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p21, an important mediator of quiescence during pituitary tumor formation, is dispensable for normal pituitary development during embryogenesis.

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Abstract

A delicate balance between proliferation and differentiation must be maintained in the developing pituitary to ensure the formation of the appropriate number of hormone producing cells. In the adult, proliferation is actively restrained to prevent tumor formation. The cyclin dependent kinase inhibitors (CDKIs) of the CIP/KIP family, p21, p27 and p57, mediate cell cycle inhibition. Although p21 is induced in the pituitary upon loss of Notch signaling or initiation of tumor formation to halt cell cycle progression, its role in normal pituitary organogenesis has not been explored. In wildtype pituitaries, expression of p21 is limited to a subset of cells embryonically as well as during the postnatal proliferative phase. Mice lacking *p21* do not have altered cell proliferation during early embryogenesis, but do show a slight delay in separation of proliferating progenitors from the oral ectoderm. By embryonic day 16.5, *p21* mutants have an alteration in the spatial distribution of proliferating pituitary progenitors, however there is no overall change in proliferation. At postnatal day 21, there appears to be no change in proliferation, as assessed by cells expressing Ki67 protein. However, *p21* mutant pituitaries have significantly less mRNA of *Myc* and the cyclins *Ccnb1*, *Ccnd1*, *Ccnd2* and *Ccne1* than wildtype pituitaries. Interestingly, unlike the redundant role in cell cycle inhibition uncovered in *p27/p57* double mutants, the pituitary of *p21/p27* double mutants has a similar proliferation profile to *p27* single mutants at the time points examined. Taken together, these studies demonstrate that unlike p27 or p57, p21 does not play a major role in control of progenitor proliferation in the developing pituitary. However, p21 may be required to maintain normal levels of cell cycle components.

Keywords

pituitary; cell cycle; p21; p27

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1. Introduction

Pituitary gland development is reliant on the coordination of signaling pathways and molecular mechanisms that direct the regulated appearance of the six major endocrine cell types: corticotropes, thyrotropes, gonadotropes, somatotropes, lactotropes and melanotropes. The pituitary is induced from the oral ectoderm at embryonic day 9.5 (e9.5) in the mouse. It consists of a population of highly proliferative progenitors located in a structure known as Rathke's pouch (RP) (Ikeda and Yoshimoto, 1991). Initially, RP cells contain SOX2, a marker of progenitor and stem cell populations (Fauquier, et al. 2008). As development proceeds, cells exit the cell cycle, extinguish SOX2 and begin to express hormones. By e18.5, although a small population of SOX2 containing proliferative cells remains, the pituitary is equipped with all hormone cell types and is ready for secretory function (Fauquier, et al. 2008; Garcia-Lavandeira, et al. 2009). In the postnatal gland it is less clear what cell type is proliferating to expand the pituitary to adult proportions. However, there is evidence to indicate that the SOX2 expressing cells, which line the lumen that separates the anterior and intermediate lobes and are scattered throughout the anterior lobe, play a role in this process (Fauquier, et al. 2008). Alternatively, in the rat, mitosis of cells that have already differentiated to contain hormones has been described (Taniguchi, et al. 2002, 2001a, 2001b, 2001c, 2000). Regardless of cell type or timing of differentiation, it is clear that the highly orchestrated expansion of pituitary progenitors and their subsequent differentiation is reliant on tightly regulated fluctuations in components of the cell cycle.

Recent studies have shown that direct regulation of cell cycle molecules is the mechanism by which the cell fate choice of proliferation versus differentiation is modulated in the developing pituitary. PITX2, a transcription factor necessary for pituitary formation, participates in promoting proliferation by activating *Ccnd2* transcription, a molecule needed to transition cycling cells from the G1 to the S phase of the cell cycle (Kioussi, et al. 2002). Furthermore, Notch signaling is essential for maintaining proliferative progenitors in RP (Monahan, et al. 2009; Raetzman, et al. 2004; Zhu, et al. 2006). Recent evidence shows that the Notch target HES1 is a transcriptional repressor essential for preventing Cyclin Dependent Kinase Inhibitor (CDKI) expression in pituitary progenitors, and that loss of *Hes1* increases CDKI expression and subsequently depletes the progenitor pool (Monahan, et al. 2009).

