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Regulation of Pituitary Stem Cells by Epithelial to Mesenchymal Transition Events and Signaling Pathways

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Abstract

The anterior pituitary gland is comprised of specialized cell-types that produce and secrete polypeptide hormones in response to hypothalamic input and feedback from target organs. These specialized cells arise from stem cells that express SOX2 and the pituitary transcription factor PROP1, which is necessary to establish the stem cell pool and promote an epithelial to mesenchymal-like transition, releasing progenitors from the niche. The adult anterior pituitary responds to physiological challenge by mobilizing the SOX2-expressing progenitor pool and producing additional hormone-producing cells. Knowledge of the role of signaling pathways and extracellular matrix components in these processes may lead to improvements in the efficiency of differentiation of embryonic stem cells or induced pluripotent stem cells into hormone producing cells *in vitro.* Advances in our basic understanding of pituitary stem cell regulation and differentiation may lead to improved diagnosis and treatment for patients with hypopituitarism.

Keywords

Pituitary; Stem Cells; SOX2; PROP1; EMT; Extracellular Matrix

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Introduction

The ability of the pituitary gland to increase hormone production in times of demand had long suggested the existence of postnatal pituitary stem cells, and interest in pituitary stem cell biology has intensified since 2008 when SOX2-expressing cells in adult pituitary glands were first shown to possess stem cell capacity to self-renew and differentiate into all of the major hormone-producing cell types of the pituitary gland *in vitro* (1). Since then, several major advances have taken place, and these have been the focus of recent review articles (2–6). *In vitro* differentiation of both human and mouse embryonic stem (ES) cells into pituitary hormone producing cells in culture has been successfully developed and improved upon in recent years, shedding light on mechanisms which could stimulate differentiation of pituitary stem cells *in vivo* (7–10). The composition of the stem cell niche is being investigated, and an epithelial to mesenchymal (EMT-like process was shown to drive migration of progenitors out of the niche (11). Pathways regulating cell turnover are being identified (12), and the limitations of self-renewal over the life of the animal are emerging (13). In this review, after an overview of pituitary development, we concentrate on the most recent advances regarding the role of the transcription factor PROP1 in establishing stem cell pools and driving the EM-like transition, the role of signaling pathways, and the potential role of mesenchymal stem cells. We also cover the challenges of stem cell therapeutics and unanswered questions that may be the focus of future studies. The role of cancer stem cells in pituitary adenomas are the subject of a separate review in this issue (J.P. Martinez-Barbera and colleagues, this Issue).

Pituitary Organogenesis: Formation of the Mature Gland from Multiple Embryonic Origins

Contributions of Surface Ectoderm and Neural Ectoderm

The pituitary gland is an endocrine organ found only in vertebrates, and aspects of its development and the nature of the specialized hormone-producing cell types are evolutionarily conserved across vertebrate species (14). Thus, studies in birds, amphibians, fish, and mammals have informed our understanding of cell specification and pituitary development (15–18). For example, the roles of FGF, BMP, SHH and WNT signaling pathways in pituitary development have been established in multiple species. Fate mapping studies in several different species have revealed that the mature pituitary gland is composed of cells that originate from the surface (oral) ectoderm, the neural ectoderm, and the cranial mesenchyme. The oral ectoderm forms the anterior and intermediate lobes, and the neural ectoderm forms the posterior lobe, while the cranial mesenchyme forms vasculature and connective tissue within and surrounding the mature gland.

Craniofacial placodes are specific regions of the non-neural surface ectoderm that thicken in relation to the adjacent ectoderm, and will give rise to the pituitary gland as well as several other craniofacial structures. The pituitary, lens, olfactory, otic, trigeminal and epibranchial placodes (in mammals) arise from the preplacodal region, which is a region of the surface ectoderm adjacent to the neural ectoderm (Figure 1). The early placodes utilize common signaling pathways and genetic networks as they form the initial placode stages, before

diverging and activating unique placode-specific programs to form the distinct tissue systems (19–21). Thus, these placode expression studies provide candidate genes for regulation of pituitary development, and some of the mechanisms that underlie stem cell regulation in other craniofacial placodes may apply to the pituitary gland.

The pituitary (or adenohypophyseal) placode is a thickening of the oral ectoderm at the roof of the mouth that invaginates to produce Rathke's pouch (reviewed in (18)) (Figure 2). It is in juxtaposition to the neural ectoderm of the ventral diencephalon, which evaginates to form the infundibulum (pituitary stalk) and posterior lobe (neurohypophysis or *pars nervosa)* (22). The infundibular region of the ventral diencephalon expresses the morphogenetic proteins BMP4, FGF8, and FGF10, which act as a signaling center, or pituitary organizer, for the induction and proliferation of Rathke's pouch (reviewed in(23)). SHH is initially expressed in the ventral midline throughout the neural tube; however, in the infundibular region of the ventral diencephalon, SHH expression is inhibited by BMP signaling and by TBX2 and TBX3. Persistent SHH expression in the infundibular region results in loss of the pituitary organizer and reduction or loss of Rathke's pouch (24,25). The pituitary organizer, especially FGF signaling, also acts as a chemoattractant for axons of oxytocin and vasopressin expressing neurons located in the paraventricular nucleus of the hypothalamus (26). The progenitor cells within the infundibular region give rise to the pituicytes, the glial-like cells of the posterior lobe, and Notch signaling through *Hes1* and *Hes5* is necessary for promoting pituicyte fate specification (27). The posterior lobe and pituitary stalk are continuous with the median eminence of the hypothalamus, which releases hypothalamic hormones into the hypophyseal portal system and regulates anterior pituitary hormone secretion.

FGF signaling from the pituitary organizer promotes the survival of the pituitary progenitor cells in Rathke's pouch (28–30). The area of highest proliferation in Rathke's pouch is at the dorsal aspect, closest to the infundibular signaling center. This region of Rathke's pouch contains the SOX2-expressing stem cells at E12.5 and E14.5 in mouse development. As the Rathke's pouch continues to invaginate upwards, the base of the pouch will close off and completely separate from the oral ectoderm, followed by the condensation of the sphenoid cartilage between the two ectodermal structures. The dorsal (top) side of the pouch forms the intermediate lobe while the ventral (bottom) side forms the anterior lobe. The residue of Rathke's cleft defines the separation between the lobes in rodents, and it can appear as a lumen in histological sections due to the shrinkage of the tissue during processing. In humans and some other species, the intermediate lobe is not distinct, but residual cleft tissue can be found. This tissue is thought to be the stem cell niche and is referred to as the marginal zone. There is an EMT-like transition, including a reduction in E-cadherin expression at the ventral aspect of the Rathke's cleft, as anterior lobe progenitor cells begin to differentiate and delaminate ventrally and migrate laterally from the lumen, causing the expansion of the parenchyma of the anterior lobe (11,31,32).

