NOTCH SIGNALING AND CELL CYCLE INHIBITOR REGULATION IN PITUITARY ORGANOGENESIS

BY

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DISSEATION

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Abstract

The pituitary is a plastic organ whose hormone cell number can fluctuate depending on physiologic need. Alterations in pituitary cell expansion can lead to disorders such as pituitary hyperplasia or tumors, which have a high prevalence in the human population. Precise control of cell cycle progression is the molecular mechanism by which pituitary cell expansion is regulated. We have investigated signaling pathways active during development, a time of rapid organ growth, to understand the process of pituitary progenitor cell division. In development, progenitors choose between proliferation and cell cycle exit, followed by differentiation. Notch signaling has been shown to promote progenitor preservation, both through repression of differentiation as well as by directing proliferation. We show that the activated Notch pathway, through HES1, serves as a proliferative signal that promotes progenitor expansion, with its loss resulting in a severely hypomorphic pituitary. HES1 transcriptional repression of Cyclin Dependent Kinase Inhibitors (CDKI) is the mechanism by which HES1 promotes cell cycle progression. Loss of Hes1 in the developing pituitary, results in increased expression of the CDKIs p21, p27 and p57. We hypothesize that this aberrant increase in expression may be a molecular mechanism contributing to progenitor pool depletion. Increased expression of CDKIs has been shown to be a hallmark of differentiating cells and loss of CDKIs has been shown to potentiate tumorigenesis of the pituitary in adult life in humans and rodent models. Studies by other groups have shown that p57 and p27 are CDKIs that modulate proliferation in the growing embryonic pituitary. With our studies now show that these molecules are not the sole contributors and that p21 may also be
responsible for controlling pituitary proliferation. To uncover the individual and overlapping roles of CDKIs in regulating pituitary cell number, we examined the pituitaries of mice lacking p21 and p27 alone, as well as double mutants. Loss of both p21 and p27 drastically increases proliferation throughout the developing pituitary, indicating that expression of these molecules is crucial to controlling pituitary progenitor cell expansion. Taken together, these studies suggest that Notch regulatory pathway activation is necessary for fine-tuning a balance between proliferation and differentiation by modulating CDKI expression during embryonic pituitary and that CDKI action is needed to restrain proliferative signaling preventing excess proliferation or even tumor formation.
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Chapter 1: Literature Review

1.1 Background and Significance

The pituitary is the master regulator, which provides for a central control of multiple endocrine tissues. The pituitary is located just below the brain and in close contact with the hypothalamus. The human pituitary is composed of posterior and anterior lobes. The posterior pituitary is comprised of axonal projections emanating from the paraventricular and supraoptic nuclei of the hypothalamus, which secretes the hormones arginine vasopressin and oxytocin. The anterior pituitary is comprised of secretory endocrine cells that originate from the oral ectoderm during development. Hypothalamus-derived releasing hormones are secreted into the primary plexus of the hypophyseal portal system, which reach the anterior pituitary and signal for anterior pituitary hormone release. Hormones are then released into the secondary plexus portal systems, carrying the hormones to responsive endocrine tissues throughout the body. The anterior pituitary is comprised of five hormonal cell types. Two hormone cell types initially express αGSU during development: thyrotropes, which express Thyroid Stimulating Hormone (TSHβ) and gonadotrope, which express Follicle Stimulating Hormone (FSHβ) and Lutenizing Hormone (LHβ). The other hormone cell types present in the adult pituitary are: lactotropes which express Prolactin (PRL), somatotropes which express Growth Hormone (GH), corticotropes which express Proopiomelanocortin (POMC) cleaved into Adrenocorticotropic Hormone (ACTH). Melanotropes, which express POMC cleaved into Melanocyte Stimulating Hormone (MSH), are a cell type found in a special subset cells in the intermediate lobe of the mouse pituitary, a structure only rudimentarily present in humans (1).

Altered pituitary cell number, leading to dysfunction, is one of the major causes of endocrine disease. Disorders display such far-reaching ramifications as dwarfism or gigantism,
metabolic dysregulation as well as reproductive disorders that lead to infertility in both males and females (2, 3). Proliferation defects during embryonic or adult life represent one major category resulting in either a hypoplastic or hyperplastic pituitary. One inherited form of hypopituitarism that manifests as a loss of growth hormone and at least one other hormone is Combined Pituitary Hormone Deficiency (CPHD). Genetic linkage studies in humans with CPHD revealed mutations in the transcription factor *PROP1* (4). Mice lacking *Prop1* have a phenotype strikingly similar to human CPHD and have been used to show that loss of the Pit-1 lineage of hormone-producing cells, including somatotropes, thyrotropes and lactotropes is the cause of the disease (5). Additionally, loss of the transcription factors *Lhx3, Lhx4* and *Hesx1* have been found to be necessary for pituitary induction and embryonic pituitary development, as well as contributing to the occurrence of CPHD (6-9).

Hyperplasia of the pituitary is a disorder that arises from uncontrolled proliferation of cells, which can result in increased hormone secretions (3, 10). Studies have shown that approximately 16.7% -35% (varying studies) of the population, over the age of 50 postmortem, will either have a hyperplastic pituitary or a pituitary tumor (2, 11). Hyperplasia of the pituitary or subsequent adenoma formation may be the result of loss of target hormone negative feedback, mutation in tumor suppressor genes, or even estrogen induction of growth factors (3). Taken together, these various forms of pituitary disease indicate that tight regulation of proliferation during pituitary development is necessary for the prevention multiple forms of pituitary dysfunction. Further investigation, utilizing transgenic and knockout mouse models, into the mechanisms that control proliferation in the pituitary is needed and the subsequent information will provide background into the studies presented in later chapters.
**Pituitary development in the mouse**

In the mouse, pituitary development begins as an invagination of the oral ectoderm around embryonic day 9.5 (e9.5). At this stage, the majority of the cells that reside in the primordial pituitary are a highly proliferative population of pluripotent pituitary progenitors. As these progenitors divide, the growing pituitary eventually pinches off from the underlying oral ectoderm to form the developmental structure call Rathke’s pouch (RP). By e12.5 some cells within RP cease proliferating and migrate ventrally to form an outcropping that will eventually form the mature anterior lobe of the pituitary, containing hormone secreting cell types (Figure 1.1). As the embryo enters its 12th day of gestation (e12.5), distinct hormone secreting cell types begin to appear in the developing anterior lobe. First to initiate hormone expression are the corticotropes whose detection can be seen with ACTH expression as well as cells with αGSU expression. By e14.5, TSHβ expressing thyrotropes can be detected. At e15.5, somatotrope GH expression is detected. Cell types expressing PRL are apparent at e15.5, with gonadotropes expressing the LHβ and FSHβ being apparent at e16.5. In the mouse, MSH secreting cells are the last to arise and appear in the intermediate lobe, which at this stage is a distinct structure from the anterior lobe (12, 13).

**Signaling Pathways and Cell fate decisions in the developing pituitary**

Various signaling pathways are involved in the genesis of the pituitary. The anatomical location of the pituitary, in the center of the head and juxtaposed to tissues such as the developing diencephalon, leave it subject to signaling pathways that emanate from the brain, underlying oral ectoderm as well as the mesenchymal tissue surround the developing pituitary. Sonic hedgehog (SHH) secreted from the oral ectoderm, has been shown to induce pituitary formation (14). FGF signaling from the adjacent infundibulum has been shown to influence cell
proliferation during early pituitary development as well as contribute to cell specification. BMP signaling from the juxtapituitary mesenchyme, contributes to early cell fate selection between thyrotrope and corticotrope lineages. Additionally, the integration of FGF and BMP signaling combined is necessary for dorsal-ventral delineation (15, 16). Wnt signaling has also been shown to play a role in the developing pituitary. Wnt4 produced in the ventral diencephalon influences pituitary growth, with a loss of function mutation leading to a slightly hyperplastic pituitary, while loss of Wnt5a leads to a larger intermediate lobe and increased POMC cells (17-19). Interestingly inactivation of Tcf4 causes anterior lobe hyperplasia, with no other apparent defects in pituitary development (20). Taken together these studies indicate that pituitary gland morphogenesis is under tight regulatory control by various developmental signaling pathways.

**Notch signaling in development and its role in cell fate determination**

Recent evidence has shown that the Notch signaling pathway plays an integral role in determining cell fate choice between maintenance of pituitary progenitors and differentiation into hormone cell types (21-23). Notch signaling is an evolutionarily conserved pathway that controls proliferation, differentiation and cell death during development in many different organ systems. Originally identified in *Drosophila*, Notch signaling is induced by the binding of the Notch receptor with its ligand Delta-like/Jagged, found on the membrane of an adjacent cell. Following binding, the receptor undergoes a conformational change allowing for the intracellular domain (NICD) to be cleaved by γ-secretase. This cleavage frees the NICD from the membrane bound receptor, allowing for translocation into the cell nucleus. Within the nucleus, the NICD can then bind to RBPJ-κ turning the complex into a transcriptional activator inducing transcription of Notch downstream factors such as *Hes* and *Hey* genes (Figure 1.2). Hes and Hey proteins are then accumulated within the nucleus to either induce or prevent transcription of
genes involved in various cellular processes that are involved in cell fate determination (6, 22, 24, 25).

Studies in other organ systems have already highlighted the role of Notch signaling in the balance between progenitor maintenance and cell specific differentiation. During development in the pancreas, Notch signaling is needed to prevent premature cell differentiation as well as specification into either exocrine and endocrine cell fates. In animals lacking either the Dll1 ligand or RBPJ-κ coactivator, premature cellular differentiation depletes progenitors in the pancreatic bud (26). In gonadal development, blockage of Notch signaling by γ-secretase inhibitors promotes early Leydig cell differentiation in the XY gonad. Additionally, Hes1 mutant gonads have increased numbers of Leydig cells, indicating that Notch is integral to maintaining gonadal progenitors in order to expand cell populations before terminal differentiation is completed (27). Notch appears to have a similar role in neural development, as loss of Hes1 in the brain results in premature neurogenesis (28) and prevents differentiation of human neuronal stem cells (29). These studies together show the importance of Notch signaling in maintaining progenitor populations and progenitor proliferation, for a window of developmental time, expanding cell numbers before terminal differentiation into functional cell types.

In the mouse pituitary, Notch 2 and 3 receptors and Delta-like 1 ligand are expressed in the RP of pituitary progenitors early in development, e10.5-e14.5 (23). Downstream targets such as Hes1 and Hey1 have also been found in the pituitary at these developmental stages (21, 30) {Figure 1.3}. Another Notch factor, Hes6 is found in a reciprocal expression pattern compared to other Notch molecules, where it is located in the area of anterior lobe induction (31). Prop1, a pituitary specific transcription factor whose expression is controlled by Notch signaling, is expressed in the pituitary beginning at e10.5 and is down regulated by e15.5-16.5 (31). As the
pituitary matures and hormone cell types begin to emerge, Notch signaling factors are
downregulated while differentiation factors such as Pit1 and Sf1 are upregulated (32, 33).

Notch molecule expression patterns indicate that Notch signaling is needed to promote
progenitor maintenance and participates in cell fate decisions. Loss of function of Notch
signaling in the pituitary has revealed defects in progenitor expansion resulting in decreased
numbers of hormone cell type as well as dysregulation of cell fate specification pathways that
hinder normal pituitary organogenesis. Loss of Rbp-jκ in Pit1 expressing cells reveals a decrease
in the Notch downstream molecules, *Hes1* and *Hey1* as well as alterations in Pit1 lineage
commitment indicating that Notch is needed to promote Pit1 lineage specification (22). Absence
of *Hes1* in the developing pituitary leads to the formation of a hypoplastic pituitary with a
reduction in all hormone cell types, further supporting the hypothesis that Notch is important in
preserving pituitary progenitors and populating the pituitary with the proper number of cells
before differentiation occurs (21, 22, 30, 32). Studies have also shown that disruption of HES1
binding on the *GH* promoter can impair pituitary progenitor maintenance resulting in a
hypopituitarism disorder called, Growth hormone neurosecretory dysfunction (GHND) (34).
Additionally transcriptional repression of *hGHR v2* by HES1 may elucidate Hes1’s role in
regulating pituitary actions on growth hormone responsive tissues (34). Loss of *Prop1* has been
shown to result in pituitary hypoplasia, as it is needed for lineage specification of the
somatotrophs, lactotrophs and thyrotrophs in both humans and mice (31, 35). Furthermore, loss
of *Hes1* and *Prop1* results in premature differentiation within RP, revealing that Notch molecules
are needed to repress differentiation in the area where progenitor proliferation is maintained (36).
Notch is also needed to promote lineage specification in the mouse and zebrafish pituitary. Loss
of *Hes1* in the mouse results in the cell fate switch of melanotropes to somatotropes in the
embryonic intermediate lobe (30). Although Notch is not active in promoting early placode formation and induction of pituitary tissues in the zebrafish, Notch is necessary to promote precursor specification. Loss of Notch in the zebrafish has been shown to result in increased prolactin and thyrotrope secreting cells and the complete loss or reduction in the somatotrope, melanotrope and corticotrope lineages (37), further highlighting the conserved nature of Notch signaling in pituitary formation across species.

Reintroduction or sustained Notch signaling can impair cell specification, resulting in loss of pituitary hormone cell types. In the mouse, persistent Notch 2 expression under the control of the $\alpha$GSU promoter leads to delayed gonadotrope cell specification (23). Interestingly, overexpression of Prop1 under $\alpha$-GSU-cre also leads to a delay in gonadotrope specification. Persistent expression of the Notch1 intracellular domain in Pitx1 cells leads to a significant decrease in somatotropes and thyrotropes cell numbers, a decrease in pro-differentiation transcription factors and a hypoplastic pituitary similar to that seen in Prop1 loss of function pituitaries. Overexpression of the Notch1 intracellular domain under the influence of Pit1 promoter reveals increases in Hes1 and Hey1 transcriptions with a decrease in pro-differentiation markers Mash1, Math3 and NeuroD1, indicating that reintroduction of Notch signaling in Pit1 precursor cells inhibits differentiation of the Pit1 lineage (22). Interestingly, zebrafish studies show that Notch gain of function leads to loss of lactotrope and thyrotrpoe lineages with an increase in corticotrope, melanotrope and gonadotrope cell numbers (37). Taken together, these studies on Notch signaling effects on pituitary gland formation in the mouse and fish reveal that tightly regulated signaling is needed to populate and specify the pituitary during organ development.
Stem cells in the adult pituitary

Although specification of pituitary lineages is complete before birth, the pituitary continues to substantially grow in size and retains the ability to generate new hormone cell types throughout adult life. The presence of a stem cell population and niche in the pituitary has been the topic of several recent studies. Different strategies as well as various known stem cell markers have been used to track down the stem cell niche in the adult pituitary. Nestin, a marker of neuronal stem cells, has been found to localize to cells surrounding the periluminal region of the adult mouse pituitary and fate mapping studies show that these cells give rise to all six hormone cell types in the pituitary (38). Studies have also shown that Pax7 may mark a putative Nestin negative intermediate progenitor population. Fate mapping studies have shown Pax7 positive cells are capable of generating all hormone cell types of the pituitary, elucidating their progenitor potential (39). Another group has shown that isolated Sox2 expressing cells have the capability to produce primary, as well as secondary, pituispheres that can differentiate into all hormone cell types (40). Taken together, these studies demonstrate that the pituitary does retain a population of cells that possesses some hallmarks of stem cells: self-renewal and the ability to generate the different pituitary cell types.

Recent studies have highlighted the presence of Notch signaling in adult pituitary stem cells. The Notch receptor Notch1 and downstream target Hes1, have been found to be present in a ‘side population’ of pituitary cells in the anterior lobe of the adult pituitary that express Sca1, a factor found in stem cell populations (41). Furthermore this ‘side population’ has been shown to express Sox2 and Sox9, common factors in stem cell populations of other organ systems (40, 42). Gain and loss of Notch signaling in isolated pituitary aggregate cultures has the ability to increase or decrease, respectively, the number of side population cells present (41). Taken
together it is tempting to hypothesize that Notch signaling may be integral to stem cell preservation and expansion in the adult.

**Cell Cycle Control of Cell Fate**

Notch signaling clearly plays a role in progenitor differentiation but the question remains as to how Notch participates in regulation of proliferation in the pituitary. Regulation of the cell cycle is integral to cell fate determination and organ size. Dysregulation of key components and steps of the cell cycle can lead to gross morphological changes that can alter or even impede organ function. The majority of studies that have shown that cell cycle components are important to regulation of organogenesis focus on key elements of the G1 to S transition phase of the cell cycle. In order for cells to progress through this checkpoint, cyclin/cyclin dependent kinase complexes must hyper-phosphorylate Rb, deactivating its repressive activity (Figure 1.4) (43, 44). Proliferating cells are then transitioned into late G2 and into M phase where mitotic division ultimately expands the progenitor pool. In order for cells to undergo differentiation, this progressive movement through the cell cycle must be halted. A class of proteins called cyclin dependent kinase inhibitors (CDKIs) is responsible for binding to cyclin/cyclin dependent kinase complexes and preventing their phosphorylation activities. As these complexes are inhibited, cells exit the cell cycle and enter into a quiescent phase (G0). In this state, cells are poised to determine cell fate by reentering the cell cycle or undergoing differentiation.