Induction of CDKI expression has been shown to be the hallmark of differentiating tissues, which need to enter into a non-proliferative state before cell specification. In the pituitary, p21, p27 and p57, members of the CIP/KIP family of CDKIs, are found in RP cells. p57 expression is localized to non-cycling cells during stages of anterior lobe cell specification, likely serving as the critical mediator of progenitor cell cycle exit. Loss of *p57* results in pituitary hyperplasia due to an increase in proliferating progenitors seen as early as e12.5. Conversely, overexpression of *p57* results in pituitary hypoplasia, indicating that there are fewer proliferating progenitors (Bilodeau, et al. 2009). p27 expression is detected in the pituitary starting at e12.5, an age when hormone cell types begin to emerge (Brinkmeier, et al. 2007). Loss of both *p27* and *p57* results in increased proliferation of pituitary progenitors at e14.5, suggesting that proper regulation of these molecules is needed to restrain progenitor expansion (Bilodeau, et al. 2009). Although p21 is present in RP at e10.5 and its expression is strongly induced upon loss of *Hes1*, its role in pituitary development is unknown (Monahan, et al. 2009).

In addition to influencing development, CDKIs also play a part in maintaining adult tissue quiescence. Dysregulation of CDKIs has been shown to be prevalent in many types of tumors and cancers (Musat, et al. 2004; Quereda and Malumbres, 2009). Pituitary tumors are detected incidentally in 12% of the population (Buurman and Saeger, 2006). The prevalence

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of clinically detected tumors is close to 1:1000 (Daly, et al. 2006; Fernandez, et al. 2010). Although mutations in *p21* are not common in pituitary tumors (Burrow, et al. 1981; Ezzat, et al. 2004), p21 expression is induced in GH producing human pituitary tumors, and this induction has been shown to be essential to limit pituitary tumor size in mice (Chesnokova, et al. 2005, 2008). Interestingly, p21 is not expressed in null cell adenomas, which do not express hormone and generally grow larger than their hormone secreting counterparts (Neto, et al. 2005). *p21* mutant mice exhibit impaired G1 checkpoint progression (Brugarolas, et al. 2002; Deng, et al. 1995) which leads to spontaneous tumor formation seen at approximately 16 months of age, however pituitary tumors have not been reported (Martin-Caballero, et al. 2001). Unlike p21, p27 is commonly dysregulated in a variety of human cancers (Bamberger, et al. 1999). Loss of functional p27 has been identified as a rare cause of the disorder Multiple Endocrine Neoplasia (MEN), which includes pituitary tumor formation, in both humans and rats (Pellegata, et al. 2006; Georgitsi, et al. 2007). Loss of *p27* in the mouse results in overall pituitary hyperplasia and intermediate lobe tumor formation (Fero, et al. 1996; Kiyokawa, et al. 1996; Nakayama, et al. 1996).

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 shorter latency when either *p21* or *p27* is also lost (Brugarolas, et al. 1998; Park, et al. 1999,). This synergistic action may 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 decreased tumor latency indicating that expression of CDKIs is necessary to restrain tumor growth by either a synergistic or redundant manner (Franklin, et al. 2000, 1998).

In addition to the role of p21 in cell cycle control, 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 (Fotedar, et al. 1999). Additionally, *p21* overexpression has been shown to induce apoptosis *in vitro* in esophageal and cervical cancer cell lines (Kadowaki, et al. 1999; Tsao, et al. 1999,). In p53-dependent human hepatoma cell lines, overexpression of p21 results in an induction of Bax, a proapoptotic protein (Kang, et al. 1999). 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 (Monahan, et al. 2009). 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 forelimb in the rodent (Vasey, et al. 2010). Together these studies suggest that p21 activation may be part of the mechanism regulating properly controlled cell death.