Contributions of the Notochord and Mesenchyme

Studies of pituitary development have focused on the nature of the signaling between the ventral diencephalon and Rathke's pouch. However, both the notochord and the

mesenchyme that surrounds the developing pituitary gland are additional sources of important signaling molecules, which have the potential to influence development of stem cell pools and stimulation of differentiation. The tip of the notochord, which is a critical signaling center, terminates on the caudal side of Rathke's pouch (33). In chick explant studies the notochord can induce the invagination of surface ectoderm, producing Rathke's pouch-like structures (34). The BMP inhibitors CHORDIN and NOGGIN are expressed in the notochord and prechordal plate and are likely critical factors for regulating the induction of Rathke's pouch. In support of this idea, *Chrd*$^{-/-}$; *Nog*$^{+/-}$ mouse embryos fail to express *Nkx2.1* in the ventral diencephalon, which results in the loss of the pituitary organizer and Rathke's pouch (35). The mesenchyme on the rostral side of Rathke's pouch is derived from neural crest, while definitive mesoderm contributes to the tissue caudal to the pouch (36,37). The cranial mesenchyme has a role in supporting cell specification. It provides a permissive signal for corticotrope differentiation in the chick (34). The head mesenchyme expresses *Foxd1*, and *Foxd1*$^{-/-}$ mouse embryos have reduced LHβ expression (38). Thus, signaling from the notochord and mesenchyme plays a role pituitary development. Additional studies are needed to define the cellular targets of these signaling pathways and the detailed molecular mechanisms whereby ectoderm and mesenchyme regulate pituitary stem cell behavior and differentiation.

Both the neural crest and definitive mesoderm derived head mesenchyme make direct contribution to the vasculature of the developing pituitary. Pituitary angiogenesis begins as the vessel network from the surrounding tissues enters between Rathke's pouch and the ventral diencephalon at E10.5 in the mouse (Figure 3). The infundibulum is vascularized first, as seen by PECAM staining at E11.5, followed by the anterior lobe at E13.5 (39).

The neural crest produces pituitary pericytes, contractile cells that wrap around endothelial cells controlling blood flow (37,40). Endothelial cells in the head are derived from the definitive mesoderm, and the endothelial precursors migrate throughout the head, penetrating regions where the head mesenchyme is primarily neural crest derived (41,42). The head mesenchyme that invades the pituitary anterior lobe from the rostral side is, therefore, a mix of neural crest and definitive mesoderm derived progenitor cells that form both the pericytes and endothelial cells of the pituitary vasculature. It expresses genes associated with stem cells, including *Prrx1, Prrx2*, and *Nestin* (43). Some of this head mesenchyme is likely to maintain stem cell potential in the mature pituitary gland. Recent studies suggest that pituitary adenomas contain a stem cell population, known as mesenchymal stem cells or mesenchymal stromal cells (MSCs), which can produce mesenchymal derivatives, as opposed to the hormone producing cell types generated from pituitary sphere forming colonies (44,45).

MSCs are defined by their ability to differentiate into adipocytes, osteoblasts, and chondrocytes when cultured *in vitro* (46). They were originally isolated from bone marrow based on their osteogenic, instead of hematopoietic, potential (47), but have been isolated from most tissues, including umbilical cord blood, adipose tissue, dental pulp, and breast milk (48–52). MSCs typically represent a heterogeneous population, and recent results demonstrate that both neural crest and mesodermal lineages produce the mixed MSC population derived from bone marrow (53,54). Both neural crest derived and bone marrow

derived MSCs have the capacity to form adipocytes, chondrocytes, and osteoblasts in culture, as well as ectodermal lineages, including neurons (55). The isolation of MSCs from pituitary adenomas suggests the presence of a mesenchymal cell population in pituitary neoplasms that likely originates from the neural crest derived pericytes, a hypothesis that awaits confirmation using mouse lineage tracing studies.

Mechanisms of endothelial and vascular contribution to cellular differentiation in other endocrine organs may serve as a paradigm for understanding pituitary development and developing treatments. For example, endothelial cells migrate into the pancreas to form the vasculature in response to angiogenic factors produced by the developing islet, and the endothelial cells produce factors that stimulate subsequent islet cell differentiation (56). It is not yet clear whether the invasion of the vasculature has a direct role in stimulating pituitary differentiation like it does in the pancreas, and future studies may focus on identifying the mechanisms that regulate normal vascularization and to deciphering the influence of vascularization on pituitary differentiation.

Pituitary Stem Cells: Postnatal Pituitary Cell Turnover and Responses to Hormone Demand

The ability and requirement of the postnatal pituitary gland to increase hormone production in response to physiological and pathological stimuli, and the natural turnover of cells, had historically been suggestive of the existence of postnatal pituitary stem cells. The rodent pituitary gland increases in size during the postnatal period, and once the animal reaches adulthood, the rate of cell proliferation is quite low; about 0.2% of the cells are in S phase (57). The natural cell turnover of a pituitary cell is estimated to be 60–70 days (58), but the pituitary does not decrease in size over time, meaning that cells must be replenished somehow. The pituitary gland has to increase hormone production under normal physiological events such as puberty and pregnancy, as well as under extreme conditions such as end organ disease, surgical adrenalectomy and gonadectomy, or pharmacological ablation of the thyroid gland.

During pregnancy and lactation in both humans and rats, there is an increase in the size of the pituitary gland and the proportion of lactotropes in the pituitary (59–61), and it was long thought that new lactotropes are generated in order to match the need for more prolactin. However, cell lineage tracing studies in transgenic mice have shown that enlargement of the organ mostly results from a ~20% increase (hypertrophy) in the volume of prolactin producing cells, and that there is no increase in the proportion of lactotropes during pregnancy in mice (62). Other specialized pituitary cell types also undergo hypertrophy under increased hormone demand: corticotrope volume increases 2.6-fold after adrenalectomy (63), gonadotropes increase dramatically in surface area after gonadectomy (64), and thyrotropes do so following pharmacological ablation of the thyroid (65).

At the same time, a significant portion of postnatal proliferating anterior pituitary cells are hormone-positive, suggesting that some differentiated cells have re-entered the cell cycle (66,67). At postnatal day 8–10 the majority of POU1F1 expressing cells are labeled with Ki67, indicative of active progression through the cell cycle (68,69). It is not known what

triggers differentiated cells to re-enter the cell cycle, but the hypothalamic releasing hormones GHRH, TRH and GNRH are essential for expansion of the somatotropes, thyrotropes and gonadotropes in the postnatal period, respectively (70–72). Therefore, hypertrophy and proliferation of existing differentiated endocrine cells contribute to pituitary homeostasis and plasticity together with the activity of pituitary stem cells.

Definitive evidence of adult pituitary stem cells came from Vankelecom and colleagues (73), who identified a side population of about 2% of anterior pituitary cells that are resistant to Hoechst 33342 dye uptake, express stem cell factors, and are able to form clonal spheres *in vitro*, demonstrating proliferative capacity. Nolan and Levy (74) separately identified that there is a nonhormonal pituitary cell population that proliferated and differentiated into new gonadotropes and corticotropes in response to gonadectomy or adrenalectomy, and that the same population gave rise to both (i.e. were multipotent). The identity of postnatal pituitary stem cells was subsequently identified in 2008, when Fauquier *et al.* showed that SOX2-expressing cells, found in the region lining the Rathke's cleft (marginal zone) and the parenchyma of the anterior lobe (Figure 4), were the cells able to form clonal spheres in culture, and that these spheres were then able to differentiate into all five hormone producing cell types of the anterior lobe (1).

