85].

Many of the transcription factors that act early in pituitary development have effects on multiple pituitary cell types. In contrast to PROP1, most of these genes are not pituitary specific and affect multiple developing structures when mutated, resulting in syndromic hypopituitarism (Table 2), (Reviewed in [76]). Loss of function mutations in some genes are likely lethal in humans because of their pleiotropic effects. Highly variable craniofacial and pituitary phenotypes are observed, possibly because there is functional overlap between members of the same gene family such as PITX1, PITX2; LHX2, LHX3, LHX4; and OTX1, OTX2. Mutations in many of these genes are associated with reduced proliferation and increased apoptosis [49, 87-89].

OTX1, OTX2, EMX1 and EMX2 have overlapping patterns of expression in the head of the developing mouse embryo [90]. Gene targeting experiments revealed essential roles for each of these transcription factors and demonstrated compensation by members of the gene family during embryogenesis. *Otx2*$^{-/-}$ mice lack head structures anterior to rhombomere 3, while *Otx2*$^{+/-}$ heterozygotes have variable craniofacial phenotypes that range from pituitary aplasia to striking pituitary dysmorphology and hypoplasia [91]. *Otx1*$^{-/-}$ mutants have an even milder phenotype, resulting in a transient delay in growth and puberty [92], supporting the idea that both *Otx1* and *Otx2* have roles in pituitary development, with *Otx2* being the most critical for normal organ morphology and function. *Otx2* is prominently expressed in the neural ectoderm, which produces FGF and stimulates the growth of Rathke's pouch [79]. *Otx2* expression in Rathke's pouch is very low and transient, and little or no expression is apparent when *Pou1f1* transcription is initiated. This suggests that the hypopituitarism characteristic of OTX2 mutations in humans and mice occurs because OTX2 is required for development of the posterior lobe and pituitary stalk. The anterior lobe hypoplasia is likely to be secondary to the neural ectoderm defect, resulting from reduced inductive signals that normally emanate from the organizing center.

Compensation and functional overlap are not limited to members of the same gene family. For example, in addition to the EMX and OTX genes, there are multiple genes that enhance or suppress the *Otx2* mutant phenotype, and they vary among different inbred strains [91]. The C57BL/6 background enhances the susceptibility of *Otx2* heterozygotes to severe craniofacial defects, while CBA is protective [93, 94]. Late in gestation, on a mixed background of B6 and CBA, *Otx2* heterozygotes range from normal appearance to extreme acephalic. Classic genetic mapping of the modifier genes revealed contributing loci on several chromosomes, although the specific genes are not yet known. These types of mouse studies may uncover genes that influence the severity of craniofacial defects in human carriers for *OTX2* mutations. In cases where the transmission of *OTX2* variants has been studied in pedigrees, the heterozygous mutations are not completely penetrant [95]. Sequencing the genomic DNA of these human patients could identify genes with deleterious variants that contribute to the penetrance of the *OTX2* mutant phenotype. This has been employed successfully in identifying genes that contribute to hypogonadotropic hypogonadism, and uncovered multiple examples of digenic or oligogenic disease [96, 97].

Emerging roles for additional transcription factor families: the forkheads

Transcriptome studies reveal that there are many different transcription factor genes expressed in the pituitary gland with unknown functions [28]. The SIX gene family has been implicated in pituitary development [98, 99], and the common effects of this family on eye and pituitary development support the idea that organs developing from placodes utilize similar regulatory pathways ref Bonner-Fraser review. There are many examples of homeobox, HMG box, helix-loop-helix and orphan nuclear receptors that remain to be analyzed Brinkmeier and Davis papers. Developmental expression studies are the first step in understanding the role of these novel genes. The role of forkhead genes in pituitary growth is beginning to emerge, and it serves as an example of the complexity that may characterize other gene families that remain to be explored.

Forkheads are a family of transcription factors that contain a conserved, winged helix DNA binding domain and were named from the phenotype of the *Drosophila* mutant that founded the group. Forkheads are implicated in many physiological processes including development, metabolism, cell cycle progression, and chromatin remodeling [100, 101]. To date 50 forkhead factors have been identified in humans and 44 in mice. A unified nomenclature has been adopted for forkhead factors. FOX (for forkhead box) a letter to designate the subfamily to which the factor belongs, and a number to identify each member of the subfamily [102, 103]. Mutations in forkhead genes often result in autosomal dominant conditions in humans, with haploinsufficiency likely. Several forkhead genes are expressed in the pituitary gland, and the best known is FOXL2 (Table 3).

FOXL2, also known as *Pfrk*, was the first forkhead to be described in the pituitary [21]. FOXL2 is important for ovarian development and function [104, 105], and it promotes female sex determination [100, 106, 107]. Mutations in the human *FOXL2* gene result in an autosomal dominant, loss of function disease called blepharophimosis, ptosis, and epicanthus inversus syndrome (BEPS), which causes eyelid abnormalities and premature ovarian failure [108]. Humans with BPES do not exhibit pituitary abnormalities, but homozygous mutant mice

reveal the role of FOXL2 in pituitary function, suggesting that loss of both *Foxl2* alleles is required to alter pituitary function [109-112].