The 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, unlike p27 and p57, is not needed to restrain progenitor cell expansion in the embryonic pituitary. However, by the third postnatal week, although there is no overall change in pituitary size, loss of *p21* results in aberrant expression of specific cell cycle components.

2. Results

2.1 p21 expression is found in embryonic and postnatal pituitaries

p21 is present in isolated cells in the pituitary throughout development as well as during postnatal life. At e10.5, a time when cells throughout RP are highly proliferative progenitors, p21 expression is located in cells concentrated at the juncture between RP and

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the underlying oral ectoderm (Figure 1A). At e11.5, p21 is localized to scattered cells in the ventral aspect of RP as well as in the oral ectoderm (OE) cells directly below RP (Figure 1B). By e12.5, the pituitary cells begin to form an outcropping in the ventral RP, forming the future anterior lobe. By this stage, p21 expression is present in a few cells within RP (Figure 1C). Similar sporadic detection of p21 at e14.5 (Figure 1D) and e16.5 (Figure 1E) is also seen, mainly in cells within the anterior lobe . At postnatal day 21 (pd21), a time near the end of the postnatal proliferative phase, there are scattered p21 positive cells in the intermediate lobe (Figure 1F, black arrowhead) and anterior lobe (Figure 1G, black arrowhead).

2.2 Loss of *p21*subtly delays closure of Rathke's pouch but does not alter cell death

During early pituitary development (e10.5-e11.5), 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 (Monahan, et al. 2009), 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, pouch closure of wildtype and *p21* mutant pituitaries was carefully examined. Embryos were somite staged to more precisely determine embryonic age, with somite stage 18 (ss18) denoting e11.5. At ss16, both wildtype and *p21* mutant pituitaries appear to be connected to oral ectoderm, forming a structure that remains open to the oral cavity in the three wildtypes and three p21 mutants that were examined (Figure 2A, E). By ss17, wildtype (Figure 2B) and *p21* mutant pituitaries (Figure 2F) begin to close, although this closure is not complete in all embryos (two of five are closed in wildtype, one of five is closed in mutant). At ss18, the stereotypical e11.5, most of the wildtype (Figure 2C) pouches are no longer open (three of four are closed), forming a RP that is autonomous from the oral ectoderm. *p21* mutant (Figure 2G) pituitaries at this stage are open in two of the four examined. By ss19, all three of the wildtype (Figure 2D) pouches examined are closed. *p21* mutants (Figure 2H), on the other hand, still exhibit variable pouch closure (one of four are closed at this stage). Interestingly, by e12.5, both wildtype and *p21* mutant pituitaries appear to have closed to the oral cavity.

Since the loss of *p21* delays pouch separation to some extent, we hypothesized 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 (Vasey, et al. 2010). 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 in the junction between the RP and the oral ectoderm in both wildtype (Figure 2I, arrowhead) and *p21* mutants (Figure 2M, arrowhead). 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 2J, arrowhead). *p21* mutant pituitaries at this somite stage show that when pouch closure is incomplete, cell death still occurs (Figure 2N, arrowhead). Yet, at ss18 and ss19 in both wildtype (Figure 2K, L) and *p21* mutant pituitaries when pouch separation has occurred, changes in cell death are not detected (Figure 2O, P). By e12.5 pouch closure is complete in both animals and cell death is no longer apparent (data not shown). These data indicate that p21 is not mediating cell death in the developing pituitary.

2.3 p21 does not appear to regulate proliferation of progenitors early in development

In addition to its role in cell death, p21 is also 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 including Ki67, which marks cells in all active phases of the cell cycle, and phosphohistone