The Cip/Kip family of CDKIs, and their roles in controlling proliferation and promoting differentiation in developing tissues, has been extensively investigated. The inhibitor p21, the founding member of the Cip/Kip family, is upregulated during MyoD induced myocyte terminal differentiation (45). p21, along with p27, has also been implicated in control of granulosa cell expansion and differentiation of the corpus lutea (46). Yet, p21 knockout mice reveal no gross
developmental abnormalities with the exception that their cells have an impaired G1 cell cycle arrest in response to DNA damage (47). A specific role for p21 in pituitary gland development has not yet been determined. Unlike p21, evidence suggests that p27 and p57 are necessary during development and in maintenance of adult tissue. Loss of the CDKI p27 in the mouse results in multiple organ hyperplasia of the thymus, testis, ovaries, T-cell populations as well as the pituitary. Additionally these animals are overall larger in size than their litter mates indicating the need for tight control of p27 is necessary to achieve proper organ and body size during mouse development (48-50). In the pancreas, p27 accumulates in terminally differentiated β-cells during embryogenesis and loss of p27 results in increased proliferation (51). p27 has also been linked to terminal differentiation of keratinocytes and astrocytes in mouse development (52, 53). The role of p57 in development has only begun to be investigated. Ablation of p57 in the mouse results in increased apoptosis as well as delayed differentiation for chondrocytes resulting in the development of shorter limbs. Loss of both p27 and p57 results in defective cell cycle exit and differentiation of lens fiber cells (54). These results indicate that upregulation of CDKIs is important for differentiation and specification of cell types in multiple organ tissues.

CDKI regulation of differentiation has been shown to be a shared mechanism in tissues throughout the body. Yet, the role of CDKIs in pituitary development has only recently been explored. p27 expression has also been shown to prevent pituitary cell proliferation by repressing differentiated cell proliferation in the pituitary (55). Loss of p57 results in accumulation of proliferating progenitors and subsequent hyperplasia of the pituitary, while overexpression of p57 leads to pituitary hypoplasia (56). Furthermore, recent studies have shown that p57 expression is needed to promote cell cycle exit of proliferating pituitary progenitors in
conjunction with p27 (55). These studies highlight the importance of cell cycle inhibitors in controlling proliferation and differentiation of progenitors in the pituitary.

Notch signaling can transcriptionally inhibit the expression of cell cycle inhibitors, modulating cell fate choice between proliferating progenitor and quiescent differentiated cell types. For instance, HES1, has been shown to directly repress transcription of p27 in embryonic carcinoma cells (57). Inactivation of the receptors, Notch1 and Notch2, is accompanied by the derepression of p27 and p57 in intestinal crypt progenitors (58). Inactivation of Hes1 has also been shown to lead to upregulation of p57 leading to precocious differentiation of pancreatic progenitors (59). Blocking Hes1 expression is associated with induced p21 expression and differentiation of neural stem cells into neurons (29). Furthermore p21 is a direct transcriptional target of Notch1/RBPJ-κ mediated cell cycle withdrawal of primary keratinocytes (60). These studies indicate that Notch control of progenitor maintenance can be the result of direct modulation of cell cycle inhibitors, in addition to its role in repression of differentiation, and may be an additional function for Notch signaling in the pituitary.

**Cell cycle regulation and pituitary tumor formation**

Hyperplasia of the pituitary is a normal physiological event that is under tight regulatory control. During pregnancy, pituitary lactotropes expand 2 fold, increasing the amount of circulating prolactin that is needed to enhance lactation for nursing (61). This instance of pituitary hyperplasia is regulated by the physiological process of nursing and as nursing decreases, negative feedback signaling reduces lactotrope cell numbers back to normal physiological proportions. The occurrence of non-physiologically necessary hyperplasia or pituitary adenoma formation and its ontogeny are beginning to be unraveled. The instance of hyperplasia or tumor formation in the pituitary accounts for ~15% of intracranial tumors and is
found in 16.7% -35% of the population upon nonselective autopsy (2, 11). Recent studies reveal many molecular mechanisms that are altered during tumor formation and growth. Deletions in chromosomal loci of 11q13, 9p, 10q and 13q14 have been shown to be present in 12-30% of invasive tumors. Heritable diseases such as MEN (Multiple Endocrine Neoplasia) reveal a high instance of pituitary tumor formation (62). This high occurrence as well as diseases that show a high preponderance of tumor susceptibility reveals the necessity for understanding what events propagate tumor formation in the pituitary.

Various mouse models, mirroring human disease, form pituitary tumors during adult life, providing for the discovery of the molecular mechanisms by which pituitary tumors arise. Frequently, tumor formation and advancement is the result of the “2 hits” model. Mutation or dysregulation of multiple tumor suppressor genes increases the likelihood of tumor survival and progression. \(p21\) loss alone in mice has shown to display a tumor phenotype at later stages in life, ~16 months of age, although tumors are not detected in the pituitary, indicating that its loss maybe compensated by other CDKI family members (63). Additional studies have postulated that \(p21\) loss may act in a synergistic manner when lost with other CDKI’s, enhancing the tumor phenotypes of single CDKI loss. For example, loss of \(p21\) on a \(p18\) null background result in a increased frequency of pituitary tumors seen with \(p18\) loss alone (64). Induction of p21 has been found in animals lacking \(Pttg\), a protein frequently dysregulated in pituitary tumors (65, 66). Loss of \(Pttg\) has been shown to recover \(Rb\) mutant pituitary tumor formation in part by increasing p21 senescence activity. When \(p21\) is additionally removed from these animals, a pituitary tumor phenotype returns (66). Interestingly, when \(p21\) is lost in \(Rb\) mutant mice tumor formation is accelerated (67). These results together support the hypothesis that \(p21\) is necessary to restrain tumor formation.
Mutation in the \( p27 \) gene was found to result in tumor formation of the intermediate lobe of mice, an area that is the vestiges of Rathke’s pouch. In fact, loss of function mutations of \( p27 \) in both rodents and humans can result in the disease multiple endocrine neoplasia (MEN) (68). It has also been shown that \( p27 \) is under expressed or absent in the majority of human pituitary tumors, indicating a role in molecular control of tissue overgrowth (69, 70). In the pituitary, murine studies show that when \( p27 \) and \( Rb \) are both lost, tumor growth is enhanced (71).

Mutation of another CDKI, \( p18 \), also results in pituitary tumor formation as well as widespread organomegaly in the mouse and mutation of both \( p18 \) and \( p27 \) accelerates pituitary tumorigenesis (72). Significant alterations in regulation of the cell cycle, via CDKIs reveal potent changes that destabilize cellular machinery needed to properly transport cells through the cell cycle. These studies provide insight into potential molecular mechanisms that promote pituitary tumor formation.

Studies have begun to show that tumor cells can exploit developmental mechanisms and signaling pathways to promote proliferation. A significant link between Notch signaling and control of cell cycle inhibitors during embryonic development provides a potential pathway that tumor cells may utilize for tumor cell proliferation during pituitary tumorigenesis. In fact, uncontrolled Notch signaling has been uncovered in human T-cell neoplasia, resulting from a translocation of the \( NOTCH1 \) gene to the TCR\( \beta \) locus (73). Mouse models created with this altered form of NOTCH develop T-cell leukemia (74). Other studies have shown active Notch signaling aberrantly deleted in breast cancers as well as human colon adenocarcinomas (17, 75, 76). In mice, Notch signaling has been shown to play a role in epithelial tumor formation. These studies characterized the frequent insertion site of the MMTV (mouse mammary tumor virus) into the Notch4 locus resulting in a truncated protein of the intracellular domain of the receptor.
causing overexpression of Notch4 (77-79). These animals formed poorly differentiated mammary and salivary-gland adenocarcinomas at 7 months of ages. These studies showed that misregulated Notch signaling could maintain mammary ductal cells in a proliferative state as well as preventing terminal differentiation with would eventually lead to adenocarcinoma formation (80, 81).

Altered Notch signaling has also been linked to tumors initiated during embryonic development. Medulloblastoma, a common brain cancer seen in children, is characterized by primitive neuroectodermal tumors thought to arise from stem or precursor cells (82). Studies show that the Notch molecules, NOTCH2 and HES1 are increased in these tumors in humans (83). In mouse model studies, Notch2 and Hes5 are increased, indicating that in both humans and mouse models that Notch signaling may be an important mechanism for potentiating and/or propagating a tumor phenotype (84).

In the pituitary, there is also evidence to suggest Notch signaling may be involved in tumor formation or progression. Prolactinomas are the most commonly occurring hormone secreting tumors found in humans. Gene profiling of these tumors has revealed alterations of Notch molecules, including NOTCH3, DLK1 and HES1 (85). Nonfunctional pituitary adenomas have been shown to cause hypopituitarism by blocking portal system access of hormone secreting cells of the pituitary. Folate receptor (FR) overexpression has been found in these nonfunctional adenomas and cell culture studies have shown that overexpression of FR in pituitary tumor cell line alphaT3-1 can lead to tumor cell growth and an leads to alterations in NOTCH3, HES-1, and TLE2 (86, 87). These studies highlight a contribution of Notch signaling in proliferation of tumor and cancer cells in humans and mice.
Notch signaling is an evolutionarily conserved pathway necessary for proper development of the pituitary as well as multiple organ systems. Preservation of progenitors during a concerted window of time is needed to promote proper pituitary development as well as preventing excessive proliferation that can lead to tumor formation. Notch signaling regulation of key elements of the cell cycle machinery provide for a potential molecular mechanism by which Notch signaling can control progenitor propagation as well as cell fate determination. With these following studies we hope to elucidate how Notch signaling promotes progenitor proliferation in the pituitary by controlling expression and subsequent actions of cell cycle inhibitors.
1.2 Figures and Figure Legends

Figure 1.1. Schematic of pituitary gland development.
Timeline of morphology of embryonic pituitary gland development, during e10.5-e16.5. Green arrows indicate age of each hormone cell emergence in the anterior lobe.
Figure 1.2. Schematic of the Notch signaling pathway.
Notch signaling begins with binding of the Notch receptor with Delta/Jagged ligands on the cell surface. γ-secretase then cleaves the intracellular domain of the Notch receptor (NICD) and allows translocation to the nucleus where NICD binds to RBP-Jκ and induces transcription of *Hes* and *Hey* genes.
Figure 1.3. Timelines and expression patterns of Notch signaling molecules expression in the developing pituitary. The Notch signaling molecules: Notch 2 and 3, Delta-like1, Jagged 1, Hey1 and Hes1 are all expressed in the embryonic pituitary. Each molecule is expressed during early embryonic development (e10.5-e12.5) in RP progenitor cells. As hormone cell types emerge (e14.5) Notch molecules are down regulated and by late development (e16.5) few Notch molecule expressing cells are present.
Figure 1.4. Schematic of key players in the G1/S phase of the cell cycle.
Progression past the G1 phase of the cell cycle is regulated by repressive hyper-phosphorylation of Rb by Cyclin/Cdk complexes. To prevent G1/S phase transition, the cyclin dependent kinase inhibitors (CDKI), p21, p27, p57 bind to Cyclin/Cdk complexes and repress their kinase activity. This repression shuttles cells in to G0.
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Chapter 2

The Notch target gene HES1 regulates cell cycle inhibitor expression in the developing pituitary.¹

2.1 Abstract

The pituitary is an endocrine gland responsible for the release of hormones, which regulate growth, metabolism and reproduction. Diseases such as hypopituitarism or pituitary adenomas are able to disrupt pituitary function leading to suboptimal function of the entire endocrine system. Growth of the pituitary during development and adulthood is a tightly regulated process. HES1, a transcription factor whose expression is initiated by the Notch signaling pathway, is a repressor of cell cycle inhibitors. We hypothesize that with the loss of Hes1, pituitary progenitors are no longer maintained in a proliferative state choosing instead to exit the cell cycle. To test this hypothesis, we examined the expression of cell cycle regulators in wildtype and Hes1 deficient pituitaries. Our studies indicate that in early pituitary development (e10.5), cells contained in the Rathke’s pouch (RP) of Hes1 mutants have decreased proliferation, indicated by changes in phosphohistone H3 expression. Furthermore, pituitaries lacking Hes1 have increased cell cycle exit, shown by significant increases in the cyclin dependent kinase inhibitors, p27 and p57, from e10.5-e14.5. Additionally, Hes1 mutant pituitaries have ectopic expression of p21 in RP progenitors, an area coincident with increased cell death. These observations taken together indicate a role for HES1 in the control of cell cycle exit and in mediating the balance between proliferation and differentiation, allowing for the properly timed emergence of hormone secreting cell types.

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2.2 Introduction

Properly timed and tightly regulated development of the pituitary gland is critical to the function of the adult endocrine system. Situated in the head and in intimate contact with the hypothalamus, the pituitary’s origin lies both in neural and oral ectoderm. Beginning at embryonic day 9.5 (e9.5) the pituitary begins as an invagination in the oral ectoderm. As the embryo develops, the rudimentary pituitary pinches off from the underlying ectoderm to form the developmental structure called Rathke’s pouch (RP). Within this structure reside the pituitary progenitor cells, which are a highly proliferative cell population. As progenitors exit the cell cycle, they differentiate into the hormone secreting cells in the ventral aspect of the developing pituitary (1-3).

Pituitary organogenesis involves the interplay of various signaling pathways. Early in development, Sonic Hedgehog (SHH) proteins emanating from the oral ectoderm signal to the surrounding tissue leading to pituitary induction (4). FGF8 and FGF10 from the infundibulum have been found to promote cell proliferation in the early pituitary and contribute to cell specification. BMP2 and 7 signaling originating from the ventral juxtapituitary mesenchyme, contribute to early cell fate selection between thyrotrope and corticotrope lineages (5, 6). The integration of FGF and BMP actions culminate in dorsal-ventral delineation of the developing gland. Additionally, the Wnt signaling molecules Wnt4 and Wnt5a are expressed in the ventral diencephalon and within RP. Wnt4 mutants display a slightly hypomorphic pituitary indicating its role in proliferation of the early pituitary (7-9). Yet the question remains, how do progenitors present in early development integrate these extrinsic signals to control proliferation and differentiation to form a gland containing the proper number of cells?
Recent studies postulate that Notch signaling in the pituitary maintains a proliferative zone of cells lining RP (10, 11). Notch signaling begins with the binding of the transmembrane Notch receptor with its ligand Delta-like/Jagged. Following binding, the Notch receptor’s intracellular domain (NICD) is cleaved, allowing the NICD to translocate to the nucleus, where along with a co-activator complex drives transcription of factors that determine cell fate (12, 13). Notch 2 and 3 receptors, Delta-like 1 ligands and the Hes and Hey genes are present in the pituitary beginning around e9.5 but expression begins to wane around day e13.5-e14.5 (14). As the pituitary matures, cells exit the progenitor state and migrate ventrally before fully differentiating. These maturing cells, no longer express Notch receptors and factors necessary for lineage specification such as PIT1 and SF1 are upregulated (15, 16).

The Notch target gene, Hes1, encodes a basic helix-loop-helix transcriptional repressor that is necessary to maintain progenitor cell populations in various endocrine organ systems such as the pancreas and intestines. Compared to wildtype (WT), Hes1 mutant pituitaries are hypomorphic, with reductions in all hormone cell types. Additionally, Hes1 mutants lack the αMSH producing cells that are found in the late stages of pituitary development (11, 17, 18). These phenotypes together indicate the necessity for Hes1 in controlling cell number and cell specification in the pituitary. Yet, what mechanisms does Notch signaling employ to control these vital developmental events?

Regulation of the cell cycle is a key component in cell fate determination and organ size. Evidence suggests that Notch signaling may control progenitor differentiation by this mechanism. Several studies have shown that HES1 can bind promoter regions of the cyclin dependent kinase inhibitors (CDKIs) of the Cip/Kip family (p21, p27 and p57) and repress their expression (19-21). CDKIs modulate cell cycle progression by binding to the cyclin/cyclin
dependent kinase complex, preventing cells from transitioning into the DNA synthesis phase. This action directs cells to a quiescent state where differentiation can potentially occur. Levels of CDKIs are tightly regulated and pituitary cell numbers are sensitive to alterations in CDKI expression and activity. Mutation of \( p27 \) predisposes rodents and humans to develop endocrine tumors. Mice lacking \( p27 \) are prone to pituitary tumors of the intermediate lobe, an area that is the vestige of Rathke’s pouch (22-24). In humans, the loss of \( p27 \) results in Multiple Endocrine Neoplasia (MEN) whose symptoms include a predisposition to form pituitary adenomas (25). These data indicate that tight control of \( p27 \) during pituitary formation and in adulthood is necessary to prevent pituitary overgrowth. Unlike \( p27 \), \( p21 \) mutant mice do not form pituitary tumors or have noticeable developmental abnormalities. Yet, when eliminated in conjunction with other tumor suppressor genes such as, \( p18 \) or \( Rb \), double mutant mice display decreased pituitary tumor latency and larger intermediate lobe cell tumors (26, 27).