FOXL2 expression is reported in mouse gonadotropes and thyrotropes and human gonadotropes [113, 114]. FOXL2 is expressed in most null cell and gonadotropin-subunit-producing adenomas, suggesting that FOXL2 contributes to gonadotrope differentiation and possibly influences proliferation, as FOXL2 cooperates with clusterin to regulate gonadotroph adenoma growth [115]. FOXL2 regulates activin-responsiveness of follistatin (*Fst*) in cooperation with SMAD3 [116], and it stimulates the activin responsive element of the *Gnrhr* gene promoter in αT3-1 cells [117]. FOXL2 is not necessary for *Gnrhr* expression, however, suggesting the possibility of genetic overlap and/or compensation [109]. Ectopic FOXL2 expression in transgenic mice is sufficient to drive ectopic expression of the gene encoding the glycoprotein hormone α-subunit (αGSU), *Cga* [113]. The necessity of FOXL2 for *Cga* expression is unclear because *Cga* expression is reduced in *Foxl2* knockout mice but transcripts are normal in a pituitary-specific deletion of *Foxl2* [109, 118]. This apparent discrepancy could be due to hypothalamic contributions of FOXL2 to regulation of *Cga* expression, or to the timing or efficiency of *Foxl2* deletion in conditional knockout animals.

The follicle-stimulating hormone (*Fshb*) gene is the most well studied FOXL2 target gene [Reviewed in: [119-121]]. Gonadotrope cell specification occurs in both systemic and pituitary-specific deletions of *Foxl2*, but basal and activin-stimulated FSH levels are severely impaired in both male and females. Pituitary-specific *Foxl2* knockout male mice have reduced testis size and spermatogenesis, and females have reduced ovarian weight and oogenesis [109]. Consistent with these studies, activin does not stimulate FSH secretion from primary pituitary cells from *Foxl2* mutant mice [118]. Several studies provide mechanistic insight about the regulation of *Fshb* expression by FOXL2. FOXL2 synergizes with SMADs to mediate activin stimulation of the murine and porcine *Fshb* genes [122-124]. FOXL2 is also involved in the synergy between activin and progestins on the *Fshb* promoter [125].

Less is known about the roles of other forkhead transcription factors during pituitary development. FOXO1 is expressed in many tissues including pancreas, liver, brain, adipose, and ovary [103, 126]. FOXO1 is present in quiescent cells of the developing pituitary, consistent with a role in suppressing cell cycle progression [127]. The cell specificity of FOXO1 expression in the pituitary is not clear. FOXO1 is reported in approximately half of somatotrope cells and one-tenth of gonadotropes in one study [127], but another reports expression primarily in gonadotropes and functional inhibition of *Lhb* expression [128]. Further studies are needed to establish the requirement for FOXO1 in pituitary development.

FOXE1 is important for thyroid organogenesis and exhibits transient expression at e9.5 and e10.5 in the oral ectoderm that will form Rathke's pouch [129, 130]. No pituitary defects have been detected in *Foxe1* null mice, however, suggesting that this gene may not be required for normal pituitary development [129].

Autoimmune hypophysitis is a rare disease of pituitary inflammation that leads to reduced hormone production. Some forkhead genes affect the immune system and influence pituitary hormone production, but the mechanisms are not yet understood. For example, FOXP3 and

FOXD1 are not expressed in the developing pituitary gland, but both affect pituitary hormone production [131, 132]. FOXP3 is necessary for normal development and function of regulatory T-cells, and FOXP3 deficiency causes severe autoimmune disease [133, 134]. Mice with an inactivating *Foxp3* mutation (scurfy mice) have reduced expression of the gonadotropins *Lhb*, *Fshb*, and *Cga*, suggesting that FOXP3 is indirectly important for gonadotrope function [132]. Besides its renal expression, *Foxd1* is expressed in the kidney and the mesenchyme surrounding the developing pituitary at e10.5. Mice deficient in *Foxd1* die within 24 hours after birth due to renal failure [135, 136], and there is a significant reduction in *Lhb* expression, specifically. These mice also exhibit failure of the sella turcica to form properly [131]. Thus, both FOXB3 and FOXD1 affect pituitary function.

These studies indicate that forkhead factors play an important role in pituitary development and function. There is much more to be done before we can truly appreciate the contribution of this family of factors to pituitary organogenesis and hormone production. Similar to many other transcription factor families, there may be functional overlap amongst the members of the forkhead family.

Pituitary progenitors: stem cells and the niche

Differentiated hormone producing cell types are detectable at birth in rodents [137]. Expansion of each population occurs after birth as the gland grows, driven by the hypothalamic releasing hormones, and by physiological demands [138-143]. This postnatal organ growth involves re-entry of some hormone-producing cells into the cell cycle [144-146]. The adult pituitary gland has some capacity to regenerate after tissue injury [147-149]. The renewal of growth hormone production after ablation is slow, and little or no renewal of prolactin cells is observed [150]. Thus, the extent to which regeneration is possible and the underlying mechanisms are not entirely clear. Pituitary adaptation to physiological demand has been shown to occur in three different ways: proliferation of terminally differentiated cells; trans-differentiation of differentiated cells, such as conversion of somatotrophs to lactotrophs, and/or differentiation of progenitors/stem cells.

Stem cells were first identified in adult organs with high regenerative capacity and/or turnover, including skin, liver, intestine and bone marrow [151]. In addition, stem cells are found in organs where most of the cells are post-mitotic, such as the brain [152] and heart [153]. In all these organs, stem cells share three fundamental characteristics: capacity to proliferate and self-renew; differentiation potential and ability to regenerate tissue after cell loss. The pituitary gland is an organ with low cell turnover [150], and while differentiated cells can re-enter the cell cycle, most hormone producing cells are not dividing [42]. A great deal has been learned about anterior pituitary stem cells in the last several years (reviewed in [154]). Advances are being made in identifying the niche, which appears to be associated with Rathke's cleft in humans and mice [155]. Other areas of active investigation are the cellular interactions necessary to preserve stem cells in the niche and to induce differentiation. A future challenge is to further define the steps in regulation of multi-potent progenitors and the mechanisms for guidance to specific cell fates.