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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 Ki67 expression (Figure 3A, B). PH3 staining in wildtype (Figure 3C) and *p21* mutants (Figure 3D) 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. Ki67 and PH3 expression is mirrored in e11.5 pituitaries of both wildtype (Figure 3E, G) and *p21* mutants (Figures 3F, H), with the majority of cells proliferating and lumen-restricted cells progressing through M phase. Additionally, cell count quantification of sections throughout the pituitary did not reveal significant alterations in PH3 positive cells in the *p21* mutant when compared to the wildtype. Wildtype pituitaries have 19.00±4.06% while *p21* mutants have 20.47±2.72% of PH3 positive cells (p=0.375). At e12.5 the majority of RP progenitors in wildtype (Figure 3I) and *p21* mutants (Figure 3J) are still marked by Ki67 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 3K) and *p21* mutants (Figure 3L). Additional examination of Cyclin E and phosphorylated Rb expression did not reveal any differences between wildtype and *p21* mutants (Supplemental Figure 1). These results indicate that p21 expression in the early pituitary gland may not play a major role in controlling proliferation. Furthermore, these data indicate that compensatory mechanisms could mask proliferation changes when *p21* is lost.

2.4 Loss of *p21* in addition to *p27* does not change proliferation at e16.5

To determine if another member of the CIP/KIP family of CDKIs, p27, has any overlapping functions with p21 during embryonic development, we examined pituitaries of mice lacking each inhibitor in isolation or as double mutants. Proliferation was analyzed in e16.5 pituitaries using the cell cycle markers PH3, Ki67 and BrdU incorporation. Wildtype pituitaries have PH3 positive cells scattered throughout the anterior lobe and intermediate lobe (Figure 4A). Furthermore, immunohistological analysis with Ki67 (Figure 4E) and BrdU (Figure 4I), which marks cells in the S-phase of the cell cycle, indicates that proliferating populations are scattered throughout the anterior lobe and developing intermediate lobe. Upon immunohistological examination, *p21* mutant pituitaries appear to have an increase in PH3 positive cells (Figure 4B). Ki67 (Figure 4F) and BrdU (Figure 4J) also appear to be increased in the *p21* mutant pituitary. Additionally, cell counts on midsagittal sections confirm that there are significantly more PH3 positive cells when compared to wildtype pituitaries (Figure 4M). Upon more stringent examination, where PH3 positive cell quantitation was conducted throughout the entire e16.5 pituitary and not only on midsagittal sections, there is no significant change compared to normalized wildtype pituitaries (Figure 4N). Further analysis of *Ki67* and *Myc* mRNA, as well as cell cycle regulators *Ccnd2* and *Ccne1*, demonstrates that in whole pituitaries at e16.5, there is no overall change in cycling cells (Figure 5). Taken together, this indicates that loss of *p21* may alter proliferation in a localized area but total pituitary proliferation remains unchanged compared to wildtype. When compared to wildtype pituitaries, *p27* mutant pituitaries, as expected, appear to have more proliferating cells in the anterior lobe as marked by PH3 (Figure 4C), Ki67 (Figure 4G) and BrdU labeling (Figure 4K). Midsagittal quantification of PH3 positive cells as well as quantification throughout the pituitary show significantly more positive cells in the *p27* mutant when compared to the normalized wildtype pituitaries (Figure 4M, N), indicating that there is a significant increase in proliferating cells when *p27* is lost. Like the *p21* and *p27* single mutant pituitaries, loss of both *p21* and *p27* results in an apparent increase in PH3 (Figure 4D), Ki67 (Figure 4H) and BrdU (Figure 4L) immunoreactive cells assessed by histological examination compared to wildtype pituitaries. Indeed, an increase in proliferating cells is further supported by quantification of PH3 in

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midsagittal sections as well as sections throughout the pituitary compared to normalized wildtype pituitaries (Figure 4M, N). However, there is not a significant difference between proliferation indices between *p27* mutants and *p21/p27* double mutants.