Mutation studies of each of the Cip/Kip family members have shown the necessity for the proper regulation of CDKIs for repression of cellular overgrowth. We predict that in the developing pituitary Notch signaling through HES1 regulates cell number by controlling the decision between progenitor cell maintenance and differentiation through regulation of CDKIs. By analyzing key regulators of proliferation and cell cycle exit throughout development we show that in \( Hes1 \) deficient mice, the phenotype of a hypomorphic pituitary is the result of decreased proliferation at early ages as well as an increase in cell cycle exit.

2.3 Materials and Methods

**Mice and Embryo collection**

\( Hes1 \) mutant mice were previously generated by replacing the first 3 exons with a neomycin-resistance cassette (28). A breeding colony was generated at the University of Illinois
at Urbana-Champaign and maintained on a mixed genetic background of C57Bl/6J and CD1. The University of Illinois IACUC approved all procedures involving mice. Heterozygous males and females were mated to generate mixed genotype litters. Mice were genotyped as previously described (29). Embryos were collected at e10.5 through e16.5 and fixed in 3.8% formaldehyde solution (Fisher) in phosphate buffered saline, PBS, (Fisher). Embryos where dehydrated through a graded series of ethanol and placed in paraffin for sectioning. Sections measuring 6 microns thick were then affixed to positively charged slides. For bromo-deoxyuridine (BrdU) experiments, animals were treated as previously published (18).

**Immunohistochemistry**

Embryo sections affixed to slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and washed in PBS solution. Slides were subjected to antigen retrieval using 0.01M Citrate buffer, pH 6.0, for 10 minutes for samples treated with anti-p57, anti-Ki67, anti-BrdU and anti-phosphohistone H3 (PH3). All slides were blocked for 10 minutes using 5% Normal Donkey Serum (NDS, Jackson Immunoresearch) in an immunohistochemistry blocking solution (IHC block) containing 5% BSA, 0.1% TritonX-100 and PBS. Primary antibodies were diluted in IHC blocking solution at various dilutions: rabbit anti-CyclinD2 (M-20: sc-593; Santa Cruz Biotechnology) 1:250; rabbit anti-p27 (C-19: sc-528 Santa Cruz Biotechnology) 1:250; mouse anti-p57 (Ab-3 (Clone KP39) Neomarkers) 1:750; rabbit anti-phosphohistone H3 (Ser 10, #06-570 Upstate Cell Signaling Solutions) 1:300; rat anti-Ki67 (DAKO) 1:100; mouse anti-BrdU (#555627 BD Pharmingen) 1:50; mouse anti-p21 (#556431 BD Pharmingen) 1:200; rabbit anti-αGSU (NHPP) 1:1500; rabbit anti-ACTH (NHPP) 1:1500. Donkey derived mouse and rabbit secondary antibodies conjugated to biotin (Jackson Immunoresearch) were diluted to 1:200 and incubated with sections for one hour. Slides were then incubated with tertiary antibodies,
Streptavidin conjugated to either cy2 or cy3 fluorophore (Jackson Immunoresearch), for one hour. Ki67 was detected with a secondary rat antibody conjugated to the fluorophore TRITC. Cell death was assessed by TUNEL as previously described (19). All sides were counterstained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma 28718-90-3) at 1:1000 (Stock 1mg/ml) and mounted using aqueous fluorescence mounting media. Samples were then visualized at 200x magnification utilizing a Leica DM 2560 microscope and images were obtained using Q Capture Pro software and processed using Adobe Photoshop software.

**Cell count quantification: Phosphohistone H3, p21, and TUNEL**

Slides containing midsagittal sections from e10.5 and e11.5 WT and Hes1 mutant embryos were stained and imaged as previously described. Images were taken at 200x magnification. Cells that were solid in nature were counted. A DAPI counterstaining was utilized to obtain an overall cell count for the whole 6-micron thick pituitary section. For each embryo at e10.5, 100-200 cells were examined and at e11.5, 200-300 cells for at least 3 embryos for each genotype. The proportion of immunoreactive cells was compared to the total number of DAPI positive cells contained within RP. A percentage of positive cells per pituitary section was determined, then tested for statistical significance using a Student t-test (SAS 9.1 Software).

**Immunostaining intensity quantification**

Slides containing e14.5 embryo sections were stained for the presence of p27 or p57 proteins and visualized using previously mentioned methods. At least 3 separate embryos, and two sections per embryo were processed and analyzed in parallel. 400X magnified images of the anterior lobe of the pituitary were converted to grey scale and contrasted to 90% using Adobe Photoshop. These images were then processed through NIH Image J software to provide for a gradation of pixel intensity (Red, Dark orange, Light orange, and Yellow denoting positive
signal for either p27 or p57 protein) by pseudo-color appointment to intensity within the image. Pseudo-colored images were then again processed in Adobe Photoshop to provide pixel counts for positive signal. Positive pixel counts were then divided by total pixel amounts for the image (120,000), resulting in a percentage of positive signal per section of anterior lobe of pituitary denoted by relative absorbance units similar to previously published protocols (30). Percentages were then tested for statistical significance using a Student t-test (SAS 9.1 Software).

2.4 Results

**Rathke’s pouch progenitors have decreased proliferation in Hes1 mutants**

*Hes1* mutant pituitaries are smaller than wildtype (WT) pituitaries at e18.5. We hypothesize that *Hes1* is necessary to maintain progenitor proliferation during early embryonic development in order to generate sufficient quantities of cells to populate the e18.5 pituitary. At e10.5, the majority of RP cells are highly proliferative progenitor cells. Pituitaries were first analyzed with markers of the cell cycle, such as Ki67, which marks cells in all active phases of the cell cycle. In e10.5 pituitaries, Ki67 is present throughout RP of both WT (Figure 2.1A) and *Hes1* mutants (Figure 2.1F). Cyclin D2 is a protein required for the G1 to S transition. Without cyclin proteins, cells are unable to enter the synthesis phase and will exit the cell cycle. The majority of cells in the pituitary express Cyclin D2 in a similar pattern in both WT (Figure 2.1B) and *Hes1* mutant embryos (Figure 2.1G). BrdU incorporation indicates active DNA synthesis during the S phase of the cell cycle. BrdU labeling in WT pituitaries show active DNA synthesis throughout RP (Figure 2.1C). *Hes1* mutants have a decrease in BrdU positive cells in the caudal region of RP (Figure 2.1H), similar to previously published data (11, 17, 18). To clarify if cells are progressing though the cell cycle and entering into the mitosis and G2 phase, we utilized phosphohistone H3 labeling. WT embryos have a mean percentage of 6.50% ±0.964 (n=5) of
mitosing progenitors in RP (Figure 2.1D), while Hes1 mutant pituitaries (Figure 2.1I) have significantly fewer mitosing cells (mean percentage $4.77\% \pm 0.890$, n=5, $p \leq 0.0092$). Although cells in the pituitaries of both genotypes are actively in the G1 stage there is a lack of progression to the next stages of the cell cycle in Hes1 mutants.

Instead of progressing through the cell cycle, cells may enter a quiescent phase, a critical decision marked by the increase in cyclin dependent kinase inhibitors (CDKI). HES1 has been shown to transcriptionally repress certain members of these inhibitors, specifically p27 and p57 (20, 21). Consequently, increased expression of CDKIs may have led to the decrease in mitosis that was observed in Hes1 mutants. p57 expression in WT pituitaries is restricted to the dorsal aspect of the pituitary with few cells extending ventrally down RP (Figure 2.1E). In Hes1 mutant pituitaries, the presence of p57 expressing cells can be found throughout RP (Figure 2.1J) with similar findings in p27 expression (data not shown). Taken together these data show that Hes1 mutants at e10.5 have decreased progression through the cell cycle that could be attributed to cells being signaled to exit the cell cycle.

**Pituitary progenitor proliferation is altered after pituitary induction in Hes1 mutants**

By e11.5, the pituitary is distinct from the underlying oral ectoderm. At this age, cells contained within RP still exhibit characteristics of progenitors: highly proliferative with no evidence of differentiation into hormone producing cells. Cells in G1 phase are apparent in the majority of Rathke’s pouch, although a cohort of cells toward the ventral region are not actively expressing Cyclin D2 in WT pituitaries (Figure 2.2A). This observation is also consistent in the Hes1 mutant pituitaries (Figure 2.2E). Cells entering into the mitosis phase of the cell cycle remain restricted to the inner lumen of RP in both WT (Figure 2.2B) and Hes1 mutant pituitaries (Figure 2.2F). By e11.5 there are no apparent changes in proliferation based on numbers of
cycling cells, like that of e10.5, but relative numbers may be changed due to prior pituitary progenitor loss through cell death or reduced proliferation at e10.5.

Although we observed no overall change in the proportion of cells undergoing proliferation, there is still evidence for cell cycle alteration, as reflected in the expression patterns of the CDKIs. p27 expression in both WT (Figure 2.2C) and Hes1 mutant (Figure 2.2G) pituitaries show a dispersed pattern of expression in midsagittal sections of RP, with an increased concentration of cells expressing CDKIs in the ventral region of RP when the pouch is in close contact with the oral ectoderm. p57 expression reiterates the observation that cells of the Hes1 mutant are readily exiting the cell cycle and progressing toward differentiation. In WT pituitaries, few cells express p57, with positive cells found in the periphery of RP (Figure 2.2D). Hes1 mutant pituitaries, however, display an increase in p57 positive cells, localized ventrally (Figure 2.2H).

These data at e10.5 and e11.5 paint a picture of early pituitary progenitor exit from the cell cycle, possibly providing a mechanism for development of the hypomorphic pituitary seen in Hes1 mutants.

**Cell cycle inhibitor expression is affected by loss of Hes1**

As the embryo ages, hormone producing cell types begin to emerge in the spatially restricted anterior lobe. Proliferation at e14.5, as seen by Cyclin D2 positive cells, is restricted to the dorsal aspect of RP and few cells detectable in the expanding anterior lobe (Figure 2.3A). Hes1 mutants similarly show the restriction of CyclinD2 expression to the dorsal part of RP (Figure 2.3D). With this decrease in proliferation there is an increase in cells expressing CDKIs, mainly p27 and p57. Both p27 and p57 protein expression in WT tissues are concentrated away from the proliferative zone and extend into the anterior lobe, where hormone-producing cells
have begun to emerge (Figure 2.3B and C). *Hes1* mutant pituitaries have a larger population of cells that express these inhibitors within the developing anterior lobe (Figure 2.3E and F).

Further supporting this theory, NIH Image J quantification has revealed a significant increase of the intensity of p57 expression in *Hes1* mutants anterior pituitaries compared to WT (Figure 2.3G). Further, p27 expression intensity in *Hes1* mutant pituitaries was also higher than WT tissues (Figure 2.3H). These data indicate that pituitary progenitors, in the absence of *Hes1*, are signaled to exit the cell cycle before a proper pituitary size is achieved.

**Few hormone producing cell types at e14.5 show colocalization with cell cycle inhibitors**

At e14.5, fully differentiated hormone producing cell types are present in the anterior lobe. We have already shown that p27 and p57 expression is increased in *Hes1* mutant pituitaries at this age. Yet, we questioned whether cells expressing CDKIs have begun to express hormones. Cells expressing the CDKI, p27 (solid arrow, pink), colocalize with few cells also expressing the hormone ACTH (open arrow, hormone in green) in the anterior lobe of both WT (Figure 2.4A and A’) and *Hes1* mutant pituitaries (Figure 2.4E). Although there appears to be more p27 and αGSU colocalization in WT (Figure 2.4B and B’) and *Hes1* mutant pituitaries (Figure 2.4F), the majority of the overlap is localized in the rostral tip thyrotrope lineage (denoted by bracket), a lineage of undetermined origin that does not secrete hormone. ACTH colocalization with p57 also reveals no overlap in both WT (Figure 2.4C and C’) and *Hes1* mutant pituitaries (Figure 2.4G). Again, colocalization of p57 and αGSU shows that the vast majority of overlapping expression patterns are found in cells of the rostral tip thyrotrope lineage in WT (Figure 2.4D and D’) and *Hes1* mutants pituitaries (Figure 2.4H). Taken together, this may indicate that although *Hes1* mutant pituitaries have a significant increase in the amount of cells expressing
cell cycle inhibitors there does not seem to be an acceleration of these exiting cells to form fully differentiated cells.

**Pituitary progenitor proliferation wanes in e16.5 embryos**

By e16.5, the remnant of RP begins to differentiate into melanotropes. Few cells in the WT RP are proliferating, as seen by Cyclin D2 and PH3 expression (Figure 2.5A and B). Similar expression patterns are observed in the *Hes1* mutant RP (Figure 2.5E and F). The inhibitors, p27 and p57, also show a decrease in expression in WT (Figure 2.5C and D) and *Hes1* mutant pituitaries (Figure 2.5G and H). Only a few p27 expressing cells colocalize with the hormone producing cells, αGSU and ACTH (Figure 2.7). By this age it is clear that without the action of HES1 to repress CDKI activity, a hypomorphic pituitary can develop due to the loss of early pituitary progenitors.

**Ectopic p21 expression is seen in Rathke’s pouch during early development in *Hes1* mutants**

HES1 can repress transcription of *p21* (19), so we examined p21 expression during pituitary ontogeny. In WT pituitaries at e10.5, a small cohort of p21 expressing cells can be found at the interface between Rathke’s pouch and the surrounding oral ectoderm (Figure 2.6B, arrow). Interestingly, this expression correlates with the area where cell death occurs (Figure 2.6A, arrow). By e11.5, and through the rest of embryonic development, p21 expression is undetectable in mid-sagittal sections of the pituitary (Figure 2.6D and data not shown), while cell death is restricted to the underlying oral ectoderm (Figure 2.6C, arrow). *Hes1* mutant pituitaries at e10.5 show a significant increase in cells expressing p21 compared to WT, although cells are not restricted to the interface between pituitary and oral ectoderm and instead can be found extending dorsally into RP (Figure 2.6F, bracket, and Table). Additionally *Hes1* mutant
pituitaries have a significant increase in the amount of cell death present in RP at e10.5 compared to WT (Figure 2.6E, arrows, and Table). At e11.5, ectopic p21 expression is evident with cells concentrated on the caudal side of RP in Hes1 mutants (Figure 2.6H) with quantification revealing a significant increase of p21 containing cells in Hes1 mutants compared to WT (Figure 2.6 Table). Additionally cell death is present in RP even at e11.5 in Hes1 mutants (Figure 2.6G and Table). By the next day and throughout the rest of development however, p21 expression and cell death is no longer detected in either genotype (data not shown). With the increase in p21 expression and an increase in apoptosis, the pituitary progenitor population early on may be significantly reduced, and with additional misregulation of other CDKIs, could lead to the hypomorphic pituitary seen in Hes1 mutant mice at e18.5.

2.5 Discussion

The maintenance of pituitary progenitors is essential to the development of a pituitary containing an optimal number of hormone secreting cells that control growth, fertility and metabolism. Proliferation of RP progenitors generates the population of cells that will comprise the functioning adult anterior lobe. Alterations in signaling factors, such as SHH, FGF and BMP that control proliferation of pituitary progenitors can lead to disorders of reduced pituitary function. The Notch signaling pathway has also been shown to be critical in the maintenance of pituitary progenitors through the action of the transcriptional repressor HES1 (11, 17, 18). We now show that in the absence of Hes1, pituitary progenitors have decreased proliferation and increased expression of the cell cycle inhibitors, p27, p57 and p21 which leads to early cell cycle exit and depletion of the progenitor pool. In addition, Hes1 mutants have increased cell death at e10.5 and e11.5, a pattern not seen in wildtype pituitaries. These data indicate that Notch
signaling control over progenitor proliferation and cell death is a critical regulator of pituitary cell number (Model, Figure 2.8).

HES1 is normally found in RP progenitor cells. Additionally, Notch receptors, ligands, and downstream molecules have been found in putative stem cell populations in the adult pituitary (31). As differentiation begins, Hes1 expression wanes and cells migrate ventrally to form the anterior lobe (10, 11, 18). Pituitaries of mice lacking Hes1 have decreased numbers of proliferative progenitors. Although the RP contains many cells that are actively in the G1 phase of the cell cycle, there are reduced numbers of cells in S-phase and undergoing mitosis, which are important for replenishing the progenitor pool. The decrease in proliferation that we see in Hes1 mutant pituitaries indicates that progenitor proliferation is impaired by the G1-S phase check point, instead shuttling cells into cell cycle exit.

As progenitors differentiate, cells must first exit from a proliferative state. This transition is mediated by the actions of a class of CDKIs of the Cip/Kip family: p21, p27, p57. As these proteins are upregulated, they inhibit cyclin/cyclin dependent kinase complexes, causing cells to cease proliferation and enter into a quiescent state. Recent p27/p57 double mutant studies revealed that in the absence of these inhibitors, there is continued unchecked proliferation of pituitary progenitors (32). HES1 has been shown to transcriptionally repress the inhibitor p27. Hes1 deficient mice have increased expression of p27 in brain, liver and thymus tissues during development. Additionally, repression of endogenous Hes1 expression in embryonic carcinoma cells results in enhanced p27 expression followed by cell cycle arrest (21). Interestingly, adult p27 mutant animals have a preponderance to form pituitary tumors, indicating that tight control of p27 throughout development is critical to the maintenance of proper pituitary size (22-24). In the pituitary, p27 expression indicates that progenitors must exit the cell cycle to fully
differentiate. *Hes1* mutant mice have increased numbers of cells that express p27 in the developing anterior lobe, indicating that RP progenitors without HES1, exit the cell cycle in increasing numbers.