To appreciate the current state of the art, we review some of the foundation studies. In 1969, a group of hormone negative cells, chromophobes, were described as pituitary stem cells [156]. Chromophobes transplanted into the hypothalamus of hypophysectomized rats underwent proliferation and differentiation into mature basophils (thyrotrophs, gonadotrophs and corticotrophs) and acidophils (somatotrophs and lactotrophs). Shortly thereafter a protocol was developed for differentiating chromophobes into basophils and acidophils *in vitro*[157]. Recent studies suggest that chromophobes are progenitors or stem cells in the pituitary that respond to hypothalamic signaling hormones [158]. Different groups, using diverse approaches, have demonstrated the presence of cells in the pituitary with progenitor or stem cell capacities such as self-renewal and differentiation into multiple cell types. More work needs to be done to characterize the pituitary stem cells, progenitors, and transit amplifying cells and to understand the regulation of progression through these steps. In this review, we outline the varied approaches to identifying pituitary stem cells.

Folliculo-stellate cells are non-granular cells with long cytoplasmic projections that confer a star-like morphology. They are located in the parenchymal tissue of the anterior pituitary gland. Folliculo-stellate cells are immunopositive for S100 and for glial fibrillary acidic protein, and they constitute 5–10% of the pituitary cells in the adult gland. They are organized in a functional network with endocrine cells, which they regulate in a paracrine manner by producing growth factors and cytokines. Their long cytoplasmic processes and gap junctions facilitate inter-cellular communication. They also act as scavenger cells with phagocytic activity [159]. A subset of the folliculo-stellate cells may be a source of pituitary stem cells, and another subset may be involved in creating a niche or a nurturing role. Additional markers are necessary to resolve the different populations of folliculo-stellate cells and assess their function more directly.

One characteristic of progenitors and stem cells is the ability to form colonies *in vitro*. Thomas's group was the first to demonstrate this for the pituitary [160]. The murine colony-forming cells (CFC) represent 0.2% of the anterior pituitary cells and may be a subpopulation of folliculo-stellate cells, based on the expression of S100 and GFAP, and on their capacity to take up the fluorescent dipeptide β-Ala-Lys-Ne-AMCA [160]. Only AMCA-positive cells, which constitute 3.7% of the pituitary cells, were able to form CFC, but only 12.3% of them did, consistent with the apparent heterogeneity of the folliculo-stellate cell population [160]. Angiotensin-converting enzyme is expressed in cells lining the remnant of Rathke's cleft and in the subluminal zone, which are areas proposed to comprise the niche and a source of precursor cells in the adult pituitary [161]. Cells sorted for angiotensin converting enzyme, but not SCA1, enriched the AMCA positive population in CFC [161]. Moreover, 6 weeks after implantation of AMCA-positive, GH-negative cells, 3.3% of them could differentiate *in vivo* and express GH [162]. These studies have confirmed the progenitor potential of a subpopulation of folliculo-stellate cells, based on their ability to form colonies *in vitro* and to differentiate *in vivo*. Evidence of self-renewal and differentiation into other pituitary lineages is necessary to conclude that CFC with these markers are truly pituitary stem cells.

Using different approaches Vankelecom and colleagues found adult pituitary cells with progenitor or stem cells characteristics [158]. One method is based on the concept that stem

cells exclude harmful components, such as rapid efflux of Hoechst dye, and the other method relies on clonal sphere formation. Cell sorting of bone marrow cells incubated with Hoescht 33342 reveals a side population of cells with rapid efflux that contains multi-potential hematopoietic stem cells markers [163]. This approach has been successful in many identifying stem cells in many tissues, including the pituitary gland [158, 164]. In the pituitary, this side population is composed of cells expressing high and low levels of the stem cell marker SCA1, representing 60% and 40% of the population, respectively. Two pieces of evidence support the idea that pituitary progenitor cells are in the non-high SCA1 fraction. This latter group of cells also expresses transcription factors characteristic of Rathke's pouch progenitors, including *Hesx1, Prop1, Pax6* and *Lhx4* (Table 2). More importantly, only non-high SCA1 cells can form spheres that can give rise to all endocrine cell types of the anterior pituitary [165]. This is in agreement with the demonstration that progenitors are confined to the angiotensin-converting enzyme positive, SCA1 negative fraction [161]. Further characterization of the non-high SCA1 cells is needed to demonstrate their capacity for pluripotency and self-renewal, and to identify markers that distinguish them amongst the heterogenous side population.

In a Nobel prize-winning series of experiments, Yamanaka and colleagues demonstrated that forcing expression of a collection of transcription factors characteristic of stem cells reprograms differentiated cells to be pluripotent stem cells [166]. Expression of two of these pluripotency factors, SOX2 and OCT4, has been explored in the search for pituitary tissue stem cells [155, 167]. Evidence has emerged supporting the idea that SOX2, SOX9 and OCT4 are markers of pituitary progenitors with many characteristics of stem cells (Reviewed in [168]).