2.5 Morphology and proliferation does not appear altered in *p21* mutants, compared to *p27* and double mutants at postnatal day 21

Because expression analysis revealed there are cells expressing p21 during the period of postnatal proliferation, we examined the effect of loss of *p21* during this stage, as well as its interaction with *p27*. At postnatal day 21 (pd21) a wildtype mouse pituitary contains a posterior lobe, intermediate lobe, and anterior lobe and the histology of a *p21* mutant pituitary (Figure 6B, F) is indistinguishable from the wildtype pituitary (Figure 6A, E). The *p27* mutant pituitary also contains a posterior lobe, intermediate lobe, and anterior lobe (Figure 6C). However, we observe intermediate lobe hyperplasia with very densely packed cells and nodule-like formations already occurring at this early age (Figure 6G), a result similar to what is observed in *p21/p27* double mutants (Figure 6D, H). We then examined proliferation at pd21 using Ki67 as a marker. The wildtype pituitary contained scattered Ki67 positive cells throughout the intermediate lobe (Figure 6I) and anterior lobe (Figure 6M), as did the *p21* mutant pituitary (Figure 6J, N). Interestingly, *p27* mutant pituitaries revealed abnormal clustering of the Ki67 positive cells throughout the intermediate lobe (Figure 6K), with many positive cells in the anterior lobe (Figure 6O). Again, *p21/p27* mutant pituitaries appeared similar to p27 mutants in the intermediate lobe (Figure 6L) and the anterior lobe (Figure 6P). Taken together, these data show that *p27*, but not *p21*, is critical for proper intermediate lobe size, cell density, and proliferation during the postnatal period. Additionally, at this age, our data indicates that there appears to be no genetic interaction between *p21* and *p27*.

2.6 Alterations in cell cycle regulators occur postnatally with the loss of *p21*

To compare proliferation levels between the wildtype and *p21* mutants at pd21, we performed quantitative RT-PCR for cell cycle components using whole pituitaries. In parallel to the immunohistochemistry, *p21* mutant pituitaries had similar *Ki67* mRNA levels to wildtypes. Interestingly, when we measured the levels of *Myc*, we found that, compared to wildtype pituitaries, *p21* mutant pituitaries have significantly less *Myc* mRNA (Figure 7A).

Because we observed a decrease in *Myc* expression in *p21* mutants, we investigated other cell cycle regulators to determine if any component of the cell cycle is misregulated. Cyclin B, D, and E are all present in the pituitary and known to regulate the cell cycle either during development or tumor formation (Jordan, et al. 2000; Qian, et al. 1998; Roussel-Gervais, et al. 2010; Turner, et al. 2000). We found that compared to the wildtype pituitary, the *p21* mutants have significantly reduced mRNA levels of *Ccnb1*, *Ccnd1*, *Ccnd2* and *Ccne1* (Figure 7B).

To explore if there was a compensatory upregulation of CIP/KIP family members in the absence of *p21*, we compared the expression of *p21* (*Cdkn1a*), *p27* (*Cdkn1b*) and *p57* (*Cdkn1c*) between the wildtype and *p21* mutant. The mRNA expression of *p21* is, as expected, not detectable in the *p21* mutant pituitary. However, *p27* and *p57* are not significantly different from the wildtype in the *p21* mutant pituitary (Figure 7C). In addition, *p15*, *p18* and *p19* are not affected by loss of *p21* (data not shown). This indicates that the expression of the CDKIs examined do not rely on *p21* expression. Taken together, these data demonstrate that *p21* is required to achieve normal mRNA levels of several components of the cell cycle, however the overall number of cycling cells is likely unaffected by loss of *p21*.

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3. Discussion

Properly timed expression of cell cycle inhibitors is needed to orchestrate developmental events that lead to a pituitary containing the appropriate number of endocrine cells. Studies have shown that CDKI expression is needed during embryonic pituitary development in order to control progenitor proliferation. In fact, loss of the CDKIs *p27* and *p57* results in excess proliferation in pituitary progenitors, as well in cells differentiated to express hormones (Bilodeau, et al. 2009). The third CIP/KIP family member, p21, is also expressed in the developing pituitary. p21 has a diverse set of described functions, including regulation of cell cycle arrest, inhibition of the G1 and G2 cell cycle phases in response to differentiation signals or genotoxic stress, and modulation of cell death (Abbas and Dutta, 2009). Previous work by our group has shown that in the pituitary, Notch signaling represses p21. In the absence of the Notch target gene *Hes1*, p21 expression is induced in a broader subset of cells, correlating with decreased progenitor cell proliferation and increased cell death (Monahan, et al. 2009). We now show that p21 alone is not an essential mediator of proliferation or cell death at early stages of pituitary development. However, during the postnatal period of cell expansion, proper expression of p21 does appear to play a role in cell cycle regulation as demonstrated by the reduction in mRNA levels of M*yc* as well as *Ccnb1*, *Ccnd1*, *and Ccne1*.