HES1 has also been shown to directly modulate the transcription of *p57* during progenitor maintenance. In *Hes1* deficient mice there is a significant increase in the expression of p57 in the Pdx1-expressing progenitor cells in the dorsal bud of the developing pancreas. This indicates that the absence of *Hes1* in pancreatic progenitors causes an increase in p57, which shifts cells from the progenitor state to cell cycle arrest (20). Furthermore, studies in intestinal crypt progenitors show that inactivation of the Notch receptor and decreased levels of *Hes1* are accompanied by an increase in cells expressing both p57 and p27, resulting in a loss of progenitor maintenance (33). Similarly, in the *Hes1* deficient pituitary the reduced number of proliferative progenitors correlates with a significant increase in p57 expressing cells located ventrally in the developing anterior lobe throughout development. The critical role p57 plays in the maintenance of proper pituitary size is further highlighted in recent studies that show *p57* mutant pituitaries are hyperplastic during development while *p57* overexpression produces a profound reduction in pituitary size (32).

One striking finding from these studies is that in both WT and *Hes1* mutant pituitaries, p27 and p57 colocalize with few hormone producing cell types (αGSU and ACTH). These data indicate that for differentiation to occur, cells must first exit the cell cycle, as indicated by CDKI expression, thus allowing for cells to leave a progenitor state and progress into the final stages of differentiation. This upregulation of p27 and p57 in the *Hes1* mutant AL at e14.5 may explain why we do not observe premature differentiation of cells when *Hes1* alone is lost (29). Expression of CDKIs in anterior lobe cells may also indicate a mechanism to prevent cell lineage
expansion in prenatal development. In support of this theory, p27 mutant mice have ACTH positive cells that colocalize with Ki-67, indicating prenatal proliferation of differentiated cells, something that is never detected in WT pituitaries (32). As animals enter into postnatal development, a period of lineage specific proliferation occurs (34-38), and disruption of this expansion during later development can perturb proper pituitary cell numbers. Studies show that mice mutant for Prop1 and Pit1, have hypomorphic pituitaries that can be attributed to reduced proliferation of anterior lobe cells (39, 40). Taken together these data reiterate the need to control proliferation during precise periods in both prenatal and postnatal development in order to maintain a proper pituitary cell number.

Another mechanism to restrict the progenitor pool is to limit progenitor expansion through programmed cell death. At e10.5, pituitaries have a small population of cells at the periphery of RP that undergo apoptosis. Interestingly, this incidence of cell death is marked by the presence of p21, a factor that is under direct transcriptional control by Notch signaling (41). In the absence of HES1 repression, p21 expression is detected in a greater proportion of cells in concert with an increase in cell death. These data may indicate that in the pituitary, p21 mediates cell death in response to the loss of HES1. This could be through inducing inappropriate cell cycle arrest leading to death or through a more direct mechanism. For example, overexpression of p21 in thymocytes leads to a hypersensitivity to p53-dependent cell death in response to radiation (42). Alternatively, like p27 and p57, p21 may also cause progenitor cell cycle exit coupled with differentiation. Studies have shown that by blocking HES1 action, p21 is induced, leading to the GABAergic differentiation of neural stem cells (43). Additionally, in the Pttg/securin knockout animals, p21 expression is induced in the adult pituitary and it serves to reduce proliferation and attenuate tumor formation (44). Our current studies point toward a role for progenitor limitation
by cell cycle exit and cell death early in pituitary induction. This indicates that tight control of progenitor proliferation is necessary to control pituitary size.

Although the role of *Hes1* in progenitor expansion has been studied extensively, its role in progenitor differentiation remains to be clarified. Studies have shown that constitutive expression of *Hes1* can prevent αGSU and TSH expression, indicating that *Hes1* is needed to prevent cellular differentiation (18). Additionally, when the transcription factor *Prop1* is mutated in *Hes1* mutant pituitaries, progenitors are found to prematurely differentiate within RP (29). These data, coupled with studies that have shown that *Hes1* is necessary to specify melanotrope cell fate (18), indicate that the actions of HES1 on pituitary development include not only early events that govern progenitor expansion, but may also include late events that determine hormone cell fate.

The most significant changes in *Hes1* mutant pituitaries seen here is the misregulation of CDKIs. Indeed, it has been shown that the loss of p27 in both rats and humans results in Multiple Endocrine Neoplasia with a high prevalence of pituitary tumors (25). These data suggest that regulation of pituitary proliferation by CDKIs is essential to prevent tumor formation. HES1 expression in the developing pituitary maintains progenitors in a proliferative, undifferentiated state by transcriptionally repressing CDKIs, preventing cells from exiting the cell cycle and preventing progenitor death. It is tempting to speculate that misregulation of Notch signaling may also contribute to pituitary tumor formation. Interestingly, Notch molecules have been found to be upregulated during pituitary tumor development, reiterating that Notch molecules are needed to maintain cells in a proliferative state (45, 46). These studies provide evidence that Notch signaling is a critical regulator of pituitary organogenesis through progenitor maintenance and may modulate organ size in the adult organ.
2.6 Acknowledgements

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Figure 2.1. *Hes1* is necessary for appropriate cell cycle progression. Mid-sagittal sections at e10.5 were immunostained with Ki67 (A), which marks actively proliferating cells in Rathke’s pouch (RP bracket) progenitor cells throughout WT and *Hes1* mutant pituitaries (F). Cyclin D2 expression, marking G1 phase of the cell cycle, is found throughout WT (B) and *Hes1* mutant RP’s (G). As RP progenitors progress to S phase, as visualized by BrdU incorporation and immunostaining, cells are not readily present in the caudal section of the *Hes1* mutant pituitary (H), unlike WT RP (C). Phosphohistone H3 (PH3) marks the cells progressing into the G2 and M phase. *Hes1* mutant pituitaries (I, arrows) have significantly fewer cells entering into these later stages than their WT littermates (D). WT pituitaries have few cells expressing p57 that are present in the dorsal region of RP (E) while *Hes1* mutant pituitary cells express p57 in the rostral region (J). Scale bar indicates 50 microns. c=Caudal, d=Dorsal, r=Rostral, v=Ventral for all images.
Figure 2.2. Increased cell cycle exit is evident in Hes1 mutant pituitaries. Sagittal sections at e11.5 were stained with cell cycle markers. Cyclin D2 expression is found in Rathke’s pouch (RP) progenitors in both WT (A) and Hes1 mutant (E) pituitaries, while a small population in the ventral aspect lack Cyclin D2 in both genotypes (arrows). Phosphohistone H3 expression is found in cells lining the lumen of RP in both the WT (B) and Hes1 mutant (F) RP. p27, a cell cycle inhibitor, is found throughout RP in WT (C) and Hes1 (G) pituitaries. p57 expression is found in few cells throughout RP in WT pituitaries (D, arrows) while Hes1 mutants (H, arrows) have an increase in cells expressing p57 in the ventral region of RP, the area of the future anterior lobe.
Figure 2.3. *Hes1* mutants have significant increases in cell cycle inhibitor expression in the developing anterior lobe at e14.5. In e14.5 pituitaries, many cells are actively in the G1 phase of the cell cycle, as marked by Cyclin D2 expression. These cells can be found in RP, but rarely in the developing anterior lobe (AL) of WT (A) and *Hes1* mutant pituitaries (D). p27 expression in WT pituitaries (B) is found in few cells throughout both RP and anterior lobe cells. *Hes1* mutant pituitaries (E) have significantly more cells in the anterior lobe expressing p27, compared to WT pituitaries. p57 expression in WT pituitaries (C) is mainly concentrated in RP cells with few found in the developing anterior lobe. *Hes1* mutants (F) on the other hand have a significant increase in p57 expressing cells in the AL. (G) Image J signal quantification of p57 reveals a significant (p<0.001) increase in mean signal strength between WT (2.335, n=3) and *Hes1* mutants (5.087, n=3) (denoted by asterisk). (H) Significant (p<0.001) p27 increase between WT (12.665, n=3) and *Hes1* mutant (18.280, n=3) (denoted by asterisk).
Figure 2.4. The cell cycle inhibitors p27 and p57 localize with few fully differentiated cells at e14.5. By e14.5, the hormone producing cells (αGSU, ACTH) that reside in the developing anterior lobe are evident. The WT AL (A’) with p27 expression (solid arrows, pink) overlaid with ACTH producing cell types shows few cells that colocalize (open arrows, green), expression that is comparable to Hes1 mutant AL’s (E). This expression patterned is also reflected in αGSU hormone overlays, with few cells expressing both p27 and hormone in WT (B’) and Hes1 mutants (F, brackets mark rostral tip thyrotropes, which do contain CDKI and αGSU). p57 expression (solid arrows, pink) and ACTH do not colocalize (green) at e14.5 in WT (C’) Hes1 mutants (G), while few cells in the anterior lobe colocalize both p57 and αGSU in WT (D’) and Hes1 mutants (H, brackets mark rostral tip thyrotropes). Figures A-D indicates magnified images from WT anterior lobes that were taken for Figures A’-D’. Magnified images from Hes1 mutants (E-H) were taken from the same location as WT. Scale bar indicates 50 microns.
Figure 2.5. The cell cycle does not appear altered in *Hes1* mutants at e16.5. Cyclin D2 expression is found in the remnant of Rathke’s pouch with few cells in the developing anterior lobe in the WT (A) and *Hes1* mutant (E). Few cells are actively undergoing mitosis, as seen by Phosphohistone H3 expression (marked by arrows) in WT (B) and *Hes1* mutants (F). p27 expression is relegated to few cells in the remnant of RP and in the developing anterior lobe in WT (C) and *Hes1* mutant (G) pituitaries. p57 expression is reduced, with few cells present in the anterior lobe of both the WT (D) and *Hes1* mutant pituitaries.
Figure 2.6. Ectopic p21 expression is found in Hes1 mutant pituitaries. At e10.5, WT pituitaries (A, arrow) exhibit cells undergoing death at the junction of oral ectoderm, as assayed by TUNEL staining. These cells also appear to be the population that expresses p21 (B, arrow). In Hes1 mutants (E), there is an increase in cell death within RP (marked with arrows). This correlates with increased expression of p21 extending into RP (F, bracket). At e11.5 (C, arrow) cell death is mostly restricted to the underlying oral ectoderm in the WT pituitary but is still detectable in the Hes1 mutant RP (G, arrows). Increased p21 expression is still evident in the RP of Hes1 mutant pituitaries (Figure 6H and Table) while WT pituitaries have little to no p21 expression at e11.5 and through the rest of development (Figure 6D, data not shown and Table).

<table>
<thead>
<tr>
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<th>Mean % Expression at e10.5</th>
<th>Mean % Expression at e11.5</th>
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<tr>
<td></td>
<td>TUNEL (p≤0.0054)</td>
<td>p21 (p≤0.0079)</td>
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<tr>
<td></td>
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<td>p21 (p≤0.0004)</td>
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<td>10.647±4.5 (n=4)</td>
<td>13.118±3.5 (n=3)</td>
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Figure 2.7. The cell cycle inhibitor p27 localizes with few ACTH and αGSU cells. At e16.5, hormone producing cell types (ACTH, αGSU) are more abundant in the anterior lobe of the developing pituitary, indicating further differentiation of pituitary progenitors. Colocalization of p27 (pink) and ACTH (green) in the AL of WT pituitaries reveals no overlap (A, solid arrow), a pattern that is similar to Hes1 mutant pituitaries (C, solid arrow). Few p27 positive cells (solid arrow) colocalize with αGSU positive cells (open arrows) in both WT (B) and Hes1 mutant pituitaries (D). Rule bar indicates 50 microns.
Figure 2.8. Schematic model depicting major cell cycle changes through embryonic development in Hes1+/+ (WT) and Hes-/- (mutant) pituitaries.
2.8 References


Chapter 3

The role of p21 in pituitary organogenesis.

3.1 Abstract

Proper development of the pituitary gland is integral to endocrine system function. Early in development the pituitary is composed of a proliferating progenitor population whose expansion and differentiation is regulated by cell cycle molecules. The family of Cip/Kip cyclin dependent kinase inhibitors (CDKI’s) participate in a cell fate decision between proliferation and cell cycle exit and differentiation. Recent studies from our group have revealed a role for the Notch signaling molecule Hes1 in repressing CDKI expression during pituitary development. In the absence of Hes1, the CDKI p21 is increased in the pituitary coincident with an increase in cell death. This indicates that HES1 is needed to repress p21, preserving a progenitor state. Similarly, p21 upregulation is seen in the hypomorphic Ptgg (securin) mutant pituitaries, indicating that p21 is involved in inducing cellular senescence. Mice lacking p21 do not have alterations in pituitary morphology when examined in the adult, yet investigations into its development role have yet to be undertaken. We hypothesize that p21 has a role in regulating progenitor expansion and programmed cell death in the pituitary. In development, p21 is found in RP progenitors in the ventral region of Rathke’s Pouch (RP) at 10.5-e11.5. Although proliferation markers do not reveal an increase in proliferation, as shown by Phosphohistone H3, pRb and CyclinE expression, we do see delayed pouch closure of RP from the oral ectoderm. This observation, in conjunction with ectopic cell death seen when p21 is increased in Hes1 mutants and the theory that cell death mitigates pouch closure, caused us to postulate that p21 may modulate RP separation and plays a role in regulating pituitary formation during development.
3.2 Introduction

Pituitary tumors are a common occurrence in the human population accounting for ~15% of intracranial tumors (1). Although these tumors are thought to arise from uncontrolled cellular division, little is known about the mechanisms governing progenitor proliferation during development and in the adult. Pituitary progenitor expansion during development is under control of signaling pathway modulation of the cell cycle. Less is known about what controls the expansion of the adult pituitary, which needs to change cell number to compensate for the body’s need for fluctuating hormonal secretions. Dysregulation of cell cycle components such as Rb, p27, and Pttg have been found to be common in many types of cancers and tumors including the pituitary (2, 3). It is, therefore, imperative to determine how cell cycle molecules control progenitor expansion as well as how these molecules are needed to prevent pituitary hyperplasia and adenoma formation.

The pituitary begins as an invagination in the oral ectoderm around embryonic day 9.5 (e9.5) in the mouse. As pituitary progenitors proliferate, the pituitary pinches off from the underlying oral ectoderm, forming the developmental structure called Rathke’s pouch (RP) at e11.5. As progenitors are signaled to differentiate, cells migrate ventrally forming an outcropping of RP that will eventually form the mature anterior lobe. By e12.5, the cells populating the growing anterior lobe begin to express hormones. By e18.5 the anterior pituitary is equipped with all hormone cell types, which are ready to secrete hormones (ACTH, PRL, FSH, LH, GH, TSH) needed during postnatal and adult life (4, 5). Recent studies by our group and others have shown that throughout embryonic pituitary development p27 and p57, members of the CIP/KIP family of cell cycle inhibitors, are expressed in RP progenitors and serve to cease progenitor proliferation and prime quiescent cells to receive differentiation cues (6, 7). During
development p27 and p57 expression is widespread throughout the developing pouch and studies have revealed that this pattern may reflect progenitors ready to undergo differentiation. Interestingly, p21, another protein member of this family, has been found at e10.5 in cells between the RP and the oral ectoderm (8). This contrast in expression patterns indicates that p21 may play a differential role in pituitary development compared to its family members.

p21 is the founding member of the Cip/Kip family. Its presence has been shown to regulate cell cycle progression in the event of external insult or under differentiation cues. Early studies addressing the function of p21 have highlighted its role as a downstream effector molecule of p53, a tumor suppressor protein whose absence results in severe G1 checkpoint disruption leading to tumor formation (9). p21 alone is considered a weak tumor suppressor. When p21 is lost in the mouse, embryonic development appears to progress normally, with the exception of impaired G1 cell cycle arrest in response to DNA damage (10, 11). Although, recent studies have shown that p21 mutant mice do form spontaneous tumors at later stages of life (~16 months), implicating its role in maintaining certain cell populations in a quiescent state (12). Furthermore, p21 has been found to be upregulated in hypomorphic Pttg mutant pituitaries and is proposed to induce senescence pathways in pituitary cells. Studies have shown that loss of Pttg in Rb mutant animals protects against tumor development and that this is in part due to p21 action (13, 14). p21 is aberrantly expressed in hormonally active pituitary tumors with reduced expression levels found in non-functioning (15). It has yet to be determined if p21 expression is a cause or a consequence of tumor formation. Although p21 alone may not be responsible for maintaining cellular quiescence in the adult tissue, it is clear that p21 plays a cooperative role with other cell cycle inhibitors. Mice lacking p21 in conjunction with loss of p18 or Rb, result in increased tumor formation and incidence (16-19). These studies indicate that although p21
expression may be limited, its role in regulating cell cycle exit in a specialized subset of cells may be protecting pituitary cells from tumor formation.