SOX2, a member of the SOXB1 subfamily of HMG box transcription factors, is required for the maintenance of several stem cell populations in humans and rodents, including the central nervous system [169]. SOX2 is expressed in Rathke's pouch during development and also in approximately 3% of adult pituitary cells, where it was found lining the cleft and also scattered in the parenchyma. SOX9 belongs to the SOXE family, and is a marker for stem cells in pancreas, retina, and central nervous system [170-173]. In some organs, members of the SOXE family modulate the activity of SOXB1 family members by promoting differentiation along specific pathways [174, 175]. SOX9 is expressed in a similar pattern to SOX2 in the mature rodent pituitary gland, but in the embryo its expression is apparently later than SOX2. A small (0.03%) population of progenitors in the adult pituitary gland, that are SOX2-positve, SOX9-negative, hormone-negative, can form pituispheres *in vitro*, which can self renew, giving to rise to secondary spheres, and they can differentiate into all of the five endocrine cells of the AP, as well as folliculo-stellate cells [167]. While these characteristics comply with most of the criteria for labeling them as stem cells, the classical definition requires at least five passages to demonstrate self-renew clearly. It is possible that they have the capacity for multiple passages of self-renewal if cultured in a milieu that better mimics the niche and/or the inductive factors produced by the organizing center in the ventral diencephalon.

Cell signaling between progenitors and other cells in the niche probably regulates the decision for stem cell maintenance vs. division to produce transit-amplifying cells.

Identifying these pathways and defining the microenvironment for stem cell survival are critically important for establishing regenerative therapies. Alvarez's group discovered that the growth factor receptor GFRa2, glial cell line derived neurotrophic factor receptor alpha 2, is a pituitary stem cell marker [155]. GFRa2 is expressed in 0.9% of adult pituitary cells lining the cleft and a few cells scattered in the anterior pituitary parenchyma. These cells express several stem markers, such as SOX2 and OCT4, and interestingly, they are positive for PROP1, the early acting, pituitary-specific transcription factor that is essential for maintenance of all pituitary cell types in humans [176]. GFPRa2 positive, PROP1 positive cells are slowly proliferating cells that can form spheres *in vitro*, generate secondary pituispheres, and differentiate into the five pituitary lineages.

Additional markers are needed to define the progenitors and supporting cells. During embryonic development, proliferating cells are enriched around the remnants of Rathke's cleft. This multilayer zone is described as the marginal zone or the niche for potential pituitary stem cells. Marginal cells are not granular; they have a poorly developed endoplasmatic reticulum and an abundance of free ribosomes and polysomes (reviewed in [154]). The idea that marginal cells are stem cells came from the demonstration that nestin is expressed in cells lining the pituitary cleft adjacent to the marginal zone [177]. Using a genetic approach, an adult pituitary stem cell population was identified that expresses nestin and can generate all of the differentiated anterior pituitary cell types [178]. Nestin transgene expression appears to mark a subset of cells in Rathke's pouch that do not express endogenous nestin, however [179]. Regardless, the ability of individual progenitors to produce all anterior pituitary cell types has been demonstrated in pituisphere cultures [167].

PROP1 may be an important player in establishing and/or maintaining a pool of pituitary progenitors. Humans with *PROP1* mutations have progressive hormone deficiencies that are usually first associated with growth insufficiency and reduced production of growth hormone, TSH, and gonadotropins. If untreated, pituitary hormone levels progressively decline, and eventually all anterior pituitary hormones may be lost, including ACTH [176]. While this evolution is not obviously mimicked in mice, multiple lines of evidence suggest a role in the transition from proliferation to differentiation [180]. PROP1 expression overlaps with several stem cell markers including SOX2, OCT4, and GFRa2 [155, 181, 182]. In addition, *Prop1* is expressed in a transitional zone in between proliferating and differentiating cells during fetal pituitary organogenesis. This transitional zone is also marked by expression of cyclin E and Notch2 [44, 55]. Notch signaling stimulates *Prop1* expression, suggesting a feed forward loop [53, 55]. Definitive studies are needed to assess the potential of *Prop1* expressing cells to form pituispheres and to trace the lineages of cells that derive from *Prop1* expressing progenitors.

An elegant study by Sasai and colleagues demonstrated that embryonic stem cells could be programmed to recapitulate Rathke's pouch formation and produce functional, differentiated corticotrophs [183]. Remarkably, transplantation of these induced corticotrophs into the kidney capsule of hypophysectomized mice was sufficient to rescue their stress response. The manipulations that guided this differentiation were developed from the knowledge that anterior pituitary development is stimulated by the neural ectoderm and regulated by WNT,

Notch, BMP and FGF signaling. It would be especially exciting if protocols could be developed that would reliably direct the development of other lineages.

Cell cycle regulation

Normal organ development requires regulation of the transition from proliferation to differentiation and the maintenance of progenitors in a quiescent state while preserving the ability to recruit them to differentiation, while avoiding excess growth and adenoma formation. The events of the cell cycle have fundamental similarities in eukaryotic cells from the yeast to vertebrates, and a brief overview is valuable for the interpretation of normal pituitary development and disease states [184, 185]. The genetic material is copied in the synthetic (S) phase and divided between two daughter cells in the mitosis (M) phase. These two phases are separated by gaps (G_1 and G_2) as the cell prepares for the next phase (Fig. 2). Cell differentiation typically occurs concomitant with cell cycle exit, from G_1 to the G_0 phase. In some cases stimulation can recruit quiescent cells to re-enter the cell cycle. Such recruitment can occur during the normal tissue homeostasis, response to physiological challenges, and regeneration or wound healing [186].

Cell cycle progression is regulated by critical checkpoint surveillance mechanisms. These are related to mitotic spindle assembly and position, and DNA integrity, including complete replication and proof reading for DNA damage [187, 188]. Checkpoint blockage causes cell cycle arrest, but surveillance failure can permit uncontrolled proliferation. The length of a cell cycle varies considerably. Embryonic stem cells have a shorter cycle time than adult tissue stem cells or differentiated cell types [189]. Generally, the G1-phase lengthens substantially over time while cells progress towards differentiation, as demonstrated by sorting cells labeled with the DNA binding dye propidium iodide [190-192].