Finally, *in vivo* evidence to show that SOX2-expressing pituitary cells are postnatal stem cells came from two groups in 2013 (75,76). Firstly, inducible postnatal SOX2-lineage tracing showed that these cells naturally give rise to new hormone cells throughout life (i.e. replenishes cells over time) (75,76). Secondly, it is also these SOX2-expressing cells that proliferate in response to gonadectomy and adrenalectomy to generate new gonadotropes and corticotropes respectively (69,76), finally confirming SOX2-expressing cells as the proliferative non-endocrine cells observed by Nolan & Levy (74). In these cases, the overall number of SOX2-expressing cells does not increase, presumably because the cells are dividing to maintain the size of the stem cell pool and producing daughter cells that progress to differentiation. Together, these studies demonstrated that postnatal pituitary SOX2-expressing cells, meaning these cells can be classed as pituitary stem cells.

Ablation of terminally differentiated somatotropes and lactotropes with an inducible diphtheria toxin paradigm can stimulate regeneration of 40–60% of the lost somatotropes and lactotropes, mainly by differentiation directly from SOX2-expressing progenitors but also from proliferation and transdifferentiation of endocrine cells (13,77,78). In contrast to gonadectomy and adrenalectomy, the ablation of somatotropes and lactotropes caused a doubling of SOX2-expressing cells and clonogenic potential. The basis for this difference is not known, but it could reflect the larger population size of the somatotropes and lactotropes relative to gonadotropes and corticotropes. Taken together, these studies demonstrate that pituitary SOX2-expressing cells are the cells required for both organ maintenance and response to physiological challenge, and thus can be classified as pituitary stem cells.

Other markers expressed in the SOX2 stem cell niche include *Sox9, Oct4, Gfra2, Ret, Ly6a (Sca-1), Prop1, Prrx1, Cdh1* (E-cadherin), *S100β*, β-catenin, neuronatin, neurturin, and *Cxadr* (Coxsackievirus and adenovirus receptor) (3,73,75,76,79–81). Clonogenic potential has been demonstrated for cells expressing *Sox2, Sox9, Gfra2*, and *S100b*, but the remaining

markers have not been tested yet. These factors are expressed in various combinations in some SOX2 cells, and the differences between these different subpopulations remain unclear, but it is well established that SOX2-expressing cells are stem cells (75,76).

In mammals, the glial cell-line derived neurotrophic factor (GDNF) family is composed of four different factors that bind to GDNF receptor alpha (GFRα1–4), that act as co-receptors for the tyrosine kinase RET and facilitate ligand binding. Alvarez and colleagues made an important contribution to the field by demonstrating expression of GFRa2 in the adult rodent and human pituitary glands (79). GFRa2 expression is detected in the marginal zone and in cells scattered throughout the anterior lobe, representing 0.9% of the total cells. The GFRa2 positive cells are also positive for the pituitary specific homeodomain transcription factor PROP1. GFPRa2 positive cells are slowly proliferating and able to form spheres *in vitro*, can generate secondary pituispheres, and differentiate into the five hormone producing pituitary lineages (79).

RET is expressed in ciliated cells on both sides of the marginal zone (5). RET and GFRa1 are co-localized in somatotrophs (82), where RET acts as a dependence receptor (83). The RET dependence pathway is regulated by the transcription factor POU1F1 (PIT 1). In GH cells, RET forms a complex with CASP3 and PKC-delta which leads to increased POU1F1 levels. This, in turn, induces $p19^{Arf}$ (*Cdkn2a*) transcription, p53 accumulation and apoptosis (12). RET is also expressed in lactotropes, and it may act together with POU1F1 and CASP3 to trigger apoptosis after lactation ceases (84). Interestingly, the RET/POU1F1 apoptotic pathway is active in human somatotrope adenomas, and it is inhibited by GDNF, suggesting the possibility that RET inhibitors may be effective therapeutics for aggressive acromegaly (85).

The mechanism for regulating the choice between pituitary stem cell self-renewal and transition to differentiation is not understood, but Notch signaling is necessary to suppress premature differentiation and to regulate postnatal SOX2 stem *cells in* vivo (69). In addition, the efficiency of differentiation into hormone producing cells is enhanced by withdrawing FGF and EGF from the media and plating the spheres on an uneven matrigel matrix (1,73), which is comprised of laminin, collagen IV, heparan sulfate proteoglycans and entactin/nidogen. This suggests a supportive role of the extracellular matrix in promoting differentiation (3).

Pituitary stem cells and regenerative capacity declines with age. SOX2 positive cells are more abundant in pituitaries of newborn and early postnatal mice than in adult animals (2-month-old), and aging to 7 months is associated with a substantial decrease in clonogenic potential (73,77,86–88). When 8-month-old mice are subjected to diphtheria toxin inducible ablation, there is no recovery of somatotropes, indicating that the ability to regenerate these cells has been lost (13). The basis for this decline is not known, but expression profiling has revealed an age-related reduction in expression of multiple genes in addition to *Sox2*, including members of the SHH, WNT, TGFβ and Hippo signaling pathways and numerous chemokines and cytokines. More studies are necessary to determine whether aging ablates the ability to regenerate other pituitary cell types, and to define the critical pathways that recruit stem cells to differentiate in young adult mice.

Epithelial-to-Mesenchymal Transition (EMT) Events in Pituitary Stem Cells

PROP1 is a pituitary-specific transcription factor that is required to activate POU1F1 (89), and the subsequent differentiation of GH, PRL, and TSH cells. *Prop1* mutant mice fail to develop the cell types that make GH, TSH and PRL during embryogenesis, which results in hypopituitarism and growth insufficiency, hypothyroidism and infertility (90). PROP1 is the most commonly mutated transcription factor in human patients with congenital hypopituitarism (91), and the patients tend to undergo progressive hormone loss, which can eventually result in life threatening hypocortisolism in the third or fourth decade of life (92). We have recently shown, using *Prop1-Cre* lineage tracing, that all hormone-producing cells in the anterior and intermediate lobes of the pituitary gland are derived from *Prop1*-expressing progenitors (93). Thus, PROP1 marks an important progenitor for Rathke's pouch derivative cells and affects all pituitary cell types in humans.

The progressive hormone loss in patients and the derivation of all hormone producing cells from a PROP1-expressing progenitor in mice suggested the hypothesis that PROP1 deficiency results in depletion of stem cell pools. In support of this idea, we discovered that *Prop1* is transiently co-expressed with *Sox2* in embryonic pituitary development, and using *Prop1* mutant mice we showed that *Prop1* is required for initial establishment of adequate stem cell pools, as well as for normal stem cell colony forming behavior (11). *Prop1* mutant neonates have more SOX2-positive cells than wild-types, and they remain in the marginal zone (Figure 4). Many of the PROP1-deficient SOX2-expressing cells fail to activate expression of the transitional markers *Sox9* and *Cyclin E.* The *Prop1* mutants have substantially reduced ability to form adherent stem cell colonies, and these mutant colonies exhibit abnormal, flat cell morphology. Thus, PROP1 is required to maintain the size and stem cell characteristics of the *Sox2*-expressing stem cell pool.