Cell cycle inhibitors are composed of a N-terminal domain that binds and inhibits the phosphorylation activities of cyclin/cyclin dependent kinase complexes that progress cells through the cell cycle. p21 preferentially binds CyclinE/Cdk2 complexes resulting in G1 arrest in response to DNA damage or developmental signals. The C terminus of p21 can bind PCNA and E2F promoters regulating DNA synthesis. Furthermore, p21 can bind Cyclin B complexes arresting cells in G2 phase (20-23). This broad spectrum of activities through all phases of the cell cycle highlights p21’s integral and diverse role in controlling proliferation during development and in adult organ systems.

Accumulation of CDKIs is a hallmark of differentiating cells during development. BMP-4 induced differentiation of osteoblast-like cells is promoted through increased expression of p21 and p27 (24). XY gonadal development relies on p21 upregulation (25), while retinoic acid induced differentiation is associated with increased p21 expression in palatal mesenchymal cells and neural crest cells (26). p21 expression has also been shown to be induced during glial and neuronal differentiation in vitro (27). Studies have elucidated p21’s role in olfactory neuron and keratinocyte differentiation in culture (28, 29). Furthermore, our group has shown that loss of the transcriptional repressor Hes1, leads to induction of p21, coincident with a decrease in proliferation (8). These studies highlight p21’s active role in repressing cell cycle progression and allowing for quiescent cells to differentiate.

In addition to p21’s role in control of cell cycle mechanisms, it has also been shown to play a role in cell death regulation. Thymocytes isolated from p21 mutant animals have been found to be hypersensitive to radiation induced programmed cell death via the p53 pathway (30).
Additionally, p21 overexpression has been shown to induce apoptosis in vitro in esophageal and cervical cancer cell lines (31, 32). In p53 dependent human hepatoma cell lines, overexpression of p21 results in an induction of Bax, a proapoptotic protein (33). In addition to p21 misexpression, Hes1 mutant pituitaries have increased cell death in an area coincident with p21 expression, indicating that p21 may be mediating senescence induced cell death in the pituitary as well (8). Recent evidence has shown that a wave of p21 expression is followed by induced cell death that is required for proper separation of the digits in the hand in the rodent (34). These studies suggest that p21 activation can be part of the mechanism regulating properly controlled cell death.

p21 mutant animals have so far not revealed developmental defects that are as overt as multiple organ hyperplasia seen in mice with a loss of its family member p27 (10, 35-37). However, the tight regulation of p21 during a specific window of developmental time indicates a specific function during pituitary development. These studies have been undertaken to explore the developmental role that p21 plays during pituitary organogenesis, specifically within the window of pituitary induction, early proliferation and cell death events that define the organ from the oral tissue that it originates.

3.3 Materials and Methods

Mice and Embryo collection

p21 mutant mice, with exons 2 and 3 replaced with pgk-neo cassette, generated by Dr. Tyler Jacks Laboratory at the Massachusetts Institute of Technology (19), were obtained from Dr. Paul S. Cooke’s laboratory at the University of Illinois at Urbana-Champaign. Heterozygous males and females were mated to generate mixed genotype litters at e10.5-e12.5. Mice were genotyped using: p21 forward primer, 5’ ACT TTT GAT TGG CCT GAT GG 3’; p21 reverse
primer, 5’ TGA CGA AGT CAA AGT TCC ACC G; neo forward, 5’ GTC TTG TCG ATC AGG ATG ATC TG 3’; neo reverse, 5’ CAA TAT CAC GGG TAG CCA ACG C 3’. Breeding colonies were generated at the University of Illinois at Urbana-Champaign and maintained on a genetic background of C57Bl/6J. The University of Illinois IACUC approved all procedures involving mice. Embryos were collected and fixed in 3.8% formaldehyde solution (Fisher) in phosphate buffered saline (PBS) (Fisher). Embryos where dehydrated through a graded series of ethanol and placed in paraffin for sectioning. Sections measuring 6 microns thick were then affixed to positively charged slides.

**Immunohistochemistry**

Embryo sections affixed to slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and washed in PBS solution. Slides were subjected to antigen retrieval using 0.01M Citrate buffer, pH 6.0, for 10 minutes for samples treated with anti-p21, anti-p27, anti-p57, anti-Ki-67, anti-CyclinE, anti-pRB and anti-phosphohistone H3 (PH3). Anti-p21 treated slides were also exposed to 0.0015% hydrogen peroxide to remove endogenous peroxidase activity. All slides were blocked for 10 minutes using 5% Normal Donkey Serum (NDS, Jackson Immunoresearch) in an immunohistochemistry blocking solution (IHC block) containing 5% BSA, 0.1% TritonX-100 and PBS. Primary antibodies were diluted in IHC blocking solution at various dilutions: mouse anti-p21 (#556431 BD Pharmingen) 1:200; rabbit anti-p27 (Santa Cruz, C-19, sc-528) 1:250; mouse anti-p57 (Neomarkers, Ab-3 {clone KP39}, #MS-897-P0) 1:750; rat anti-Ki67 (DAKO) 1:100; rabbit anti-CyclinE (ab52189 AbCam) 1:500; rabbit anti-phospho-Rb (Ser807/811, Cell Signaling Technology, #9308) 1:500; rabbit anti-phosphohistone H3 (Ser 10, #06-570 Upstate Cell Signaling Solutions) 1:300. Donkey derived mouse and rabbit secondary antibodies conjugated to biotin (Jackson Immunoresearch) were
diluted to 1:200 and incubated with sections for one hour. Slides were then incubated with tertiary antibodies, Streptavidin conjugated to cy3 fluorophore (Jackson Immunoresearch), for one hour. p21 expression was detected using a PerkinElmer TSA Cyanine 3 System (NEL704A001KT0) with the tertiary antibody, Streptavidin conjugated to Horse Radish Peroxidase then visualized with cyanine 3 tyramide. Ki67 was detected with a secondary rat antibody conjugated to the fluorophore TRITC. Cell death was assessed by TUNEL as previously described (38). All sides were counterstained with 4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma 28718-90-3) at 1:1000 (Stock 1 mg/ml) and mounted using aqueous fluorescence mounting media. Samples were then visualized at 200x magnification utilizing a Leica DM 2560 microscope and images were obtained using Q Capture Pro software and processed using Adobe Photoshop software.

**Somite Staging**

Somite number was determined by counting the appearance of somites behind the hind limb bud and to the tail of each animal at e11.5. Previous groups have characterized the e11.5 developmental stage as an embryo containing 18 somites past the hind limb bud (39). Somites were counted under a dissecting microscope utilizing a dark background for ease of visualization.

**Cell Count Analysis-Phosphohistone H3**

Slides containing perisagittal to midsagittal sections from e11.5 WT and p21 mutant embryos were stained with PH3 and imaged as previously described. Images were taken at 200x magnification. For each genotype tested there was an n≥3 with at least two sections from each animal tested. All cells positive for PH3 were counted and a DAPI counterstaining was utilized to obtain an overall cell count for the whole 6-micron thick pituitary section. For each genotype,
least 3 embryos were analyzed. The proportion of immunoreactive cells was compared to the total number of DAPI positive cells contained within RP. A percentage of positive cells per pituitary section was determined, then statistically analyzed with a two tailed T-Test.

3.4 Results

**CIP/KIP cell cycle inhibitors are found in the developing pituitary.**

In the pituitary, CDKIs can be detected in RP as early as e10.5. p21 (Figure 3.1A, arrow) is located to the area where Rathke’s pouch meets the underlying oral ectoderm. p27 and p57 expression are found throughout RP (Figure 3.1E, arrows). As the pituitary continues to grow, it separates from the underlying oral ectoderm to form an autonomous structure at e11.5. At this stage, p21 expression is restricted to a small cohort of cells in the ventral aspect of RP. Additionally, cells in the oral ectoderm also express p21 (Figure 3.1B, arrows). p27 and p57 can also be found in the ventral region but also have cells scattered throughout the dorsal aspect of RP (Figure 3.1F and 1J). At e12.5, the developing anterior lobe begins to appear in the ventral region of RP and p21 expression is almost extinguished from the pouch, with only a few cells remaining (Figure 3.1C, arrow). On the other hand, p27 and p57 expression persists in the pituitary, mainly concentrated to the division between RP and the developing anterior lobe (Figure 3.1G and 1K). Little expression of p21 is found at later stages of pituitary development. At e14.5, only a couple of p21 expressing cells can be detected in RP or the more fully developed anterior lobe (Figure 3.1D, arrow). At this stage p27 expression is widespread through the developing pituitary (Figure 3.1H) while, p57 expression is mostly restricted to the dorsal portion of RP with few cells scattered in the AL (Figure 3.1L, arrows).
**p21 does not appear to regulate proliferation of progenitors early in development.**

p21 is a negative regulator of cell cycle progression. To determine if pituitary progenitors are actively proliferating in the presence and absence of p21, we performed immunohistochemistry utilizing established markers of proliferation: Ki-67 which marks cells in all phases of the cell cycle and phosphohistone H3 (PH3) which marks cells transitioning into M and late G2 phase. At e10.5 in wildtype and p21 mutant pituitaries, the majority of RP progenitors are in some phase of the cell cycle as marked by Ki-67 expression (Figure 3.2 A, D). PH3 staining in wildtype (Figure 3.2G) and p21 mutants (Figure 3.2J) reveals that although most RP progenitors are in phases of active proliferation, only the cells lining the inner lumen of RP are actually completing the cell cycle and actively dividing. Ki-67 and PH3 expression is mirrored in e11.5 pituitaries of both wildtype (Figure 3.2B, H) and p21 mutants (Figures 3.2E, K), with all cells in active proliferation and lumen restricted cells progressing through M phase. Additionally, cell count quantification at this age did not reveal significant alterations in PH3 positive cells. Wildtype pituitaries on average have 19.004±4.058% while p21 mutants have 20.466±2.722% of progenitors entering M or late G2 phase of the cell cycle detected from persagittal to midsagittal in the pituitary. At e12.5 the anterior lobe begins to form as an outcropping in the ventral aspect of RP. The majority of RP progenitors in wildtype (Figure 3.2C) and p21 mutants (Figure 3.2F) are still marked by Ki-67 while the cells in the developing anterior lobe are exiting the cell cycle and entering into a quiescent state. This again is mirrored by PH3 expression with cells restricted to the luminal portion of RP, away from the developing anterior lobe in both wildtype (Figure 3.2I) and p21 mutants (Figure 3.2L). These results indicate that p21 expression in the early pituitary gland does not regulate proliferation of pituitary progenitors.
**Loss of p21 does not affect expression of Cyclin E and Rb phosphorylation in early pituitary development.**

p21’s role in the cell cycle is necessary to inhibit the actions of cyclin/cyclin dependent kinase complexes. In particular, p21 has been shown to directly inhibit complexes containing CyclinE. With this in mind, we hypothesize that loss of p21 would result in changes in CyclinE expression. At e10.5 immunostaining shows that CyclinE expression is restricted to few cells in the inner lumen of RP in wildtype (Figure 3.3A) and p21 mutant (Figure 3.3D) pituitaries. At e11.5 an age where p21 expression is still present in normal tissues, CyclinE positive cells are in the RP lumen of wildtype (Figure 3.3B) with p21 mutant (Figure 3.3E) animals revealing no change in expression patterns. By e12.5, the age at which p21 expression normally wanes, p21 mutant (Figure 3.3F) pituitaries do not have changes in Cyclin E expression compared to wildtype (Figure 3.3C). This indicates that although p21 is known to regulate CyclinE, loss of p21 does not affect its presence in pituitary progenitors. Expression of a phosphorylated form of Rb is characteristic of cells that are capable of proliferating and this is achieved by CyclinE/cyclin dependent kinase complex phosphorylation activities. Immunostaining of pRB in RP is limited. At e10.5, few pRB positive cells are detected in RP in wildtype (Figure 3.3G) and p21 mutant (Figure 3.3J) pituitaries. Similar to CyclinE data, alterations in pRB are also undetected at e11.5-e12.5 of p21 mutants (Figure 3.3K-L) compared to wildtype (Figure 3.3H-I). This suggests that loss of p21 does not affect expression of pRB, results similarly seen with CyclinE expression patterns.

**Loss of p21 impairs timely closure of Rathke’s pouch.**

Between the ages of e10.5-e11.5 the pituitary pinches off from the underlying oral ectoderm to form Rathke’s pouch, a developmental structure containing proliferating progenitor
cells. p21 expression is restricted to a small population of cells at the border between RP progenitors and the underlying ectoderm. This limited expression, as well as its correlation to a small amount of cell death in the region, led us to speculate whether p21 may play a role in pouch separation from oral tissue. To determine if loss of p21 impairs timely separation of RP progenitors from the underlying oral ectoderm, wildtype and p21 mutant pituitaries embryos were somite staged to more precisely determine embryonic age. Somite stage 18 (ss18) is the stereotypical e11.5 and somite stages surrounding this stage were utilized to determine the time frame of pouch closure. At ss16, both wildtype and p21 mutant pituitaries appear to still be connected to oral ectoderm, forming a structure still open to the oral cavity (Figure 3.4A, E, I). By ss17, wildtype (Figure 3.4B, I) and p21 mutants (Figure 3.4F, I) begin to close, although this closure is not complete in all embryos. At ss18, the stereotypical e11.5, we can see that most of the wildtype (Figure 3.4C, I) pouches are no longer open, forming a RP autonomous from the oral ectoderm. p21 mutant (Figure 3.4G, I) pituitaries at this stage are open in some individuals and closed in others. By ss19, all wildtype (Figure 3.4D) pouches are closed. p21 mutants (Figure 3.4H, I), on the other hand, still may remain open. This indicates that p21 may play a role in timely pouch closure. Interestingly, by e12.5 both wildtype and p21 mutant pituitaries appear normal and have closed to the oral cavity indicating that although p21 may participate in pouch closure activities, it is likely that redundant or additional mechanisms compensate for p21’s loss.

**Loss of p21 does not alter cell death detected by TUNEL.**

Since the loss of p21 delays pouch separation, we hypothesize that p21 may promote cell death. In fact, a wave of p21 expression appears before induction of cell death seen in digit separation during embryonic development in the rodent (34). To investigate this hypothesis, we
utilized TUNEL staining to detect areas of cell death in the developing pituitary. At e10.5, when the pituitary still remains open to the oral cavity we can see small amounts of cell death the in the junction between the RP and the oral ectoderm in both wildtype (Figure 3.5A) and \textit{p21} mutants (Figure 3.5C). At e11.5, pituitary separation occurs and in wildtype pituitaries at ss17, cell death is not found in RP but can be detected in the oral ectoderm directly beneath RP (Figure 3.5B, arrows). \textit{p21} mutant pituitaries at this somite stage show that when pouch closure is incomplete, cell death still occurs (Figure 3.5G, arrow). Yet, at ss18 and ss19, in both wildtype and \textit{p21} mutant pituitaries, when pouch separation has occurred, changes in cell death are not detected (Figure 3.5C-D and 3.5H-I). By e12.5 pouch closure is complete in both animals and cell death is no longer apparent (Figure 3.5C, F). These data indicate that although p21 may regulate pouch closure (Figure 3.5I) it is not the only mechanism that can induce cell death and induce separation.

3.5 Discussion

Properly timed expression of cell cycle inhibitors is needed to orchestrate developmental events that lead to a functioning pituitary. Studies have shown that CDKI expression is needed during embryonic pituitary development for controlling progenitor proliferation. Loss of the CDKIs \textit{p27} and \textit{p57} results in excess proliferation in pituitary progenitors, as well as specific hormone cell types (7). Furthermore loss of \textit{p27} in \textit{Brg1} mutants is correlated with an increase in CyclinE expression, leading to tumor formation (6). Since its discovery, the actions of p21 have been shown to be diverse, including cellular senescence in adult tissue, control of all phases of the cell cycle, as well as modulation of cell death. We hypothesized that p21 may operate similarly to \textit{p27} and \textit{p57} in pituitary gland development. Previous work by our group has shown that in the pituitary, Notch signaling represses \textit{p21} expression (8). This repression of p21
correlates with progenitor cell proliferation and cell survival. We now show that p21 may not influence detectable changes of proliferation at early stages and that cell death does not appear altered.

p21 has been shown to be a potent modulator of cell cycle progression. G1/S phase transition is a critical step in cycling progenitors, providing a checkpoint for cells that have undergone cellular insult or DNA damage, allowing for repair. p21 has been shown to repress the activity of the CyclinE/Cdk2 complex, which is needed to phosphorylate Rb allowing for progression into S phase (21). Radiation and chemical insult of p21 deficient cells has been shown to impair the G1/S checkpoint (11). p21 has also been shown to modulate DNA transcription. The N-terminal domain of p21 has a PCNA binding capability, which can in turn regulate DNA synthesis (22). Furthermore, studies show that p21 can modulate the later G2/M phase transition by binding to and repressing Cyclin B activity (23). In the pituitary, Cyclin E, pRb and PH3 expression patterns remain unchanged in p21 mutant pituitaries in comparison to wildtype pituitaries. Additionally cell count quantitation of cells entering into M phase and late G2, as assessed by PH3, reveals no significant changes in proliferating progenitors in p21 mutants compared to wildtype pituitaries. This indicates that in the absence of p21, repressive phosphorylation of Rb is not altered and progenitors pass though the G1/S checkpoint normally and unchanged PH3 expression indicates that G2/M transition is not altered. Although our data has revealed no alterations in any phases of the cell cycle at these ages, we cannot rule out that changes in detecting proliferating cells may occur at later developmental time points. Proliferation of pituitary cells is not restricted to embryonic development. During the second week of prenatal life a second wave of proliferation expanding the population of hormone cell
types present in the pituitary (40-44). It is possible that p21 action may regulate this later stage of pituitary cell expansion.