Mammalian cells have evolved a high degree of molecular regulation of the cell cycle in which multiple controller protein heterodimers provide checks and balances [184]. The presence of cyclins (*Ccn*) and cyclin-dependent kinases (*Cdk*) can dominate distinct cell cycle phases. Multiple signaling pathways regulate these proteins for endogenous checkpoint surveillance and response to external stimuli [193]. Phosphorylation and de-phosphorylation events and controlled protein degradation are significant parts of this process. One example is the phosphorylation of the tumor-suppressor retinoblastoma protein in late G_1 phase, which allows dissociation from the E2F1 transcription factor, and induction of gene expression necessary for G_1 to S phase progression [194]. Retinoblastoma remains phosphorylated and E2F1 dissociated, until M-phase is completed.

In humans and mice there at least 30 cyclin and 25 cyclin-dependent kinase and kinase-like genes known (http://www.ncbi.nlm.nih.gov/gene/), which illustrates the complexity and the potential for redundancy in cell cycle regulation. The cyclin-dependent kinase inhibitors (*Cdkns*) form inhibitory protein complexes with their phase representative counterparts. Many of these *Cdkns* affect at least G1 specific *Ccns* and *Cdks*, including *Ccnd1-3, Ccne1-2* and *Cdk2/4/6* [184, 195, 196]. The CIP/KIP group includes *Cdkn1a*, *Cdkn1b* and *Cdkn1c*, which are also known as *p21, p27* and *p57*, respectively. A different group is comprised of INK4

(inhibitor of CDK4) and *Cdkn2a*, *Cdkn2b*, *Cdkn2c*, and *Cdkn2d* or *p16*, *p15*, *p18* and *p19*, respectively [44].

During pituitary development, $p57^{Kip2}$ (*Cdkn1c*) and cyclin E (*Ccne*) mark the exit of proliferating progenitors from the cell cycle, yielding non-cycling, undifferentiated precursors [100]. Differentiation is accompanied by extinction of $p57^{Kip2}$ and cyclin E expression and activation of $p27^{Kip1}$ (*Cdkn1b*) expression. $p57^{Kip2}$ deficiency causes pituitary overgrowth, possibly because it normally limits progenitor expansion, thereby controlling the size of the progenitor niche and the organ. $p57^{Kip2}$ and $p27^{Kip1}$ probably have redundant activities as inhibitors of the cyclin E complex. The redundancy of cell cycle regulators poses a challenge in understanding how the gateway to differentiation is regulated, and how transcription factor deficiencies result in mis-regulation of this process.

Single gene global knock out of cell cycle regulators rarely leads to a pituitary phenotype (Table 4) (reviewed in [197]). The intermediate lobe is most frequently affected, which contains melanotropes in mice and is rudimentary in humans [77]. Anterior pituitary hyperplasia is detected in *p57*$^{-/-}$ mice during embryonic life, in contrast to other knockouts in which the hyperplasia appears much later. The study of pituitary adenomas in mice can be confounded by the normally high incidence of adenomas at advanced ages, a characteristic that is genetic background dependent [198]. Pituitary hypoplasia is characteristic of *Pttg1* and *Cdk4* knockouts [199-201]. Because cell cycle regulators have overlapping functions, double and triple loss of function mutations usually exhibit more severe phenotypes [44, 199, 202-205]. For example, triple knockouts of *Cdk2*, *Cdk4*, and *Cdk6* die at E14.5, underlining the indispensability of *Cdk1* for the cell cycle [206]. There is a great deal of functional overlap and compensation amongst cell cycle regulators because these pathways are so important.

The role of cell cycle regulators, oncogenes and tumor suppressors in pituitary adenomas is beginning to emerge [207, 208]. Most pituitary adenomas are benign and sporadic, although some familial types exist [18, 209]. These include multiple endocrine neoplasia, due to mutations in menin (MEN1) or cyclin-dependent kinase inhibitor 1B (p27, MEN4), Carney Complex caused by mutations in protein kinase A regulatory subunit-1-alpha, *PRKAR1A*, and aryl hydrocarbon receptor interacting protein (AIP). An active area of investigation involves studying the therapeutic potential of drugs that affect cell cycle regulators, like the histone deacylase inhibitors (HDACs) that affect the *p53*, *p21* DNA-damage pathway [187, 210].

Vascularization and the hypophyseal portal system

Normal pituitary function is dependent upon development of the hypophyseal portal system. The steps in anatomical development have been catalogued using India ink, fluorescent gelatin, and immunostaining for markers like platelet endothelial cell adhesion molecule, yet the molecular mechanisms that regulate development of the vascular system are mostly unknown [15, 139, 211]. Moreover, it is not clear whether the invasion of the vasculature has a direct role in stimulating pituitary differentiation. A variety of angiogenic and anti-angiogenic factors are expressed in normal pituitary gland, and VEGF and FGF are amongst the best-studied [212]. VEGFA is expressed at the appropriate time to have a role in

stimulating the vascularization of the pars distalis. Expression coincides with penetration of the portal vessels into the pars distalis and connection with the secondary capillary plexus, at e15.5 in the rat [211]. VEGFA expression is detectable in folliculostellate cells and some hormone-positive cells of the pars distalis. Normally, the pars distalis is much more vascularized than the pars intermedia. Ectopic expression of VEGFA in the pars intermedia causes reduced expression of the differentiation markers MSH and prohormone convertase 2 and increased growth of the lobe [213]. Does vascularization of the pars distalis affect its differentiation? Treatment with an anti-VEGFA antibody reduces pituitary growth and serum prolactin levels in mice predisposed to multiple endocrine neoplasia [214]. Radiologic studies suggest that development of the hypophyseal arteries and portal system may be abnormal in some children with hypopituitarism, but it is not clear whether this is the cause or the effect [215]. VEGFA expression is not sufficient for normal angiogenesis because *Prop1* mutant pituitaries express VEGFA, but they have poor vascularization, failed differentiation and increased apoptosis [139]. More research is necessary to identify the mechanisms that regulate normal vascularization and to decipher the influence of vascularization on pituitary differentiation.