Expression of the angiogenic factor VEGFA coincides with penetration of blood vessels into the anterior lobe (forming the secondary capillary plexus) and connection of the secondary plexus to the hypophyseal portal system. VEGFA is co-expressed with the transcription factor PROP1 at E14.5 (Figure 5), but the relationship between these two factors is not known. *Prop1* mutant pituitaries express VEGFA (Figure 6), but they have inadequate vascularization (68), and it is not clear what inhibits this process in *Prop1* mutants. During normal pituitary organogenesis an EMT-like process is initiated. There is a reduction in E-cadherin expression at the ventral aspect of Rathke's cleft, and progenitor cells switch from tightly packed, polarized, planar shape to a rounded cell morphology, and then these cells migrate into the parenchyma of the organ where they commence differentiation (31,32,94). This process fails in *Prop1* mutant mice, resulting in a highly dysmorphic and hypoplastic anterior pituitary. Gene expression profiling of adherent stem cell colonies revealed that many genes in the Notch, TGFβ, WNT and SHH signaling pathways were down-regulated in *Prop1* mutants. In addition, *Prop1* mutant colonies have up-regulated epithelial cell markers like E-cadherin, keratins and claudins, and down-regulation of mesenchymal cell markers like metalloproteinases and the EMT inducer *Zeb2* (11). Studies in intact animals confirmed that *Prop1* is required for activation of genes like *Zeb2* and *Slug* (32,95). This is consistent with a requirement for *Prop1* to trigger EMT, in addition to initially establishing an adequately sized stem cell pool.

Several groups have conducted gene expression profiling to identify candidate genes for regulating the stem cell niche and cell migration. Gene expression profiling yielded numerous markers that might be involved in regulation of the stem cells, including many signaling molecules and genes involved in EMT (88,96,97). Kato and colleagues hypothesized that genes regulating neural stem cells may also be involved in pituitary gland stem cell regulation (3). They developed a model for EMT in which the cells in the niche are expressing SOX2, *Cxadr*, E-cadherin and TGFβ receptors. TGFβ signaling triggers cells to leave the niche and migrate, and they express *Cxadr* and vimentin, and have silenced SOX2 expression. This induces expression of *Snail1, Slug, Twist and Zeb* genes, and these, in turn, drive increased expression of matrix metalloproteinases and chemokines like *Cxcl12*, and suppress expression of E-cadherin. The chemokines are proposed to orient the direction of migration. Functional studies will help clarify the requirements for individual genes in regulating migration and EMT.

Cell Signaling Pathways Regulating Pituitary Stem Cells

Studies elucidating the specific mechanisms that regulate pituitary stem cell self-renewal, proliferation, and differentiation are ongoing. In light of their expression of SOX2, adult pituitary stem cells are likely to resemble embryonic SOX2-expressing cells and be regulated by similar signaling factors, such as BMPs, FGFs, SHH, Notch, and Wnt. Currently, only the Wnt, Notch, and SHH signaling pathways have been directly implicated in adult pituitary stem cells (86,98,99).

Transmembrane Notch receptors and their cognate ligands are expressed in the developing pituitary gland, with their expression reflecting the location of progenitor cells, suggesting that they play a role in their regulation (100,101). Studies have shown that Notch signaling is required to maintain the progenitor state and inhibit specification of certain pituitary endocrine lineages (32,69,98,101–108), akin to the role of Notch signaling in pancreatic endocrine differentiation (109). In the juvenile postnatal pituitary, conditional deletion of *Notch2* from early pituitary development causes a reduction in postnatal SOX2- and SOX9-progenitor cells and a reduction in cell proliferation (98). Similarly, conditional deletion of *Rbpjk* in *Prop1*-lineage cells causes a reduction and continued depletion of SOX2-expressing cells, and a loss of their sphere-forming capacity (69). This indicates that Notch signaling is likely involved in the maintenance of self-renewal and the pluripotent state in pituitary stem cells.

Many Wnt pathway molecules are expressed in the embryonic pituitary, and attenuation of Wnt signaling leads to reduced proliferation and reduced POU1F1 expression (89,110,111). Therefore, Wnt signaling stimulates endocrine specification during pituitary development (reviewed in (112)). However, this does not appear to be the case in adult pituitary stem cells. Over-activation of Wnt signaling in embryonic HESX1-expressing pituitary cells, encompassing most anterior pituitary cells, causes hyperplasia and formation of tumors resembling human adamaninomatous craniopharyngioma (86). Human adamaninomatous craniopharyngiomas often have increased expression of β-catenin (113). Furthermore, postnatal induction of Wnt signaling in adult SOX2-expressing cells also causes the same adamaninomatous craniopharyngioma tumor formation, indicating that pituitary stem cells

respond to Wnt signaling. However, the tumors are hormone-negative, and in fact, the affected SOX2-cells do not themselves form the tumors, and instead send out signals to induce the transformation of neighboring cells. Pituitary craniopharyngiomas and cancer stem cells are further detailed in an accompanying review in this Issue by J.P. Martinez-Barbera and colleagues.

SHH signaling also regulates pituitary stem cells. Inducible deletion of the SHH receptor PTCH in adult mouse pituitary causes over-activated SHH signaling, increased proliferation of SOX2- and SOX9-expressing cells, and increased expression of ACTH, GH, and PRL (114). This study also found high SHH and GLI1 expression in ACTH-, GH-, and PRL-expressing human pituitary adenomas, suggesting that SHH activity could be involved in stimulating pituitary stem cells into tumorigenesis.

The Hippo signaling pathway is a recently-identified candidate for regulating the pituitary stem cell population. Hippo pathway molecules are expressed during pituitary development, and its effectors YAZ/TAZ are expressed in the postnatal stem cell niche (115), suggesting they play a role there. Accordingly, Hippo pathway factors are up-regulated in the population of stem cells that proliferate in response to somatotrope ablation by diphtheria toxin (13). This is of particular note given that the Hippo pathway is interconnected with EMT, and EMT is a critical step in transitioning stem cells to differentiation (11).

Recent studies suggested that cell adhesion has an important role in the regulation of pituitary stem cell function, similar to its role in intestinal stem cell differentiation (116). In some tissues, like intestines and skin, the stem cell niche participates in ephrin and ephrin receptor signaling. Eph/ephrin signaling is involved in the regulation of many important developmental process, like cell adhesion, migration and morphology. They are expressed in almost all tissues of a developing embryo, and are crucial for maintaining stem cell properties (117).

The role of Ephs in the pituitary is not yet known. The ephrin-A5 and ephrin-B2 are expressed in pituitary organogenesis (118,119). Ephrin-A5 is expressed in the ventral hypothalamus and developing posterior lobe. Ephrin-B2 is co-expressed with SOX2 in Rathke's pouch, suggesting a role in the formation of the anterior pituitary stem niche. We found altered expression of ephrins and ephrin receptors in adherent stem cell colonies derived from *Prop1*$^{+/+}$ and *Prop1*$^{df/df}$ pituitaries (Table 1). Transcripts for the ligands *Epha4* and *Efna1* were elevated and the receptors *Ephb1* and *Ephb2* were substantially reduced in *Prop1* mutants. Interestingly, we found PROP1 DNA binding sites in genes of Eph family suggesting the possibility of direct regulation. Taken together, these data support the idea that ephrin signaling is involved in regulation of the pituitary stem cell niche (119), but further studies are needed to demonstrate a functional role.