p21 can be a potent inhibitor of cell cycle progression. p21 represses the activity of the CyclinD/Cdk4 and CyclinE/Cdk2 complexes, which are needed for progression into S phase (Alt, et al. 2002; Chen, et al. 2002). G1/S phase transition is a critical step in cycling progenitors, providing a checkpoint for cells to exit the cell cycle and begin differentiation. Additionally, the G1/S checkpoint serves as a mechanism for damaged cells to pause in the cell cycle, allowing for repair or apoptosis. *p21* deficient cells that have been exposed to radiation or chemical carcinogens aberrantly progress in the cell cycle (Brugarolas, et al. 2002), highlighting the importance of p21 as a tumor suppressor. In addition to its role in G1/S phase, p21 can modulate the later G2/M transition by binding to and repressing Cyclin B activity (Smits, et al. 2000). Despite the role of p21 in several phases of the cell cycle, in the developing embryonic pituitary, proliferation is not dramatically altered in the absence of *p21*. Furthermore, *p21* knockout mice have normal pituitary function, as indicated by their lack of endocrine related pathologies, which suggests that p21 is not required for controlling proliferation or differentiation in pituitary cells. A similar situation occurs in epithelial cells where loss of *p21* does not alter cell cycle parameters, however *p21* is essential for initiating the irreversible growth arrest known as senescence (Weinberg, et al. 1999; Paramio, et al. 2001). Our studies do not, however, rule out that p21 acts in specific cohorts of cells that might have been missed by examining the pituitary as a whole. For example, like the pituitary, p21 is only expressed in a small number of cells in the pancreas (Chesnokova, et al. 2009). However, a role for p21 in cell cycle inhibition is only observed when subsets of progenitors are separated and examined by flow cytometry (Miyatsuka, et al. 2011).

Proliferation of pituitary cells is not restricted to embryonic development. During the first weeks of postnatal life a second wave of proliferation expands the population of hormone cell types present in the pituitary (Taniguchi, et al. 2002, 2001b, 2001a, 2001c, 2000). Based on the fact that p21 is expressed in the pituitary at pd21, it is possible that p21 action may regulate this later stage of pituitary cell expansion. We find that although overall proliferation, as assessed by Ki67 immunohistochemistry and quantitative RTPCR, is unchanged, there is evidence to suggest cell cycle components are reduced in the absence of *p21*. These data are consistent with the reduction of Cyclin D and Cyclin D/cdk4 complexes in both osteosarcoma and vascular smooth muscle cells depleted of *p21* mRNA and in fibroblasts from *p21* knockout mice (Cheng, et al. 1999; LaBaer, et al. 1997; Weiss, et al.

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2000). These data suggest that in the right context, p21 may be necessary in the pituitary and other systems to support basal levels of the cell cycle machinery. In fact, in a model of a glial-derived brain tumor, p21 promoted tumor growth and loss of *p21* resulted in a decrease in proliferation as marked by Ki67 (Liu, et al. 2007). Another way p21 can promote the cell cycle is by preventing Cyclin D/cdk4 export from the nucleus, which allows it to remain active (Alt, et al. 2002). Taken together, although high levels of p21 inhibit the kinase activity of CDKs and prevent cell cycle progression, low levels of p21 are necessary to maintain Cyclin levels and to permit formation of Cyclin D/cdk4 complexes allowing for cell cycle progression. This phenomenon may not be unique to p21 because a similar reduction in Cyclin mRNA levels was observed in 30 week old *p27* mutant pituitaries, despite the increased organ size observed at this age (Oesterle, et al. 2011).