Conclusion

Exploiting new technologies and diverse model systems will undoubtedly advance our understanding or pituitary development. Next generation sequencing and bioinformatics make it feasible to monitor developmental and cell specific changes in gene expression and chromatin accessibility on a genome wide scale. Zebrafish provides the opportunity to enhance and suppress gene expression at various developmental times [216]. The chick and frog offer the possibility of tissue transplantation during development [217-219]. The mouse excels in genetic engineering, and has recently delivered breakthroughs in manipulating stem cells to differentiate into hormone producing cells [183]. Finally, human patients always identify the genes of relevance.

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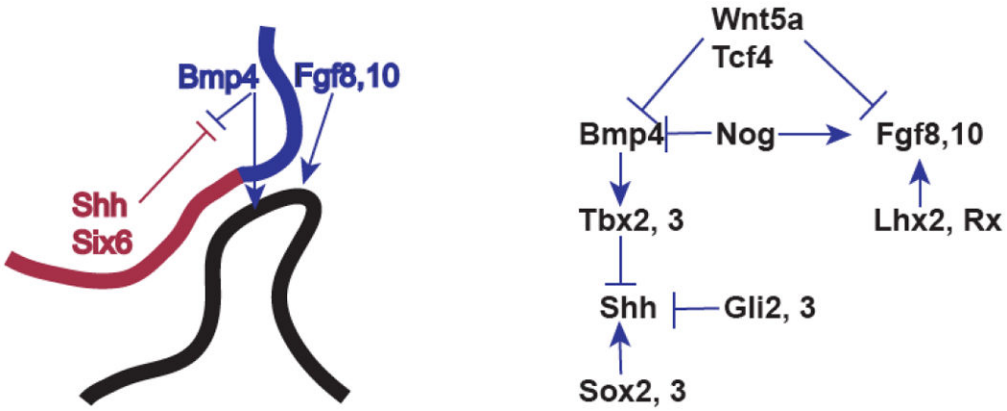

Fig. 1. Signaling pathways initiating in the organizing center regulate anterior pituitary gland growth and shape

The oral ectoderm invaginates at the roof of the mouth to produce Rathke's pouch (black). The overlying neural ectoderm is defined molecularly by expression domain of *Shh* and *Six6* and a more dorsal region expressing *Bmp* and *Fgf*. These regions exert inhibitory effects on each other, creating a balance that induces normal patterning and growth of Rathke's pouch. The interaction between stimulatory and inhibitory transcription factors and signaling molecules is diagrammed (right).

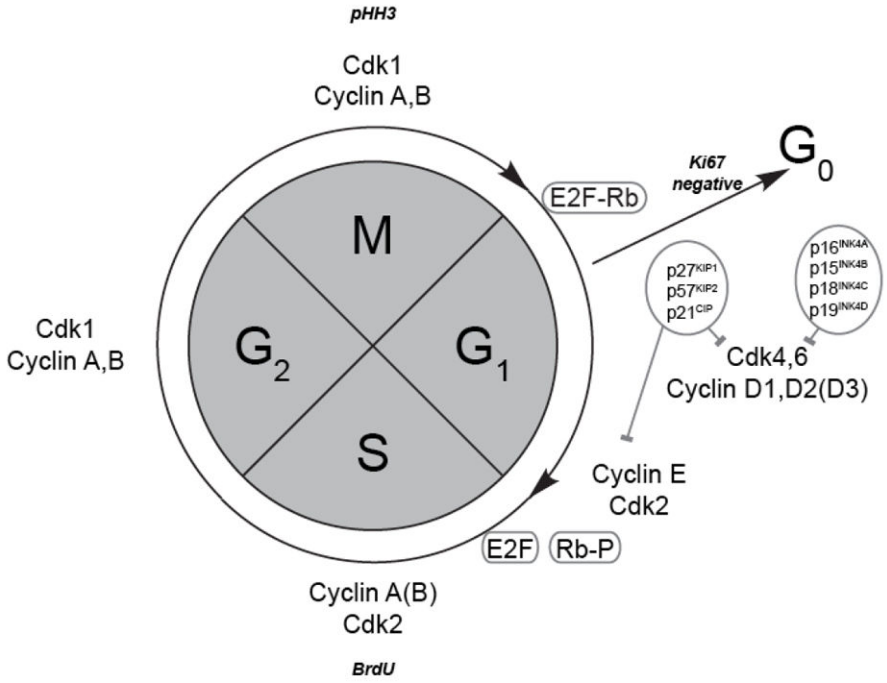

Fig. 2. Regulation of the cell cycle

Immunostaining for phospho-histone H3 (PHH3) and incorporation of bromodeoxyuridine (BrdU) marks the mitosis (M) and synthesis (S) phases of the cell cycle. Ki67 staining is absent in quiescent cells. Association of E2F with Rb is disrupted when Rb is phosphorylated at the juncture between G1 and S phase. A variety of cyclins and cyclin dependent kinases are expressed and have critical roles at specific points in the cell cycle.