Eph/ephrin signaling is involved in cell migration, and a relationship between eph/ephrin signaling and integrin signaling has been postulated. However, the connection between them is uncertain. While a number of studies showed that Eph/ephrin signaling increased integrin cell adhesion, others reported the opposite (120). Integrins are transmembrane proteins that attach cells to components of the **E**xtra**c**ellular **M**atrix (ECM), which provides an

environment for maintaining the stem cell niche properties. Major components of ECMs are laminin, collagen, fibronectin and proteoglycan. Several genes from these families were uniquely affected by *Prop1* deficiency in stem cell derived colonies (Table 1). Thus, these are candidates for involvement in migration and EMT that fails in *Prop1* mutants.

In Vitro Differentiation of Pluripotent Stem Cells into Pituitary Endocrine Cells

The elucidation of the signals active during pituitary development *in vivo* have contributed to major advances in the efficient differentiation of human and mouse pluripotent stem cells into functional pituitary endocrine cells (7–10), paving the way for translational application in treating hypopituitarism.

Sasai and collagues were the first to demonstrate the rescue of hypopituitarism in mice with differentiated pituitary endocrine cells derived from pluripotent stem cells *in vitro* (8). They established a 3D culture technique for mouse embryonic stem (ES) cells called serum-free floating culture of embryoid body-like aggregates (SFEB) (8). In these cultures, the ES cells are cultured at high density on low-adhesion plates in defined media containing chemicals modulating SHH signaling, causing them to form aggregates. These aggregates then self-organize into an inner layer resembling hypothalamic neural ectoderm and an outer layer resembling surface ectoderm, recapitulating the inductive process that takes place during normal pituitary development. Endogenous BMP and FGF signals from the inner layer induces the outer layer to invaginate and form a Rathke's pouch-like structure expressing pituitary-specific transcription factors like *Lhx3* and *Pitx2.* Further modulation of Wnt, Notch, glucocorticoid, and estradiol signaling causes cells in these pouches to differentiate into functional endocrine cells from multiple lineages (8). In a follow up study they successfully adapted the protocol to induce human ES cells to differentiate into pituitary hormone producing cells. Formation of aggregates with inner and outer ectodermal layers required additional inductive factors such as exogenous BMP and Rho kinase inhibitors to form the aggregates with inner and outer ectodermal layers. Exogenous FGF induces the invagination of the Rathke's pouch-like structures, and manipulation of signaling pathways also stimulates the differentiation of functional pituitary endocrine cells (9).

While the Sasai group performed *in vitro* pituitary cell differentiation in a 3D culture mimicking the developmental interaction between the neural and oral ectoderms, the Studer group developed a way to differentiate pituitary cells from human ES and induced pluripotent (iPS) cell monolayers (7,10). Timed withdrawal of BMP inhibitors from the culture media of human ES cells caused their progression into an early preplacodal fate (10). Treatment of these preplacodal-like cells with SHH agonists then induced their expression of pituitary-specific markers, and further inhibition of Notch signaling stimulates their differentiation into mature, functional pituitary endocrine cells. Combinations of exogenous BMP and FGF signals also cause the cells to adopt intermediate markers of differentiation seen in the developing pituitary gland (7).

Corticotropes differentiated *in vitro* from each of these methods were shown to be physiologically functional *in vivo* when grafted under the kidney capsule of

hypophysectomized hypoadrenal mice and rats (7–10). Future studies are necessary to determine if *in vitro*-derived, hormone secreting cells can be safely re-introduced into human patients to combat hypopituitarism. In recent decades, it has become clear that the safe and efficient translation of laboratory discoveries, notably gene and stem cell therapies, into therapeutic intervention can take a very long time (121). Great progress has been made in the stem cell therapies for spinal cord injury, macular degeneration, diabetes and heart disease, and the use of human iPS cells instead of ES cells has reduced the ethical controversy in investigating stem cell derived therapies in humans (reviewed in (122)). However, quality control for epigenetic regulation and somatic mutation will be required to insure efficacy and safety (123,124). There are also significant concerns about the cost of such treatments for relatively rare disorders, and it may not be realistic for a stem cell therapy to be more cost effective than hormone replacement therapy for CPHD, whether the cause is genetic or not. There is hope that a stem cell therapy would provide a better quality of life, however, if the production of hormones came under the control of physiological feedback loops allowing for better homeostasis. Traumatic head injury is a major cause of hypopituitarism, and recent studies suggest that the hypothalamic inputs to the pituitary are a major source of the dysfunction, which is daunting to repair (125), but there are many genetic and environmental causes of hypopituitarism where pituitary stem cell replacement could be a viable, permanent therapeutic if it were available.

Conclusion and Future Directions

Pituitary progenitor cells were long speculated to exist, and SOX2-expressing pituitary stem cells were formally identified in 2008. A great deal has been learned about pituitary stem cells since then, and in the future it will be important to understand the mechanisms that regulate embryonic progenitor exit from the cell cycle and postnatal cell cycle re-entry, as postnatal pituitary stem cells are likely controlled by similar mechanisms. The roles of ephrin and Hippo signaling, cytokines, chemokines, extracellular matrix, and EMT are exciting and promising areas for future investigation. The ability to monitor differentiation of pluripotent stem cells into hormone producing cells *in vitro* is an asset for testing the effects of individual genes and prioritizing them for *in vivo* studies in animals. Ultimately, better understanding of the factors regulating embryonic pituitary stem/progenitor cells may lead to the development of methods to stimulate postnatal pituitary stem cells to safely regenerate *in vivo* into the specific hormone cell-types required in human patients with different types of hypopituitarism.

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Highlights

- Pituitary gland organogenesis involves tissue contributions from oral ectoderm, neural ectoderm, and cranial mesenchyme.
- Establishing and maintaining pituitary stem cell pools requires SOX2 and the pituitary transcription factor PROP1.
- An epithelial to mesenchymal transition-like process is involved in migration of stem cells from the stem cell niche into the anterior pituitary.
- Signaling pathways and matrix components are important in stem cell maintenance and differentiation.
- The ability of the pituitary to self-renew is lost with age in rodents.