Although not usually detectable in adult pituitaries, p21 expression can be induced during tumor formation, where it plays an important function in restraining tumor growth. p21 expression is upregulated in *Pttg/Rb* mutant pituitaries (Chesnokova, et al. 2005). Loss of *p21* in these *Pttg/Rb* mutant pituitaries results in increased tumor penetrance (Chesnokova, et al. 2008). Additionally, in a *p18* mutant, additional loss of *p21* results in an increased incidence of pituitary tumor formation (Franklin, et al. 2000). These studies indicate that p21 can be used to restrain proliferation in a context dependent manner and, when lost in addition to other key cell cycle regulators, allows for unrestrained growth and tumor formation. This led us to examine if p21 and p27 acted synergistically to induce proliferation. We find that unlike the cases described above, there is no interaction of p21 and p27, at least until pd21. Although this does not preclude the fact that a synergistic interaction may be observed at a later time point, it strongly suggests that these two CDKIs control different events in the pituitary.

In addition to its role in cell cycle regulation, p21 can also influence cell death. Expression of p21 is necessary to protect against apoptosis during differentiation, in response to growth factor withdrawal or ionizing radiation induced cell death (Fotedar, et al. 1999; Jiang, et al. 2000; Ozaki and Hanazawa, 2001). Yet, there is also evidence that shows that p21 can act as a pro-apoptotic protein. *p21* overexpression has been shown to induce apoptosis in esophageal and cervical cell lines (Tsao, et al. 1999, Kadowaki, et al. 1999). Furthermore, in p53 dependent hepatoma cell lines, p21 induces expression of Bax, a known pro-apoptotic protein (Kang, et al. 1999). Highly regulated cell death occurs during development of many organs, including the brain and the pituitary. p21 expression is coincident with the location of cell death in the wildtype pituitary at e10.5 and is strikingly increased in the area of cell death observed in *Hes1* mutants (Monahan, et al. 2009). This led us to hypothesize that p21 may mediate cell death during separation of the proliferating pituitary progenitors to create a RP distinct from the oral ectoderm. 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. Wildtype pituitaries on the other hand have separated from the oral ectoderm completely by this stage. This observation implies that specifically timed p21 expression promotes pouch closure, although the mechanism has yet to be determined.

With these current studies we show that changes in p21 at early stages of embryonic development may not influence pituitary progenitor proliferation or 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 in the mutant, direct consequences may not be readily apparent. It is likely that many cell cycle inhibitors are involved in coordinating the proliferation and differentiation of pituitary cells.

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4. Materials and Methods

4.1 Mice

Cdkn1a (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 at the Massachusetts Institute of Technology (Brugarolas, et al. 1998). Heterozygous males and females for the *p21* mutant allele 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'. *Cdkn1b (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 (Fero, et al. 1996).

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 mutant; *p27* heterozygous mutant animals to generate litters of *p21* mutant and *p21/p27* 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. For genotyping *p21* mutant mice with p27 mutant alleles the following primers were used: *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 3'. For the *p27* genotyping the following primers were used to detect a wildtype band: *p27* wildtype forward primer, 5' GAT GGA CGC CAG ACA AGC 3'; *p27* wildtype reverse primer, 5' CTC CTG CCA TTC GTA TCT GC. To detect a *p27* mutant band the following primers were used: forward, 5' GGCCATTTTCCATCTCTGAA; reverse 5' CAA TAT CAC GGG TAG CCA ACG C. The University of Illinois IACUC approved all procedures involving mice.

For bromodeoxyuridine (BrdU) studies, mice were injected with 0.1 mg/g body weight BrdU intraperitoneally 30 minutes prior to sacrifice.

4.2 Sample collection

Embryos were collected and prepared for either immunohistochemistry or RNA extraction. For immunohistochemistry, the embryos were fixed in 3.8% formaldehyde solution (Fisher) in phosphate buffered saline (PBS) (Fisher), dehydrated through a graded series of ethanol and methyl salicylate, and embedded in paraffin. Sections measuring 6 microns thick were then affixed to positively charged slides. For RNA extraction, e16.5 pituitaries were removed and placed in a lysis solution. Samples were then homogenized and RNA was extracted following the protocol outlined in the Ambion RNAqueous® Micro Kit (#1931). cDNA was created utilizing the New England BioLabs ProtoScript M-MuLV First Strand cDNA Synthesis Kit (#E6300L).