Table 1

Effects of signaling pathways demonstrated in genetically engineered mice

Gene	Disruption	Phenotype	Reference
BMP related			
Bmp4	Mouse knockout	Failure to induce Rathke's pouch	21
Bmpr1a	conditional knockout, *Cga-cre*	Hypoplastic Rathke's pouch, loss of *Isl1* expression	29
Noggin	Mouse knockout	Expanded Rathke's pouch, selective expansion of organizing center	29
Cga-Bmp4	transgenic expression	Prevent terminal differentiation, get intermediate markers	21
Pitx1-Noggin	transgenic expression	Hypoplastic Rathke's pouch	21
Cga-ΔBmprII	transgenic expression	Loss of *Pou1f1* lineage, expanded ACTH, LH still present	21
FGF related			
Fgf8	*Nkx2.1* knockout, reduced *Fgf8* expression	Hypoplastic Rathke's pouch, increased apoptosis	5
Fgf8	*Fgf8* hypomorph from Neo insertion	Variable, loss of anterior lobe to normal morphology with loss of LH	24
Cga-Fgf8	aGSU-FGF8 transgenic expression	Increased proliferation, loss of all but ACTH, maitain progenitor state	21
Fgf10	Mouse knockout	Hypoplastic Rathke's pouch, increased apoptosis	23
Fgfr2	Mouse knockout	Hypoplastic Rathke's pouch, increased apoptosis	22
WNT signaling			
Wnt5a	Mouse knockout	Expanded Rathke's pouch, expanded organizing center	32
Wnt4	Mouse knockout	Reduction in GH, TSHβ, and αGSU (*Cga*)	21
Wnt4	Mouse knockout	Slight reduction in GH and TSHβ	21
Wnt6	Mouse knockout	No affect	33
Tcf7l2 (Tcf4)	Mouse knockout	Rathke's pouch hyperplasia, expanded organizing center	33
Ctnnb1 (β-catenin)	*Pitx1-cre* conditional knockout	Loss of POUF1 lineage	33
Ctnnb1	*Pou1f1-cre* conditional knockout	No affect	31
Lef1	Mouse knockout	Increased POUF1 lineage	45
Ctnnb1	*Pitx1-cre* conditional activation of *Ctnnb1*	Hypoplastic Rathke's pouch	45
Ctnnb1	*Hesx1-cre* conditional activation of *Ctnnb1*	Craniopharyngioma; increase in stem cell population	45
Pou1f1-Ctnnb1	*Pou1f1*-cre conditional activation of *Ctnnb1*	No affect	45
Gh-Ctnnb1	*Gh-cre* conditional activation of *Ctnnb1*	No affect	46
Prl-Ctnnb1	*Prl-cre* conditional activation of *Ctnnb1*	No affect	46

Gene	Disruption	Phenotype	Reference
Aes	Mouse knockout	Dysmorphic intermediate lobe	46
Shh signaling			
Hip	*Pitx1-Hip* transgenic	Hypoplastic pituitary, loss of BMP2	50
Shh	*Cga-Shh* transgenics	Increased BMP2, expanded thyrotropes and gonadotropes	50
Gli2	Mouse knockout	Loss of BMP4, reduction of FGF8 in organizing center, reduced proliferation in anterior lobe	38
Gli2, Gli3	Mouse knockout	No pituitary	38
Shh	SBE2 (SHH brain enhancer) conditional knockout	Expasnion of BMP4 and FGF10 in organizing center	35
Notch signaling			
Rbp-j	*Pitx1-cre* conditional knockout	Expanded corticotropes, loss of POU1F1 lineage	53
NICD	*Pit1-NICD* trangenics (notch intracellular domain)	Prevention of terminal differentiation	53
Hes1	Mouse knockout	Conversion of melanotropes to somatotropes	52
NICD	POMC-cre conditional activation of NICD	Inhibition of differentiation for corticotropes and melanotropes	54
Dll3	Mouse knockout	No affect	55
Dlk1	Mouse knockout	Reduction in somatotrope number	57
Dlk1	Mouse knockout	Reduction in all anterior lobe cell types, somatropes more significantly reduced	56
Pituitary organizer affects			
Sox2, Sox3	Double heterozygotes	Expansion of FGF10 in organizing center	35
Sox3	Mouse knockout	Expansion of BMP4 and FGF8 in organizing center	220
Rx	Mouse knockout	Reduction in FGF10 in organizing center	40
Tbx3	Mouse knockout	Expansion of SHH reduction in BMP4 and FGF in organizing center	36
Lhx2	Mouse knockout	Expansion of FGF8 in organizing center	39