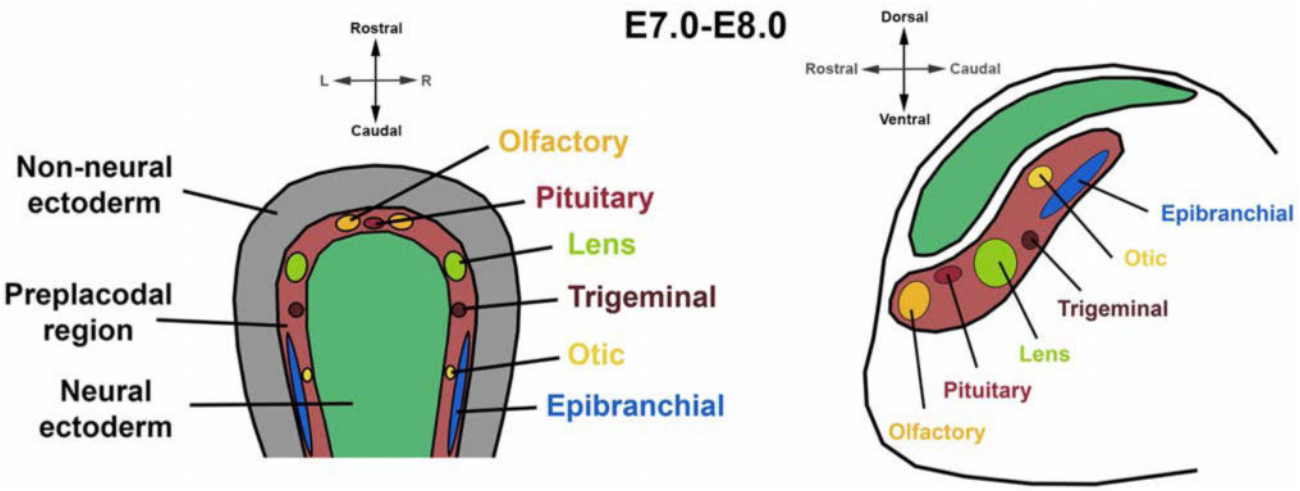

Figure 1. The Preplacodal Region is the Interface Between the Neural and Surface Ectoderms

Interaction between the surface and neural ectoderm gives rise to the preplacodal region between E7.0–E8.0 in mouse (~E15–19 in humans, ~E8.5–9.0 in rats), from which multiple craniofacial tissues are ultimately derived.

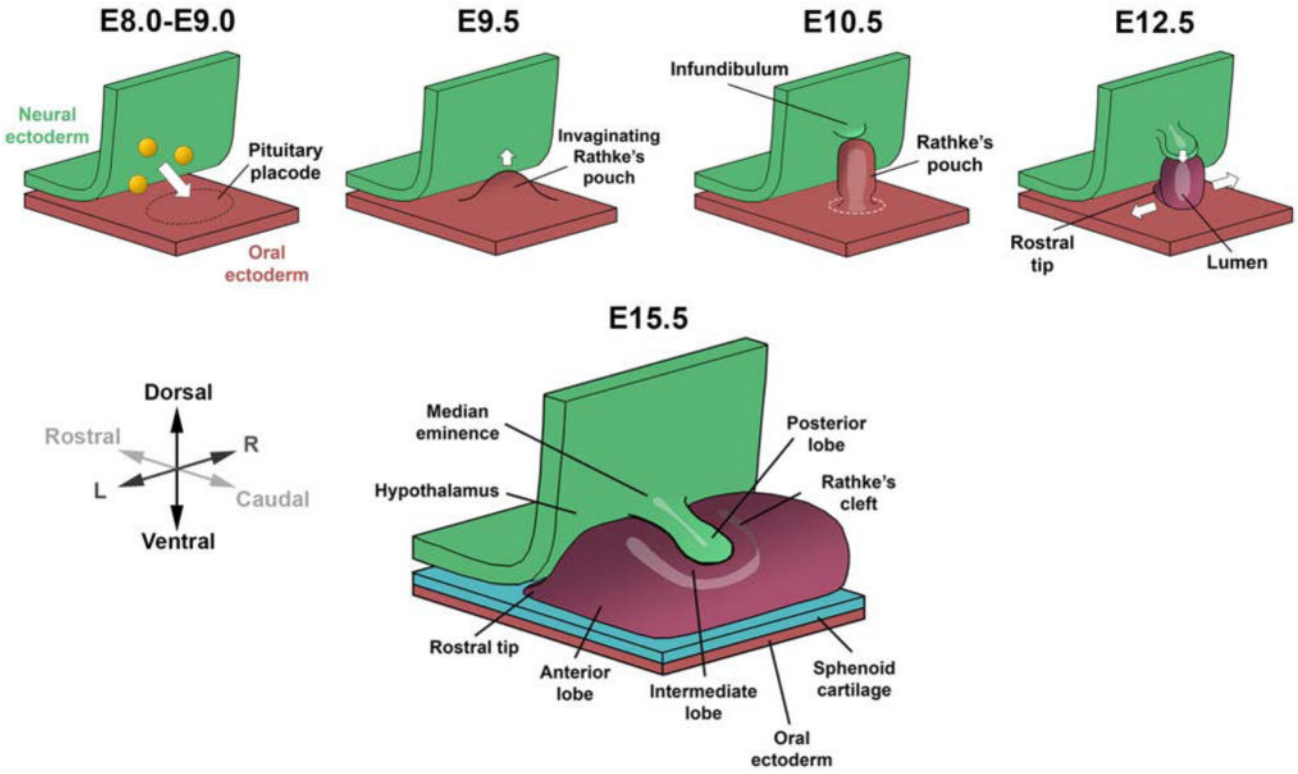

Figure 2. The Pituitary Gland Develops from Distinct Embryonic Origins

Pituitary development in the mouse begins from E8.0, when the neural ectoderm adjacent to the pituitary placode produces signaling factors (yellow) to initiate the invagination of Rathke's pouch. The pouch will begin to pinch off from the oral ectoderm at E10.5 and completely separate by E12.5. Rathke's pouch gives rise to the anterior lobe, which continues to expand ventrally and laterally. The infundibulum evaginates downwards from the neural ectoderm and forms the posterior lobe.