Apoptosis is a common event during embryonic development and expression of p21 has been shown to modulate cell death. p21 mutant thymocytes have been shown to be hypersensitive to radiation induced cell death (30). Yet, there is growing evidence that shows that p21 can also act as a pro-apoptotic protein. p21 overexpression has been shown to induce apoptosis in esophageal and cervical cell lines (31, 32). Furthermore, in p53 dependent hepatoma cell lines, p21 expression induces expression of Bax, a known pro-apoptotic protein (33). Based on expression studies that show p21 in the wildtype pituitary at the time point where cell death occurs as well as the presence of ectopic expression of p21 in Hes1 mutants, coincident with an area of cell death, we hypothesized p21 may mediate cell death during pouch separation (8). However, our studies show that loss of p21 does not result in changes in cell death, visualized by TUNEL. Nevertheless, in the absence of p21, RP fails to pinch off in a timely manner, with an open pouch phenotype appearing through ss19, a time point when wildtype pituitaries have pinched off. This observation implies that specifically timed p21 expression promotes pouch closure with a definitive mechanism yet to be determined.

We have shown that p21 expression is detected at e10.5 in a small subset of cells at the juncture between RP progenitors and the oral ectoderm (8). This narrow window of expression indicates that other CDK1 molecules are needed to control proliferation in the bulk of RP progenitors. Interestingly, p21 expression may instead be used to arrest the cell cycle in pituitary cells during cellular insult. Although not usually expressed in adult pituitaries, p21 expression can be induced during tumor formation, where it plays an important function in restraining tumor growth. p21 has been shown to be upregulated in Pttg/Rb mutant pituitaries (13). Loss of
p21 in these Pttg/Rb mutant pituitaries results in increased tumor penetrance (14). It is also possible that during pituitary tumor formation due to cell cycle dysregulation by other mechanisms, p21 may also be induced. Alone, p21 mutants display spontaneous tumor development in multiple organs late in life, ~16 months, although pituitary tumors were undetected (12). But when p21 and p18 are both lost, pituitaries have an increased incidence of tumor formation (16). These studies indicate that p21 is needed to restrain proliferation and, when mutated in addition to other key cell cycle players, allows for unrestrained growth and tumor formation.

p21 induction may also be employed during pituitary tumor formation in humans. Studies looking at hormone secreting adenomas have shown p21 is strongly expressed, indicating that in tumor cells, p21 may maintain a differentiated state and may or may not contribute to growth control (45). Interestingly, p21 is not expressed in null cell adenomas, tumors that do not express hormone and generally proliferate past stages of their growth hormone secreting counterparts (15). p21’s role as a differentiation marker has been proposed in other developing systems and it may help to maintain the differentiated state of human tumors. However, p21 expression in the developing pituitary occurs at stages before terminal hormone cell differentiation. Therefore, we can only hypothesize that p21 activity in hormonally active pituitary adenomas differs from embryonic developmental mechanisms.

With these current studies we show that changes in p21 at early stages may not immediately influence pituitary progenitor proliferation and preserve cell survival. Expression at a narrow window of time indicates p21 may have a specific developmental action in the pituitary, but due to lack of changes at this stage when p21 is lost, direct consequences may not be readily apparent. Further investigations at later stages of embryonic development as well as
postnatal proliferation may provide clues as to whether p21 modulates pituitary cell proliferation and whether changes later in life could prime pituitary tumor formation when additional cell cycle molecules are altered.

### 3.6 Acknowledgments

We would like to thank Sabina Rybak and Agata Parfieniuk with experimental assistance. This work is supported by a grant from the National Institutes of Health (R01 DK076647) to LTR.
Figure 3.1. CIP/KIP cell cycle inhibitors are found in the developing pituitary. Expression of CDKI from e10.5-e14.5 was detected by immunohistochemistry. At e10.5, p21 is found at the border between Rathke’s Pouch (RP, bracket) and the oral ectoderm (A, arrow and bracket). p27 is located throughout RP at e10.5 (B, arrow) p57 expression is found scattered throughout RP with a higher concentration dorsally (F, arrows). At e11.5, p21 expression is in few cells ventrally and in the underlying oral ectoderm(OE) (B, arrows). P27 is scattered throughout RP (F, arrow). p57 at e11.5 expression is again dispersed through RP (J, arrows). By e12.5 p21 expression is limited to few cells around RP (C, arrow) and by e14.5 it is rarely found (D, arrow). P27 at e12.5 is scattered around RP extending into the growing anterior lobe (G, arrow) and at e14.5 a large proportion of the pituitary has p27 expression (H, arrow). p57 persist in the pituitary at e12.5, with expression found throughout the growing gland and in cells between RP progenitors and the burgeoning anterior lobe (K, arrow). By e14.5, p57, expression is mostly restricted to the RP with few cells in the anterior lobe (L, arrows). n \geq 3. Scale bar denotes 50 microns.
Figure 3.2. p21 does not appear to regulate proliferation of progenitors early in development. Proliferation was detected using Ki67 to mark cells in all phases of the cell cycle and Phosphohistone H3 (PH3) showing cells in late G2 and M phase. At e10.5, the majority of RP progenitors are in some phase of the cell cycle and no change in PH3 expression in the inner lumen of RP is detected in both wildtype (A and G) and p21 mutants (D and J). By e11.5 in wildtype (B and H) and p21 mutants (E and K) most of RP is Ki67 positive with the more ventral region beginning to exit the cell cycle and PH3 expression remains in luminal cells. By e12.5 an outcropping in the lower portion of RP, the future anterior lobe, does not contain Ki67 or PH3 positive cells in either the wildtype (C and I) or the p21 mutant (F and L). Additionally changes in Ki67 or PH3 expression patterns are not observed. n≥3. Scale bar denotes 50 microns.
Figure 3.3. Loss of p21 does not affect expression of Cyclin E or RB phosphorylation in early pituitary development. Immunohistochemistry of the markers Cyclin E and pRB were performed at e10.5-e12.5. Cyclin E expression is found in few cells in the wildtype pituitary at e10.5 (A) with similar results seen in p21 mutants (D). At e 11.5 RP cells in the lumen of RP are positive for Cyclin E in both wildtype (B) and p21 mutants (E). By e12.5, cells that are Cyclin E positive are still in the RP lumen with few cells that have moved from this zone in wildtype (C) and p21 mutants (F). pRb expression is in few RP progenitors at e10.5, with no change found in p21 mutants (G) compared to wildtype (J). By e11.5, some cells lining the lumen of RP are positive for pRB with no alterations seen in p21 mutants (K) compared to wildtype (H). By e12.5 the majority of cells that are pRB positive are found lining the RP lumen in wildtype (I) compared to p21 mutants (L), with one or two cells found outside the luminal zone. n≥3. Scale bar denotes 50 microns.
Figure 3.4. Loss of p21 impairs timely closure of Rathke’s pouch. Hematoxylin and Eosin staining reveals pituitary morphology at e11.5. To determine if pouch separation occurs in a timely manner animals were divided into somite stages (ss). At ss16 all pituitaries observed appear to be open to the oral cavity in both wildtype (A) and p21 mutants (E). By ss17, pouch closure is variably detected in p21 mutants (F) and wildtype pituitaries (B). ss18 denotes the stereotypical e11.5 and pouch closure is mostly complete in wildtype (C) compared to p21 mutant (G) pituitaries. Finally one somite stage later at ss19 all pituitaries observed in the wildtype (D) are not open to the oral ectoderm in the wildtype (D), yet some p21 mutants (H) still retained an open pouch. Somite staged counts of open pouch/total ratio show delayed pouch closure in p21 mutant pituitaries (I). n≥3. Scale bar denotes 50 microns.
Figure 3.5. Loss of p21 does not alter cell death detected by TUNEL. Cell death was detected using a TUNEL assay. At e10.5 in wildtype pituitaries (A, arrow) an area of cell death is seen in the area where the oral ectoderm meets RP a pattern seen also in p21 mutants (F, arrow). Somite staged e11 pituitaries revealed TUNEL positive staining mostly restricted to the oral ectoderm (arrows) directly beneath RP in wildtype pituitaries (B-D). p21 mutants (G-I) did not show any change in cell death compared to wildtypes, although open pouches at these somite stages still had TUNEL positive cells at the juncture between RP and the oral ectoderm (arrow). By e12.5 wildtype (E) and p21 mutant (J) pituitaries have separated from the oral ectoderm and an autonomous structure is found and cell death is not apparent. n≥3. Scale bar denotes 50 microns.
3.8 References


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Chapter 4

p21 and p27 prevent excess pituitary progenitor proliferation during development.

4.1 Abstract

Pituitary gland function is dependent on proper organogenesis during embryonic life. During pituitary development, progenitor proliferation is halted as hormone cell specification begins. A class of proteins called CIP/KIP cyclin dependent kinase inhibitors (CDKI) mediates the process of turning a proliferating progenitor into a differentiated cell. Notch signaling uses transcriptional control of multiple CDKIs to prevent progenitor exit from the cell cycle therefore preventing differentiation. These observations led us to question whether CDKI expression alone or in combination is necessary to restrain proliferation and to allow differentiation during pituitary formation. Expression of p21 in the pituitary is most readily detected to embryonic days 10.5-12.5 with isolated cells detected in the adult, and pituitary tumor growth has been postulated to be restrained though p21 upregulation. We now show that loss of p21 may limit pituitary cell proliferation in a limited number of cells in a localized manner. In contrast, p27 is found throughout the developing pituitary and is needed to prevent expansion of cells that have moved from the proliferative zone to the anterior lobe, but have yet to differentiate. p27 is commonly disrupted in tumors and mice lacking p27 form intermediate lobe tumors. Our data now suggests that during embryonic development CDKIs are needed to control progenitor pituitary cell proliferation. With these studies in mind we can speculate that although hyperplasia and tumor formation normally manifests in the mature gland, a detectable change in proliferating progenitors in the embryonic pituitary when CDKIs are lost may potentiate a tumor receptive environment.
4.2 Introduction

Pituitary gland development is reliant on the coordination of signaling pathways and molecular mechanisms that direct proliferation and differentiation of progenitor cells. Induction of the pituitary begins and embryonic day 9.5 (e9.5) which is defined by a population of highly proliferative progenitors, located in a structure known as Rathke’s pouch (RP). Initially, RP cells contain Sox2, a marker of progenitor and stem cell populations (1). As the pituitary matures, Sox2 is restricted to a small population of cells lining the lumen separating the anterior and intermediate lobes. Additionally, pituitary progenitor cells that have progressed into an intermediate progenitor status are derived from the Sox2 stem-like cells. These cells, marked by Sox9 expression, also likely proliferate in a controlled manner. In the intermediate lobe of the pituitary, a unique intermediate progenitor, expressing Pax7, also contributes to cells in the intermediate and anterior lobes (2). The expansion of pituitary progenitors is reliant on tightly regulated fluctuations in components of the cell cycle. With these studies we hope to elucidate which cell cycle components are needed to drive pituitary progenitor proliferation and how alterations in this controlled mechanism can alter pituitary progenitor expansion during development.

Various molecules and signaling pathways have been shown to regulate pituitary proliferation. Growth factors such as, FGF8, emanating from the ventral diencephalon in pituitary development has been shown to be a potent proliferative signal that promotes progenitor expansion and repression of differentiation by blocking BMP2 (3). Lhx3/4 as well as Hesx1, are transcription factors induced by the signaling pathways that are needed for proliferation and pituitary gland induction (4-7). Recent studies have shown direct regulation of cell cycle molecules is the mechanism by which the cell fate choice of proliferation versus
differentiation is modulated. Pitx2, a transcription factor necessary for pituitary formation, participates in promoting pituitary progenitor proliferation by driving Cyclin D2 expression, a molecule needed to transition cycling cells from the G1 phase of the cell cycle (8). Furthermore our group has shown that Notch signaling, through the transcriptional repressor HES1, is needed to depress Cyclin Dependent Kinase Inhibitors (CDKI) expression, preserving pituitary progenitor proliferation (9).

Prior to differentiation, progenitors must first exit the cell cycle, a process driven by increases in CDKI expression. CDKI upregulation has been shown to be the hallmark of differentiating tissues, which need to enter into a non-proliferative state. The CDKI p21 has been shown to participate in neuronal and glial development and differentiation ((10, 11) Chapter 3). Epithelial tissues, such as the skin additionally require p21 induction for keratinocyte specification (10). The CDKI, p27 has also been shown to be upregulated in differentiating tissues. β-cell differentiation of the endocrine pancreas is reliant on p27 mediated cell cycle exit (12). In the pituitary, p21, p27 and p57, members of the CIP/KIP family of cell cycle inhibitors, are found in RP cells. At e10.5 p21 and p27 are expressed in a limited number of cells. p21 expression continues into e11.5 with few positive cells detected at later ages, indicating action in a specific subset of cells. p57 expression is found in a limited number of non-cycling cells during stages of anterior lobe cell specification, likely serving as the critical mediator of progenitor cell cycle exit (Chapter 3). Loss of p57 results in pituitary hyperplasia resulting from an increase in proliferating progenitors seen as early as e12.5. Conversely, overexpression of p57 results in pituitary hypoplasia, indicating fewer proliferating progenitors (13). p27 expression in RP progenitors and AL cells is detected at e14.5, an age when hormone cell types begin to emerge. Loss of both p27 and p57 together, results in increased proliferation of pituitary progenitors at
e14.5, suggesting that proper regulation of these molecules is needed to restrain progenitor expansion. Additionally, p27 expression has then been shown to replace p57 and is needed to prevent differentiated cell proliferation, a phenomenon rarely seen during embryonic development (13). By e18, the pituitary is mostly absent of cycling progenitors, although a small population of Sox2 and Pax7 containing cells remains, and the pituitary is equipped with all hormone cell types ready for adult secretary function (1, 2).

In addition to their role in development, CDKIs also play a role in maintaining adult tissue quiescence. Dysregulation of CDKIs has been shown to be prevalent in many types of tumors and cancers (14, 15). Pituitary tumors account for ~15% of all intracranial tumors and are present in 16.7-35% of the population upon autopsy. Although p21 is not normally found to be mutated in the majority of human cancers (16, 17), p21 induction has been shown to protect against pituitary tumor formation (18-20). *p21* mutant mice appear phenotypically normal during the majority of life, with the exception of impaired G1 checkpoint progression (21, 22). However, *p21* mutant animals have spontaneous tumor formation seen at ~16 months of age with no detection of pituitary tumors (23). p27 is commonly dysregulated in a variety of human cancers (24). Loss of functional p27 has been implicated in the disorder Multiple Endocrine Neoplasia (MEN) in both humans and murine models, which includes pituitary tumor formation (25). Loss of *p27* in the mouse results in overall pituitary hyperplasia and tumor formation of the intermediate lobe (26-28). It is tempting to speculate that these intermediate lobe tumors may arise from the pituitary stem cell population, as the putative stem cell niche is located lining the periluminal region of the pituitary cleft and intermediate lobe.

Consistent with a two-hit model, pituitary tumor susceptibility appears to be increased when multiple cell cycle molecules are dysregulated. Mice lacking the retinoblastoma gene, *Rb,*
form intermediate lobe pituitary tumors with increased incidence and shorted latency when either p21 or p27 are also lost (29, 30). This synergistic action may also explain why loss of multiple CDKIs also display increased tumor susceptibility. Loss of p18, another CDKI, in addition to loss of p21 or p27 again reveals decreases tumor latency indicating that CDKIs expression is necessary to restrain tumor growth by either a synergistic or redundant manner (31, 32).

The high prevalence of pituitary tumors in the population warrants investigations into what molecular mechanisms are needed to guard against excess proliferation in the embryo and the adult stem/progenitor cells. Studies have already shown the potential for CDKIs, specifically of the CIP/KIP family, to operate as tumor suppressors. Here we show that p21 and p27 are needed to restrain progenitor cell expansion in the embryonic pituitary. Loss of p21 results in increased proliferation in a localized area of the developing pituitary while loss of p27 expands anterior lobe cells. Loss of both molecules, during development, appears to increase pituitary progenitor proliferation, potentially initiating tumor susceptibility and development during embryonic life. With these studies, we hope to elucidate how CDKI’s participate in pituitary gland development and potentially how tumor formation occurs through dysregulation of cell cycle progression of pituitary progenitors.