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Table 2

Pituitary Transcription Factors

Family, Gene	Human disease	Pituitary Function	Ref. (*)
Paired homeo			
PITX1	Congenital club foot, polydactyly, Liebenberg syndrome (homeotic arm to leg transformation)	Modest, overlaps with PITX2	88, 221-223
PITX2	Rieger Syndrome, Eyes, teeth, umbilicus	Rathke's pouch expansion	48, 224
HESX1	Septo-optic dysplasia, mild to severe hypopituitarism	Affects midline and pituitary growth	47
PROP1	Evolving hypopituitarism	Silencing HESX1, OTX2 and Activating NOTCH2 and POU1F1	47, 55, 78
PAX6	Various eye and optic nerve anomalies	Increased growth, expansion of TSH cells at expense of GH and LH	225-227
PAX7	Rhabdomyosarcoma 2, alveolar	Chromatin remodeling for selecting melanotrope fate	77, 228
Pou homeo			
POU1F1 (Pit1)	Hypopituitarism	Signature factor for somatotropes, lactotropes and thyrotropes	62, 229
POU5F1 (Oct4)	Sarcoma if fused with EWS (KO mice die at gastrulation)	Pituitary stem cell marker	155, 230
OTX1	Not known (Critical role in head development)	Expressed in pituitary postnatally. Delayed growth and puberty. Functional overlap with OTX1 and EMX1, 2 in early head development.	92
OTX2	Variable, anopthalmia, micro-ophthalmia, hypopituitarism	Expressed in pituitary organizer, neural ectoderm and transiently in Rathke's pouch	79, 231, 232
LIM homeo			
ISL1	Not known (critical role in heart development)	Rathke's pouch induction	5, 233
LHX2	Not known (regulates hematopoietic stem cells and head development)	Failure to form pituitary stalk and infundibulum. Small dysmorphic anterior lobe	234
LHX3	Hypopituitarism, variable effects on cervical spine	Pouch induction, functional overlap with LHX4	235, 236
LHX4	Hypopituitarism, variable cerebellar and skull defects	Pouch induction, functional overlap with LHX3	49, 237
T-box			
TBX2	Dose dependent heart defects	Dispensable, but marks posterior lobe cells	36, 238
TBX3	Ulnar-mammary syndrome	Required to establish *Tbx2* expression, repress *Shh*, pituitary stalk formation, growth Rathke's pouch	36, 239
T-PIT (Tbx19)	Adrenocorticotropic hormone deficiency	Signature factor for corticotropes and melanotropes	67, 69, 70
Helix-loop-helix			
NEUROD1	Allelic variants cause maturity onset diabetes of the young (MODY)	Signature factor for corticotropes Delayed corticotrope development	66, 240-242

Family, Gene	Human disease	Pituitary Function	Ref. (*)
NEUROD4 (Math3)	Unknown	Stimulate somatotropes expression of POU1F1, GH and GHRHR	53
HES1	Chronic myelomonocytic leukemia	Notch target, regulates melanotrope cell fate	52, 243
Zn finger			
GATA2	Various hematopoietic defects tbx2	Suppresses gonadotrope and promotes thyrotrope fate, Pituitary KO has modest effects, Gata3 compensation	244-246
GATA3	Hypoparathyroidism, sensorineural deafness, and renal dysplasia	Not known, overlaps with *Gata2*	245, 247
Orphan nuclear receptor			
NR5A1	Hypogonadotropic hypogonadism, Sex reversal, premature ovarian insufficiency, adrenal failure	Signature factor for gonadotropes. Activates *Gnrhr*, *Lhb*, and *Fshb*	248-250
High Mobility Group			
SOX2	Microophthalmia, anterior pituitary hypoplasia, hypogonadotropic hypogonadism	Anterior pituitary growth, stimulation of *Pou1f1*, *Gh* and *Tshb* expression, progenitor proliferation	251, 252
SOX3	Hypopituitarism, mental retardation	Neural ectoderm expression (Pituitary organizer), Rathke's pouch growth and shape	220, 253
Kruppel			
GLI2	Holoprosencephaly, central incisor, hypopituitarism	Regulates expression of BMP and FGF necessary for Rathke's pouch induction	38, 254
EGR1 (*Krox24*, *Ngf1a*)	Likely tumor suppressor in acute myeloid leukemia, myelodysplastic syndrome	*Gh* and *Lhb* expression	255, 256
EGR2 (*Krox20*)	Charcot Marie Tooth disease, neuropathy	GH production	257, 258

*due to space constraints only selected references are listed. Additional references can be found in OMIM (http://omim.org) and MGI (http://www.informatics.jax.org)

TABLE 3

Forkhead genes and pituitary function

Gene	Mutation phenotypes		Pituitary Expression	
	human	**mouse**	**Adult Cell specificity**	**Other comments**
FOXL2	*BPES, POI, dominant, haploinsufficient. normal pituitary	Systemic and pituitary specific knockouts. Homozygous loss of function causes hypogonadotropic hypogonadism.	Thyrotrope and gonadotrope	first detected in CGA-positive cells around e10.5-11.5
FOXO1	Unknown. 1/90 women with POI had potentially deleterious variants in FOXO1 [259]	Knockouts die by e10.5 due to placental defects [260]	Somatotroph and gonadotroph	Expressed in quiescent pituitary cells
FOXE1 (TTF2, TITF2, FKHL15)	Bamforth-Lazarus syndrome: thyroid agenesis, cleft palate, choanal atresia, spiky hair [129]	No pituitary phenotype thyroid agenesis, cleft palate	unknown	Expressed in oral ectoderm, e9.5-e10.5
FOXP3	IPEX, X-linked severe autoimmunity that can be fatal.	Regulatory T-cells, autoimmune disorder, infertility. Reduced pituitary *Lhb*, *Fshb*, *Cga* expression	None detected	None detected
FOXD1	Unknown	Perinatal lethal, renal failure. Abnormal sella turcica, reduced *Lhb* expression	Adult pituitary (Ellsworth, unpublished)	Expressed in mesenchyme near Rathke's pouch at e10.5

*BPES: blepharophimosis, ptosis, and epicanthus inversus syndrome

POI: premature ovarian insufficiency

Table 4

Function of cell cycle regulators in pituitary gland growth

Pituitary phenotype	targeted gene	Reference
AL hyperplasia	*Cdkn1c (p57)*	44
IL tumor	*Cdkn1b (p27)*	261, 262
	Cdkn2c (p18)	204
	Rb	263, 264
Hypoplasia	*Pttg1*	199, 203
	Cdk4	200, 201
None	*Cdk6,2,1*	206, 265, 266
	Cyclin A, B, E, D	267-272
	Cdkn1a (p21)	273, 274
	Cdkn2a (p16)	275
	Cdkn2b (p15)	276
	Cdkn2d (p19)	277
	E2f1	278
	Trp53	279