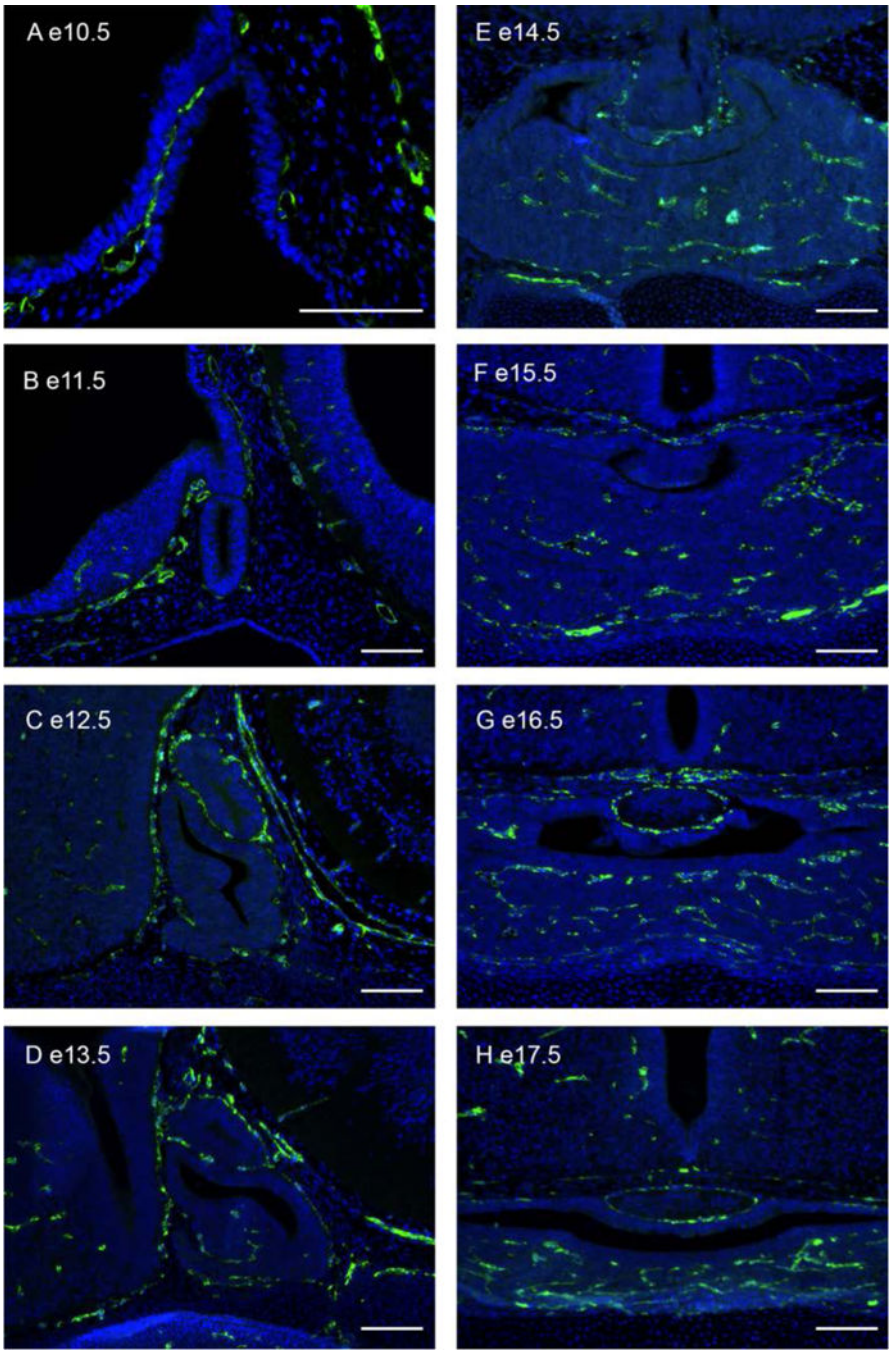

Figure 3. Vascularization of the Developing Pituitary Gland

(A–H) Immunostaining for CD-31 (PECAM, green) and counterstained with DAPI (blue). Endothelial cells surround the infundibulum at E11.5 and invade the anterior lobe beginning at E13.5. Scale bars 100 μm.

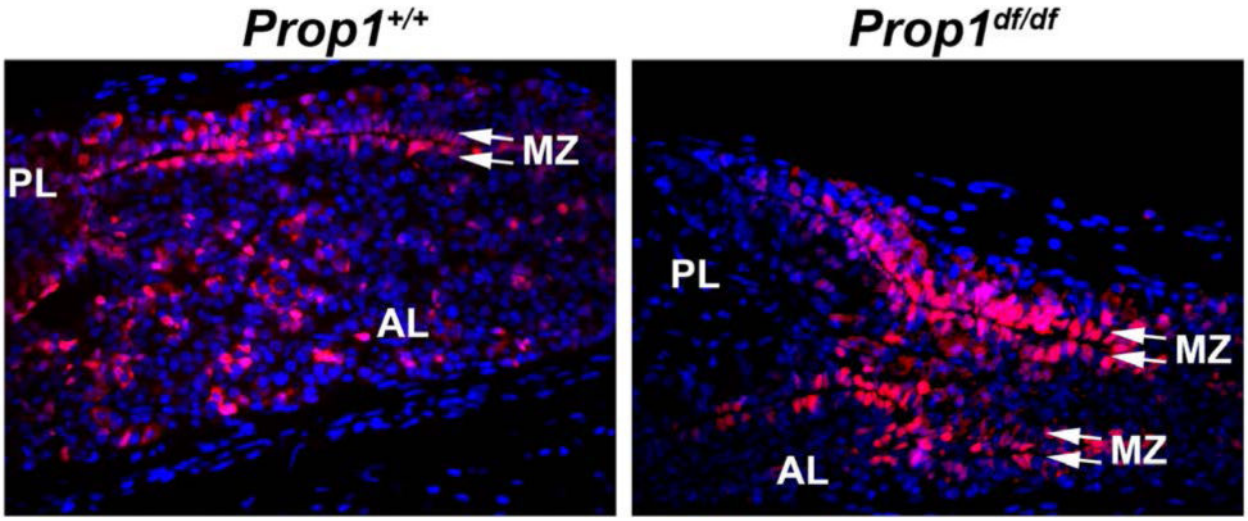

Figure 4. SOX2-Expressing Pituitary Stem Cells Line the Marginal Zone

SOX2-expressing cells (red) are found in the postnatal pituitary lining Rathke's cleft or the marginal zone and in the parenchyma of the anterior lobe. In *Prop1*$^{df/df}$ mutant mice, SOX2 stem cells cannot undergo an EMT-like process and are retained in the marginal zone, causing it to become convoluted and dysmorphic. AL=anterior lobe, PL=posterior lobe, MZ=marginal zone.