4.3 Materials and Methods

Mice and Embryo collection

p21 mutant mice, with exons 2 and 3 replaced with pgk-neo cassette, were obtained from Dr. Paul S. Cooke’s laboratory at the University of Illinois at Urbana-Champaign. These mice were originally generated by Dr. Tyler Jacks Laboratory at the Massachusetts Institute of Technology (30). p27 mutant mice, with pgk-neo cassette replacing the entire coding sequence, were obtained from the Jackson Laboratories and were generated by Dr. Matthew Fero at the
Fred Hutchinson Center (26). p21 homozygous mutant and p27 heterozygous mutant mice were first interbred for 3 generations to obtain consistent genetic background. Two separate breeding schemes were then undertaken to generate wildtype controls and p27 homozygous mutant mice, as well as p21 homozygous mutant and p21/p27 double mutant animals. The first scheme called for breeding of p21 homozygous; p27 heterozygous mutant animals to generate litters of p21 mutant and p21/p27 mutant homozygous double mutant animals. The second scheme required breeding of p21 homozygous for the wildtype allele with p27 heterozygous mutant animals to generate litters of wildtype and p27 mutant mice. Pregnant females were sacrificed at 14.5 and 16.5 days after detection of a vaginal plug. p21 genotype was determined by using: p21 wildtype forward primer, 5’ ACT TTT GAT TGG CCT GAT GG 3’; p21 wildtype reverse primer, 5’TGA CGA AGT CAA AGT TCC ACC G. p27 genotype was determined by using: p27 wildtype forward primer, 5’ GAT GGA CGC CAG ACA AGC 3’; p27 wildtype reverse primer, 5’CTC CTG CCA TTC GTA TCT GC; p27 neo mutant forward primer, 5’ GTC TTG TCG ATC AGG ATG ATC T 3’; p27 neo mutant reverse primer, 5’ GGC CAT TTT CCA TCT CTG AA 3’. The University of Illinois IACUC approved all procedures involving mice. Embryos were collected and fixed in 3.8% formaldehyde solution (Fisher) in phosphate buffered saline, PBS, (Fisher). Embryos where dehydrated through a graded series of ethanol and placed in paraffin for sectioning. Sections measuring 6 microns thick were then affixed to positively charged slides.

**Immunohistochemistry**

Embryo sections affixed to slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and washed in phosphate buffered saline (PBS) solution. Slides were subjected to antigen retrieval using 0.01M Citrate buffer, pH 6.0, for 10 minutes for samples treated with anti-Ki67, anti-CyclinE, anti-Bromodeoxyuridine (BrdU), anti-Phosphohistone H3.
(PH3), anti-Sox2 and anti-ACTH. All slides were blocked for 10 minutes using 5% Normal Donkey Serum (NDS, Jackson Immunoresearch) in an immunohistochemistry blocking solution (IHC block) containing 5% BSA, 0.1% TritonX-100 and PBS. Primary antibodies were diluted in IHC blocking solution at various dilutions: rabbit anti-Phosphohistone H3 (Ser 10, #06-570 Upstate Cell Signaling Solutions) 1:300; mouse anti-BrdU (#555627 BD Pharmingen) 1:50; rat anti-Ki67 (DAKO) 1:100; rabbit anti-ACTH (NHPP) 1:1500; rabbit anti-Pit1 (obtained from Dr. Simon Rhodes at Indiana University School of Medicine) 1:500; rabbit anti-CyclinE (ab52189 AbCam) 1:500; rabbit anti-Sox2 (AB5603) 1:750. Donkey derived mouse and rabbit secondary antibodies conjugated to biotin (Jackson Immunoresearch) were diluted to 1:200 and incubated with sections for one hour. Slides were then incubated with tertiary antibodies, Streptavidin conjugated to cy3 fluorophore (Jackson Immunoresearch), for one hour. Ki67 was detected with a secondary rat antibody conjugated to the fluorophore FITC. ACTH and Pit1 expression was detected with the secondary rabbit antibody conjugated to the fluorophore FITC. Cell death was assessed by TUNEL as previously described (33). All sides were counterstained with 4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Sigma 28718-90-3) at 1:1000 (Stock 1mg/ml) and mounted using aqueous fluorescence mounting media. Samples were then visualized at 200x.

**Cell Count Analysis-Phosphohistone H3**

**Midsagittal Cell Counts**

Slides containing midsagittal sections from e16.5 WT, p21 mutant, p27 mutant, and p21/p27 double mutant embryos were stained with PH3 and imaged as previously described (Chapter 2 and 3). Images were taken at 200x magnification. For each genotype tested there was an n≥3 with at least two sections from each animal tested. All cells positive for PH3 were
counted and a DAPI counterstaining was utilized to obtain an overall cell count for the whole 6-micron thick pituitary section. For each genotype, least 3 embryos were analyzed. The proportion of immunoreactive cells was compared to the total number of DAPI positive cells contained within RP. A percentage of positive cells per pituitary section were determined using NIH Image J software, then statistically analyzed with a two tailed T-Test.

**Total Pituitary Cell Counts**

To quantify proliferation throughout the developing pituitary at e16.5 immunohistochemistry was performed on every 4-5th slide through a sectioned pituitary. Slides containing sections from e16.5 WT, p21 mutant, p27 mutant, and p21/p27 double mutant embryos were stained with PH3 and imaged as previously described (Chapter 2 and 3). Images were taken at 200x magnification. For each genotype tested there were at least 5 embryos analyzed with 5 slides affixed with 2 sections for each animal tested. All cells positive for PH3 were counted and total pituitary section area was visualized by DAPI counterstaining of whole 6-micron thick pituitary section. The proportion of immunoreactive cells was compared to the area of RP as determined by tracing analysis on NIH Image J software. The number of positive cells per micron² area of pituitary per section was determined with total counts throughout all pituitary section being added to provide a proliferation indices for each animal tested. Statistical analysis was then performed using a two tailed T-Test to determine statistical significance.

**RT-PCR quantitation of p21 mutant e16.5 pituitaries**

In order to determine proliferation of WT and p21 mutant pituitaries, Real Time PCR analysis was performed in pituitaries extracted from e16.5 embryos. Following dissection, pituitaries were placed in a lysis solution. Samples were then homogenized and RNA was extracted following the protocol outlined in the Ambion RNAqueous® Micro Kit (#1931),
extracted RNA was then stored at -80°C until cDNA synthesis was performed. cDNA was created utilizing the New England BioLabs ProtoScript M-MuLV First Strand cDNA Synthesis Kit (#E6300L) and stored at -20°C until RT-PCR analysis was performed. To determine proliferation in collected samples, Ki67 products amplified at an annealing temperature of 64°C (Ki67 forward primer 5’ CCAGGGATCTCAGCGCAATTACAG 3’ and Ki67 reverse primer 5’ GGATAGGACAGAGGCCACATTTC 3’) were compared to GAPDH products (GAPDH forward primer 5’ GGTGAGGCCGCTGCTGAGTATG 3’ and GAPDH reverse primer 5’ GACCAGTTTTGCTCCACCCTTC 3’). 5-6 separate pituitaries were tested for each genotype and run on the BioRad iQ5 Multicolor Real-Time PCR Detection System Machine in Dr. Ann Nardulli’s laboratory. Data was collected utilizing the BioRad iQ5 Optical System Software Version 2.0 and analyzed on Excel, with a 2 tailed T-Test utilized to determine statistical significance.

4.4 Results

Loss of p21 and p27 increases proliferating progenitors at e14.5

In development, the cell cycle inhibitors p21 and p27 are utilized to shuttle proliferating progenitors out of the cell cycle, allowing for differentiation programs to determine cell fate. At e14.5, proliferating progenitors are restricted to the remnant of RP (bracket). This is visualized by immunostaining for BrdU incorporation, marking cells in S-phase (Figure 4.1A), and Phosphohistone H3 (PH3) expression, marking cells in M and late G2 phase (Figure 4.1E). Cells in the developing anterior lobe (bracket) have ceased proliferation. Pituitaries lacking p21 have similar BrdU (Figure 4.1B) and PH3 (Figure 4.1F) expression, with the majority of the positive cells found in RP. Interestingly, loss of p27 results in the appearance of more BrdU (Figure 4.1C) and PH3 (Figure 4.1G) immunoreactive cells in the anterior lobe. Loss of both p21 and p27
appears to increase proliferation throughout RP and the developing anterior lobe (Figure 4.1D and H), although double mutant pituitaries and single mutant pituitaries appear at this age morphologically similar. This indicates that loss of both cell cycle inhibitors profoundly effects proliferation in the developing pouch, resulting in cells either remaining in the cell cycle or reentering.

**Loss of p21 and p27 results in increased cells in M and G2 phase at e16.5**

Loss of p21 and p27 appears to alter the proliferative status of pituitary cells at e14.5. We therefore investigated whether proliferation changes could be detected at later stages. Wildtype pituitaries have few cells scattered throughout the anterior lobe and intermediate lobe that are positive for PH3 (Figure 4.2A, arrowhead) contrary to results seen at e14.5 where cells in the RP are still highly proliferative. Cell count quantitation reveals that 6.43% of total pituitary cells in midsaggital sections are proliferating (Figure 4.2I), while whole pituitary quantitation shows that 8.40051E-06 cells per micron$^2$ are PH3 positive (Figure 4.2J). p21 mutant pituitaries may have a slight increase in PH3 positive cells by histological examination (Figure 4.2B) and quantitation at midsaggital sections show a significant increase in PH3 positive cells (7.76%, p≤0.0465) in comparison to wildtype (Figure 4.2I). With this observation we quantified proliferation based on Ki67 mRNA using RT-PCR in wildtype and p21 mutants. When normalized to GAPDH p21 mutants had a relative fold change of 0.97002 compared to 1.16457 for wildtype pituitaries, indicating no overall change in proliferation. Upon more stringent examination where PH3 positive cell quantitation was conducted throughout the e16.5 pituitary (Figure 4.2 J) there did not seem to be a significant change (9.61108E-6 cells per micron$^2$, p≤0.2490). This may indicate that loss of p21 may alter proliferation in a localized area with few cells affected overall. p27 mutant pituitaries, a model known to have intermediate lobe tumors in adult life, appear to have
more proliferating cells in the anterior lobe (Figure 4.2C). Quantitation midsagittally shows 9.15% of cells positive for PH3, a percentage significantly higher (p ≤ 0.0480) than wildtype pituitaries (Figure 4.2I). Additionally, quantitation throughout the pituitary remains consistent with the conclusions from midsaggital counts where 1.42690E-05 cells per micron² are PH3 positive, indicating that there is a significant change (p ≤ 0.0001) in proliferating cells when p27 is lost. Loss of both p21 and p27 in the pituitary reveals an overall increase in PH3 immunoreactive cells assessed by histological examination compared to wildtype pituitaries (Figure 4.2D). Indeed, an increase in proliferating cells is further supported by quantitation at midsaggital sections of p21/p27 mutant pituitaries where 10.87% of cells are actively in M and G2 phase of the cell cycle, a significant change (p ≤ 0.0216) compared to their wildtype counterparts. Further scrutinizing analysis shows that compared to wildtype whole pituitary cells counts, loss of p21 and p27 alters proliferation in the developing pituitary with 1.26455E-0.5 cells positive for PH3 per micron² of area (p ≤ 0.004). These data indicate that p21 and p27 are both crucial to controlling progenitor proliferation in the pituitary at e16.5. Although at this age, there does not appear to be a significant difference between proliferation indices between p27 mutants and p21/p27 double mutants, a result that may changes as the pituitary continues to grow in adult life.

CDKI activity is needed to repress Cyclin/ Cyclin dependent kinase complex. We therefore investigated whether Cyclin E, a common molecular target of CDKIs, expression was altered when p21 and p27 were lost. Immunohistochemistry reveals no change in Cyclin E expression at e16.5. Wildtype pituitaries only contain a few Cyclin E positive cells scattered throughout the anterior lobe and the intermediate lobe (Figure 4.2E, arrowhead). Additionally p21 mutants (Figure 4.2F), p27 mutants (Figure 4.2G) and p21/p27 double mutants (Figure 4.2H), all display similar Cyclin E expression patterns with only a hand full of positive cells
scattered throughout the pituitary. These results indicate that although loss of \( p21 \) and \( p27 \) does not affect expression patterns of proteins they stereotypically inhibit, proliferation does appear to be increased throughout the developing pituitary at e16.5.

**Excess proliferation is not seen in Sox2 positive progenitors**

Uncontrolled proliferation in progenitor or stem cells is a hallmark of certain types of cancers such as medulloblastomas (34). We hypothesize that loss of the cell cycle inhibitors \( p21 \) and \( p27 \) can result in increased proliferation of the pluripotent progenitor population of the pituitary. Ki67 immunohistochemistry at e16.5, recapitulates the findings that loss of one or both cell cycle inhibitors, \( p21 \) and \( p27 \), results in an increase in proliferating cells seen in the pituitary. Ki67, unlike PH3, marks cells in all phases of the cell cycle, revealing which cells are in active proliferation or have the potential to divide, given the proper signals. In wildtype pituitaries cells scattered throughout the pituitary are Ki67 positive (Figure 4.3A, arrow). \( p21 \) mutants reveal increased Ki67 immunoreactive cells in the developing intermediate lobe, similar to PH3 experiments, (Figure 4.3B). \( p27 \) mutants have increased Ki67 positive cells in the anterior lobe compared to wildtype although intermediate lobe expression remains similar to wildtype (Figure 4.3C). Loss of both \( p21 \) and \( p27 \) results in an overall increase in proliferation throughout the pituitary (Figure 4.3D). To assess whether proliferating cells within the developing pituitary are multipotent progenitors, expression of Sox2, a common stem/progenitor marker, was employed. Immunohistochemistry reveals that cells in the developing intermediate lobe and cells lining the cleft are Sox2 positive (Figure 4.3E, arrow). Interestingly, the area of Sox2 positive cells in the \( p21 \) mutant does not appear to be expanded, even though proliferation marker studies revealed increased proliferation (Figure 4.3F). \( p27 \) mutants as well as \( p21/p27 \) double mutants also have unchanged Sox2 patterning (Figure 4.3G, H). To determine whether Sox2 positive cells are the
cell population that has increased proliferation in the p21/p27 mutants, Ki67 and Sox2 immunohistochemistries were merged. Wildtype pituitaries show little overlap between proliferating cells and Sox2 progenitors (Figure 4.3I and 3I’). Additionally p21 mutants (Figure 4.3J and J’) and p27 mutants (Figure 4.3K and K’) have few cells that are double positive for Ki67 (Figure 4.3L and L’). Further, p21/p27 double mutants have few Ki67 and Sox2 positive cells. These surprising results indicate that although increased proliferation is apparent, cells that have the potential to proliferate are not Sox2 progenitor cells.

**Differentiated cell proliferation is not robustly observed in CDKI mutants.**

During prenatal development, differentiated cell proliferation does not normally occur in the pituitary. Recent studies have shown that p27 expression is needed to prevent reentry of hormone producing cells into the cell cycle (13). We therefore hypothesize that that the cells undergoing ectopic proliferation in the intermediate lobe and anterior lobe of p21 and p27 mutant pituitaries may be differentiated cells. To test this, we acutely injected animals with BrdU to determine cells currently in S phase of the cell cycle and checked if these cells were also positive for the hormone marker POMC, present in the corticotrope and melanotrope lineage or the transcription factor PIT1 which marks thyrotropes, somatotropes and lactotropes. Similar to the data that we previously showed, cells immunoreactive for proliferation markers such as BrdU, are found scattered throughout the intermediate lobe and anterior lobes of e16.5 wildtype pituitaries (Figure 4.4A, E). p21 mutants have may have increased BrdU incorporation in the pituitary (Figure 4.4B, F). p27 mutants also appear to have an increase in BrdU incorporation in the developing pituitary (Figure 4.4C, G). p21/p27 mutants have increased proliferation throughout the pituitary at e16.5 (Figure 4D, H). POMC immunohistochemistry reveals that fully differentiated melanotropes and corticotropes can be found scattered throughout the IL and AL,
respectively and Pit1 immunoreactive cells are found in the majority of anterior lobe cells. Merged images of BrdU positive cells and ACTH or Pit1 positive cells reveal that few cells in the wildtype pituitary colocalize both markers, indicating that normally hormone cell types do not proliferate (Figure 4.4A and E, respectively). Similar results are seen in p21 mutant (Figure 4.4B and F), p27 mutants (Figure 4.4C and G), as well as in p21/p27 double mutants (Figure 4.4D and H). Additionally the limited number of cells that colocalize POMC and BrdU appear in an area close to the cleft between the AL and IL indicating that the cells expressing both markers may be transitional in nature. These results together show that although increased proliferation is seen in p21, p27 and p21/p27 mutant pituitaries the cells that are ectopically proliferating have yet to undergo hormone specific cell differentiation.