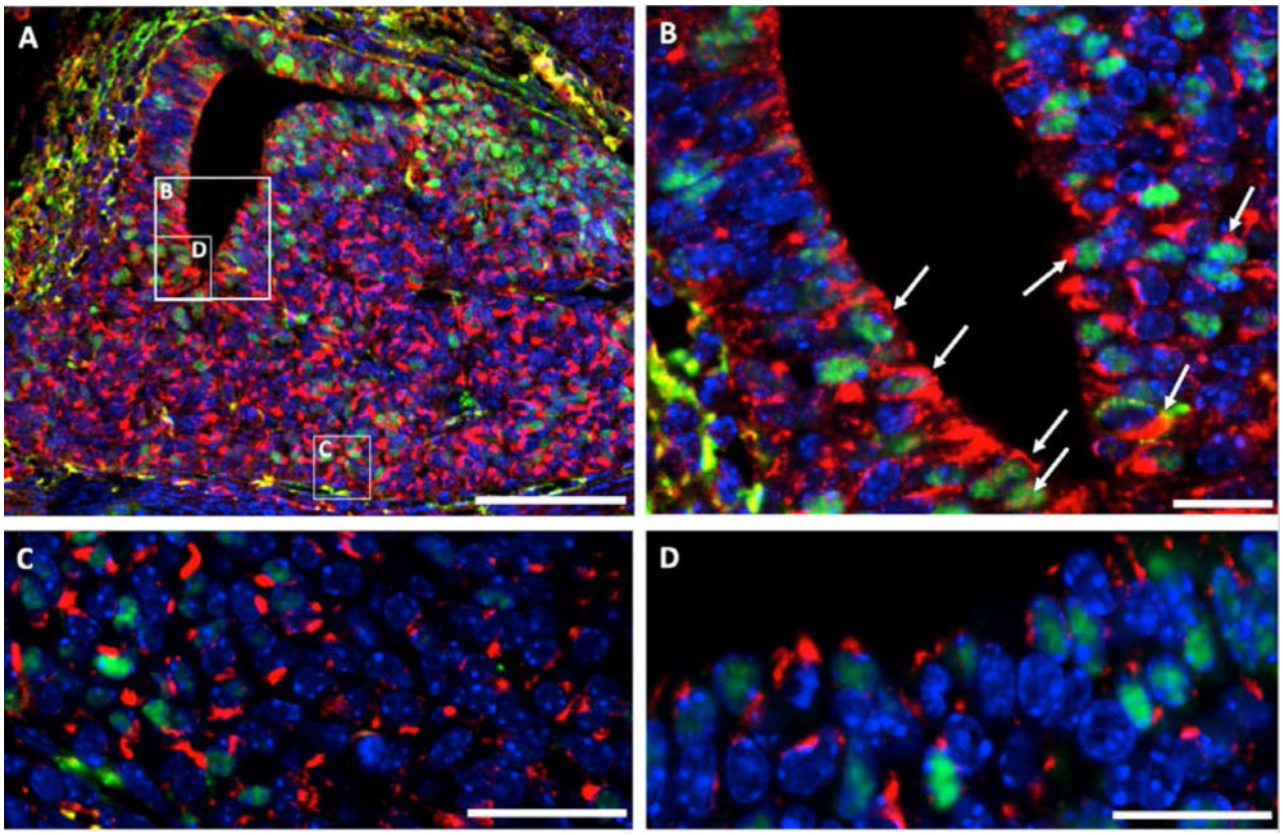

Figure 5. PROP1 and VEGFA are Co-Expressed in Pituitary Stem Cells

Double immunofluorescence reveals co-staining of PROP1 (green) and VEGFA (red) at E14.5 around the residue of Rathke's cleft (B and D) and in the anterior lobe (C). Cell nuclei were stained with DAPI (blue). The white box indicates where higher magnification photo (B) was taken. Scale bars 100 μm (A) or 20 μm (B, C and D).

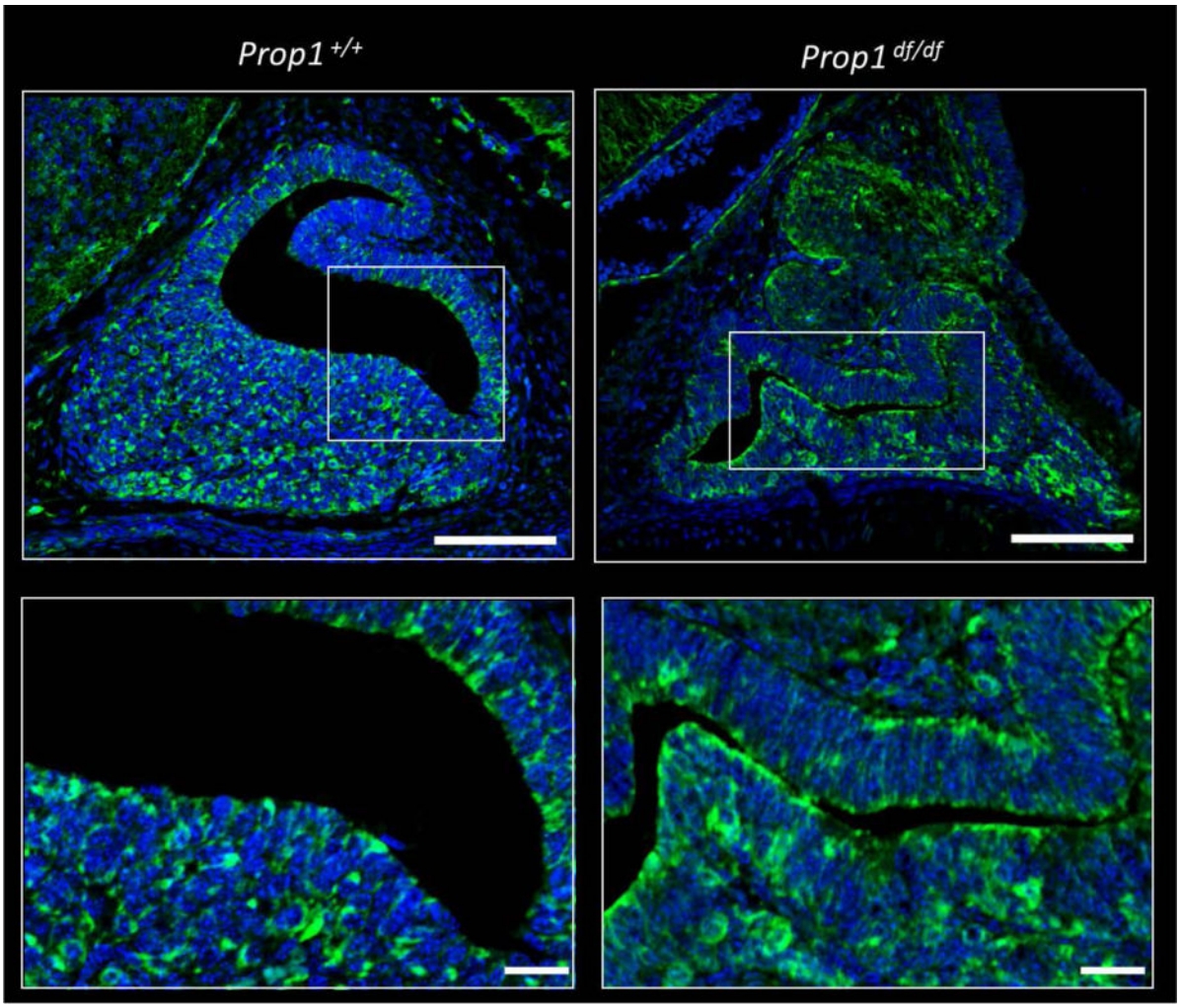

Figure 6. VEGFA Expression is Maintained in *Prop1* Mutant Pituitaries

Immunohistochemical staining for VEGFA was performed on pituitary sections from E16.5 *Prop1*$^{+/+}$ and *Prop1*$^{df/df}$ mice (green). Cell nuclei were stained with DAPI (blue). White boxes indicate where higher magnification photos were taken. Upper panel: Scale bars 100 μm. Lower panel: scale bars 20 μm.

Table 1

Prop1-Dependent Gene Expression Changes in Adherent Pituitary Stem Cell Colonies

Selected genes that were up-regulated (green) or down regulated (red) in *Prop1* but not *Pou1f1* mutant stem cell colonies relative to wild type ($p \leq 0.05$, log2FCI ≥ 1)

Gene symbol	Gene name
Ephrin and Eph Signaling	
	EPH Receptor A4
	Ephrin A1
	EPH Receptor B1
	EPH Receptor B2
Extracellular Matrix (ECM)	
	Integrin Subunit Alpha 8
	Integrin Subunit Beta 3
	Integrin Subunit Beta 4
	Integrin Subunit Beta 6
	Integrin Subuit Alpha 1
	Integrin Subuit Alpha 4
	Integrin Subuit Alpha L
	Laminin Subunit Alpha 4
	Laminin Subunit Beta 2
	Laminin Subunit Gamma 1
	Biglycan
	Osteglycin