**Ectopic cell death is not seen in CDKI mutant pituitaries**

Our studies have revealed that p21 mutant, p27 mutant and p21/p27 double mutant pituitaries have increased immunoreactive cells positive for markers of proliferation. Interestingly, p21 mutants at adult stages do not appear any larger than wildtype pituitaries. Furthermore hyperplasia of the pituitary seen in adult p27 mutants is not apparent until later stages of postnatal development. We hypothesize that this size discrepancy may be the result of cell death in the excessively proliferating cell population that we have uncovered. To determine if cell death is occurring in the developing pituitary at e16.5, TUNEL staining was utilized. Wildtype pituitaries display an absence of cell death in the anterior and intermediate lobes (Figure 4.5A). Lack of cell death can also be seen in p21 mutant (Figure 4.5B), p27 mutant (Figure 4.5C) and p21/p27 double mutants (Figure 4.5D). These results indicate that active cell death is not occurring in the developing pituitaries at e16.5 and that other regulatory mechanisms
may modulate pituitary size in the developing pouch leading to normal pituitary size, at least seen in the \textit{p21} mutant during postnatal and adult life.

4.5 Discussion

Regulation of the cell cycle is necessary for proper pituitary gland formation. Alterations in expression of key cell cycle factors can impair proper expansion of progenitors or even impede proper differentiation of hormone cell types. We now show that loss of the CDKIs, \textit{p21} and \textit{p27}, alone or together, help to restrain overproliferation of pituitary progenitors during development.

Induction of the pituitary begins at e9.5. At this stage, and throughout the rest of embryonic development, pituitary progenitor proliferation populates the growing gland with the bulk of cells needed during postnatal life. Cells in RP are a highly proliferative and undifferentiated population, and as cells begin to migrate ventrally to form the anterior lobe, progenitors exit the cell cycle. CDKI expression patterns indicate that as cells cease proliferation, they enter into a quiescent state and this exit may prime cells for differentiation (9, 13). Notch signaling through HES1 has been shown to promote progenitor preservation by restricting CDKI expression from proliferating progenitors (9). This indicates that CDKI action is tightly controlled and alterations in this patterning of pituitary cells could lead to developmental dysfunction. \textit{p57} alone has been shown to modulate proliferation in anterior lobe cells and its loss results in pituitary hyperplasia (13). \textit{p27} has been shown to impact proliferation in the pituitary with its loss resulting in tumor formation in the adult (26-28). Additionally, studies have speculated that \textit{p27} contributes to anterior lobe development in the embryo. Upregulation in \textit{p27} expression has been reported to restrict differentiated cell proliferation (13). Our studies have already shown that \textit{p27} expression does not colocalize with hormone cell types (Chapter 2) and
its loss does not reveal any changes in proliferation of hormone subtypes compared to wildtype tissues. Studies have shown that joint loss of \(p27\) and \(p57\) in the pituitary results in proliferation of all pituitary cells eliminating the ability to exit the cell cycle (13). These results speculate that these two molecules alone are the only CDKIs that are necessary to restrict proliferation in the pituitary. Our studies now show that \(p27\) and \(p57\) are not the sole regulators in cell cycle exit in the embryonic pituitary. Loss of \(p21\) results in aberrant proliferation in a localized area of the pituitary, indicating that \(p21\) may regulate a subpopulation of pituitary progenitors.

Our studies indicate that loss of \(p21\) and \(p27\) does not alter Sox2 progenitor proliferation. Sox2 is found in the majority of RP during induction and its expression becomes restricted to cells in the cleft of the pituitary, an area where the putative stem cell niche resides in the adult organ. By e16.5 cells in the intermediate lobe and lining the cleft are Sox2 positive, and this expression pattern persists even when \(p21\) and \(p27\) is lost. Loss of \(p21\) does show excess proliferation midsagittally although colabeling studies did not reveal any increase in Sox2 cell proliferation. Furthermore, few proliferating cells colabel with differentiation markers indicating that the increase in proliferation seen when CDKIs are lost is not attributed to differentiated cell expansion. These results indicate that although increased proliferation is evident, the predominate cell type has yet to be elucidated. Recent studies have shown that Pax7 marks intermediate progenitors of the pituitary, with little Sox2 overlap, in cells populating the cleft and intermediate lobe (2). Further investigations are required to determine if the cell type that is proliferating in \(p21\) and \(p27\) mutants are in fact, intermediate progenitors or a potential trans-amplifying cells that is unspecified in nature.

Proliferation of pituitary cells is not restricted to embryonic development. A wave of postnatal proliferation is found during the second week of postnatal life, which populates the
gland with cells needed for adult life. Interestingly at this stage, the subtype of cells that are proliferating are hormone cell types and Pax7 positive cells may also contribute to postnatal proliferation activities (2, 35-39). Further studies are needed to determine whether p21 and p27 are necessary to modulate this proliferative stage.

Tumors of the pituitary are a common occurrence, accounting for ~15% of intracranial tumors (16). Studies have shown that mutation or dysregulation of CDKIs are commonly found in pituitary tumors. Loss of p27 results in the disorder Multiple Endocrine Neoplasia (25). Patients and rodent models with this disorder display with pituitary tumors. p27 has been shown to be necessary to restrain proliferation in Brg1 mutant animals and its loss results in increased incidence of adenoma formation (40). With these studies our group in conjunction with other studies shows that p27 is needed to restrain pituitary cell proliferation during development and that its loss may prime the pituitary to form tumors (13).

Recent evidence has shown that p21 expression is needed to exert a protective mechanism in the event of cellular insult or aberrant cell proliferation. Loss of Pttg in Rb mutant mice results in restraint of tumor potential and upregulation of p21 may induce senescence in these abnormally cycling cells (19). Further loss of p21 in Pttg/Rb mutant pituitaries eliminates this protective mechanism and pituitary tumor formation is again apparent (18). These studies, similar to our results indicating that loss of Hes1 can induce p21 expression may further highlight p21s role in protecting the pituitary under aberrant signaling (9). Interestingly, p21 loss alone does not lead to tumors of the pituitary (22). Yet additional loss of other molecules known to induce tumors in conjunction with p21 expedites tumor initiation, indicating that p21 may in part suppress tumor growth and its loss negates it protective potential.
Loss of multiple CDKIs has been shown to increase pituitary tumor incidence and decrease latency. Loss of \(p27\) along with either \(Rb\) or \(p18\) has been shown to increase tumor formation, with similar results seen when \(p21\) is lost with these molecules (29-32). Yet, the question remains, at what stage of life does tumor initiation begin? With these studies, we show that loss of both \(p21\) and \(p27\) increases ectopic proliferation of pituitary cells leading us to postulate that these changes may potentiate tumor formation earlier than the single mutant alone. We hypothesize that a potentially embryonic intermediate progenitor cell does not stop cycling appropriately, leading to a slow expanded growth of the pituitary leading to tumor formation.

Taken together, CDKI expression and regulation is tightly controlled during pituitary development. Alterations in gene expression or protein modulation can change the normal proliferative status of the pituitary and lead to tumor formation. Our studies reveal that CDKIs are necessary to restrain progenitor expansion during development and multiple hits could propagate tumor formation during early life. Understanding the mechanism behind tumor formation and how cell cycle molecules modulate pituitary cell expansion could provide crucial information for the detection of tumors and provide insight into development biomarkers and therapeutics that specifically target tumors of the pituitary.

4.6 Acknowledgements

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Figure 4.1. Loss of p21 and p27 increases proliferating progenitors at e14.5. Proliferation of e14.5 pituitaries was assessed by immunohistochemistry using the markers BrdU to mark S phase and PH3 to mark late G2 and M phase. Proliferation, marked by BrdU and PH3 positive cells, is mostly seen in the area around RP (bracket) with few cells in the anterior lobe (bracket) of the wildtype (A and E) and p21 mutant (B and F) pituitaries. BrdU and PH3 expression in the anterior lobe of the p27 mutant may reveal a small increase in proliferation (C and G). Together when p21 and p27 is lost, there is an increase in proliferation throughout the developing pituitary at e14.5 (D and H, arrows). n≥3. Scale bar denotes 50 microns.
Figure 4.2. Loss of p21 and p27 results in increased cells in M and G2 phase at e16.5. Proliferation at e16.5 is restricted to few cells in the intermediate and anterior lobes in the wildtype pituitary as visualized by PH3 (A, arrows). p21 mutants appear to have increased intermediate lobe proliferation (D, arrows) p27 mutant may have an increase in anterior lobe proliferation and p21/p27 double mutants (D) have increased immunoreactive cells throughout the pituitary compared to wildtype. Cyclin E a molecule CDKIs have been shown to regulate is found in few cells in the in the e16.5 pouch in wildtype pituitaries (E). This expression pattern is also seen in p21 mutant (F), p27 mutant (G) and p21/p27 mutant (H) pituitaries. Quantitation of PH3 positive cells in midsaggital sections reveals changes in p21, p27 and p21/p27 mutants in comparison to wildtype pituitaries (I, * denote significant changes). Whole pituitary cell counts per micron² shows changes in p27 and p21/p27 mutant pituitaries in comparison to wildtype pituitaries (J). n≥3 (for histology samples and midsagittal counts), n≥5 for whole pituitary counts. Scale bar denotes 50 microns.
**Figure 4.3. Excess proliferation is not seen in Sox2 positive progenitors.** Proliferation of Sox2 progenitors was detected by double labeling of the proliferation marker Ki67 and Sox2 antibodies. Wildtype pituitaries have cells positive for Ki67 (A) scattered throughout the intermediate and anterior lobes and Sox2 expression (E) is found in the intermediate lobe and luminal region of the anterior lobe. Merge of these images reveals that few Sox2 positive cell is actively proliferating (I and I’, arrow). *p21* mutants appear to have increased Ki67 immunoreactive cells in the intermediate lobe (B) while Sox2 staining reveals patterns similar to wildtype (F). Merging these images shows few double reactive cells (J and J’). *p27* mutants may have more Ki67 positive cells in the anterior lobe (C) and Sox2 expression (G) remains similar to wildtype. Merge in *p27* mutant stainings again shows few double positive cells (K and K’). Ki67 positive cells appear to be increased throughout the pituitary when *p21* and *p27* are both lost (D). Although Sox2 expansion is not observed (H) and an increase in Sox2 proliferation is not seen (L and L’). n≥3. Scale bar denotes 50 microns.
Figure 4.4. Differentiated cell proliferation is not robustly observed in CDKI mutants. To detect if differentiated hormone cells are proliferating when CDKIs are lost we performed double labeling studies with POMC, Pit1 and BrdU markers. Wildtype pituitaries have BrdU positive cells scattered around the e16.5 pituitary (A, E, arrow, red). POMC positive cells are found in the intermediate and scattered in the anterior lobe (B, green). Pit1 positive cells are found throughout the anterior lobe (E). Merged images reveal that few Pit or POMC cells are colabeled with BrdU. Loss of p21 results in increased BrdU labeling but there are few cells that colabel with either POMC or Pit1 (B and F). Loss of p27 also may have more immunoreactive BrdU labeled cells in the pituitary with no change in hormone cell colabeling (C and G). Loss of both p21 and p27 reveals increased BrdU positive cells throughout the pituitary and normal hormone cell patterning with few if any colabeled cells (D and H). n≥3. Scale bar denotes 50 microns. Dotted line denotes intermediate lobe.
Figure 4.5. Ectopic cell death is not seen in CDKI mutant pituitaries. Cell death was detected by TUNEL assay. Wildtype pituitaries at e16.5 rarely have cell death in the anterior and intermediate lobes (A). Interestingly, p21 mutants (B), p27 mutants (C) and p21/27 double mutants (D) do not contain ectopic cell death in the pouch. n≥3. Scale bar denotes 50 microns.
4.8 References


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Chapter 5

Thesis Discussion

The pituitary gland is the major hormone regulator of the endocrine system. Its trophic hormone signals regulate integral and diverse endocrine functions such as somatic tissue growth, metabolic homeostasis and fertility. Disruptions in pituitary gland function, if left undetected, can have major physiologic ramifications throughout life. Understanding the signaling pathways and molecular mechanisms that develop the pituitary gland can provide insight into how pituitary dysfunctions arise and how they can be treated.

Understanding the genesis and physiologic repercussions of endocrine diseases can help lead to better screening techniques and diagnostic tools that could treat endocrine dysfunctions in a more targeted manner. Pituitary hypo- and hyperplasia can arise throughout life including during embryonic development. Craniopharyngiomas and Rathke’s Cleft Cysts have the ability to impede pituitary function during early life, a time when tightly controlled hormonal fluxes are necessary to shape the developing body and establish proper endocrine system function for adult life. Few molecular mechanisms have been found that are intrinsic to pituitary gland formation. Loss of function mutations in \textit{PROP1}, encoding a protein needed to establish somatotropes, thyrotropes and lactotrophs lineages, results in individuals that are short in stature and fail to enter puberty (1). Disorders such as Combined Pituitary Hormone Deficiency has a prevalence of 1:20,000 births and genetic testing for mutations in transcription factors such as \textit{PROP1} provide only one route in truly understanding how diseases that lead to hormone deficiencies can be treated (2). Studies in understanding pituitary gland development have only begun to highlight how growth factors such as FGF and BMP are necessary to promote pituitary
gland induction and growth and how transcription factors such as HES1 are needed to begin organ formation (3-7). Our studies have now highlighted Notch signaling, specifically through HES1, is responsible to establishing a proper pituitary size during embryonic life. Our data now shows that HES1 transcriptional repression of cell cycle inhibitors allows for progenitor proliferation. Loss of this gene in turn stunts pituitary cell proliferation, limiting the overall numbers of hormone producing cell types (8). With these observations in mind, HES1 mutation screenings of the population could uncover another contributor to hypoplastic and hormone deficient individuals.

Notch signaling molecules have been found to be present in the stem cell subpopulation of the pituitary (9). This suggests that activated Notch signaling is necessary for either stem cell survival or proliferation of these specialized cells. Recent reports have shown that Sox2 marks the putative stem cell population and future studies could elucidate what mechanisms Notch controls in these cells (10). It is still unclear as to what cell type is needed to expand hormone specific cell types in instances of physiologic need such as during pregnancy. Sox2 positive cells although pluripotent are slow cycling and we hypothesize that intermediate progenitors may be a transamplifying cell that responds to hormonal feedback and hypothalamic hormone induction. Our data has revealed that by eliminating the CDKIs, which are needed to restrain proliferation, we see excess expansion of cells that are neither differentiated nor stem/progenitor in nature. This highly suggests that intermediate progenitors could potentially be a highly proliferative subpopulation of undifferentiated cells that are responsible for normal postnatal proliferation and maybe the key in understanding how pituitary cell number is controlled in adult life. If indeed Notch plays an integral role in preserving the stemness of pituitary cells, future therapeutic
strategies could utilize this information to induce stem cell proliferation in hypoplastic pituitaries. Our lines of investigation have truly highlighted how integral Notch signaling is necessary for pituitary gland development and together with other studies in uncovering other pituitary intrinsic factors could help establish a larger genetic screen for pituitary disorders resulting in deficient hormone secretion.

The most striking statistic in pituitary diseases is that ~15% of intracranial tumors are pituitary in origin (11). Additionally, epidemiological studies have shown that 16.7-35% of the population will have hyperplasia or tumors of the pituitary, a statistic that boggles the mind (11, 12) This high occurrence warrants investigations into what genetic factors increase incidence of pituitary cell overgrowth. Studies in breast cancer research has established genetic screens for the BRCA1 and BRCA2, genes that when mutated have to ability to form aggressive and invasive breast cancers (13). Within the pituitary field, a better understanding is needed of pituitary specific factors that can lead to tumor formation, allowing for a more target approach to treatments. Multiple Endocrine Neoplasia (MEN) is rare heritable disorder found in 1:30,000 individuals that results in formation of pituitary tumors (14). Although this disorder is rare, it has provided insight into possible mechanisms that can result in excess cellular proliferation of the pituitary.

Loss of the cell cycle regulator CDKN1B (p27) results in MEN and has been shown to be disrupted in many types of pituitary tumors (15). Growth hormone adenomas can have decreased levels of p27, leading to dysfunctional G1/S checkpoint transition and excess cellular expansion (16). Our group now shows that p27 and its protein family members are necessary to restrain pituitary cell proliferation during embryonic life. From our CDKI expression studies and work done by the Drouin group, p27 and p57 appear to play an
integral role in restraining proliferation in the embryonic pituitary (17). We hypothesize that loss of these CDKIs can disrupt a proliferative balance of pituitary progenitors and this disruption can help to establish a tumorigenic environment during early stages of pituitary development.

With our data we now show that p21, another protein family member, may also play a role in controlling proliferation in the pituitary. But our data along with studies by the Melmed group suggest that p21 may also acts as a cellular protectant (18, 19). It is surprising that with the incidence of pituitary tumors being so high that the occurrence of pituitary cancers or metastatic spread of these tumors is rarely seen. Pituitary size is dynamic and can change upon physiologic need. This evidence leads us to speculate that intrinsic mechanisms that restrain spread of cells may be why pituitary tumors tend to not be cancerous. With its suggested role as a cellular protectant, p21 may be the key to understanding how pituitary tumors remain within the hypopheseal fossa.

To better understand how pituitary disorders can arise we must understand what factors and pathways establish the normal functional gland. With more insight into molecular factors that are intrinsic to the pituitary we can establish more refined lines of investigation that can help lead to better treatment and diagnostic protocols for the medical field.
5.1 References


Curriculum Vitae

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