Pituitaries were removed from postnatal day 21 (pd21) mice and were prepared for either immunohistochemistry or RNA extraction. For immunohistochemistry, the pituitaries were fixed in 3.8% formaldehyde on ice for one hour, rinsed with PBS, added to 30% sucrose at 4°C overnight, then snap frozen in OCT. The pituitaries were then sectioned with a cryostat (Leica), and the sections were adhered to charged slides. For RNA extraction, the pituitaries were snap frozen in microfuge tubes submerged in a dry ice/ethanol slurry. Prior to RNA extraction, cold RNAlater (Ambion, AM7021) was added to the tubes incubated overnight at

-20°C . The RNA extraction and cDNA synthesis were done in the same manner as for the embryo samples.

4.3 Immunohistochemistry

Embryo sections affixed to slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, and washed in PBS. Frozen pd21 pituitary slides were thawed at room temperature for 5 minutes, fixed in 3.8% formaldehyde for 10 minutes, and rinsed with PBS. Slides were subjected to antigen retrieval using 0.01M Citrate buffer, pH 6.0, for 10 minutes (paraffin) or 5 minutes of hot, non-boiling 0.01M Citrate buffer, pH 6.0 (frozen). All slides were blocked for 10-60 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) 1:500; 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-CyclinE (ab52189 AbCam) 1:500; rabbit anti-phospho-Rb (Ser807/811, Cell Signaling Technology, #9308) 1:500. Donkey-derived mouse and rabbit secondary antibodies conjugated to biotin (Jackson Immunoresearch) were diluted to 1:200 and incubated with sections for one hour followed by incubation with Streptavidin conjugated to cy3 fluorophore (Jackson Immunoresearch), for one hour. Ki67 was detected with a secondary rat antibody conjugated to the fluorophore FITC or cy3. p21 was detected in e16.5 and pd21 tissue with a Vectastain kit (Vector Laboratories) diluted in PBS followed by Sigma Fast 3,3- Diaminobenzidine tablets (Sigma) and counterstained with methyl green. Cell death was assessed by TUNEL as previously described (Raetzman, Cai, Camper. 2007). All sides immunofluorescent 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 or 400x.

4.4 Cell Count Analysis-Phosphohistone H3

4.4.1 Midsagittal Cell Counts—Slides containing midsagittal sections from e11.5 WT and *p21 mutant* and e16.5 WT, *p21* mutant, *p27* mutant, and *p21/p27* double 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. 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 using NIH Image J software, then statistically analyzed with a two tailed T-Test.

4.4.2 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 described above. 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 micron^2 area of pituitary per section was determined with total counts throughout all pituitary sections being added to provide a proliferation indices for each animal tested. To determine statistical significance, ANOVA

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was performed using Stat Plus. A two tailed T-Test was then used to determine statistical significance between groups.

4.5 Quantitative RT-PCR

The following primer sequences and annealing temperatures were used during quantitative real time PCR: *Gapdh* forward primer 5' GGTGAGGCCGGTGCTGAGTATG 3' and *Gapdh* reverse primer 5' GACCCGTTTGGCTCCACCCTTC 3'; *Ki67* (64°C) forward 5' CCAGGGATCTCAGCGCAATTACAG 3' and reverse 5' GGATAGGACAGAGGGCCACATTTC 3'; *Ccnb1* (64°C) forward 5' TTGAATTCTGACAGCCAGATGGG and reverse 5' TCCAGGTGGCATTACAAGACAGG; *Ccnb2* (64°C) forward 5' CTGGAAGTCATGCAGCACATGG and reverse 5' TGACAGCGATGAACTTGGTACGG; *Ccnd1* (64°C) forward 5' CGCGCGTACCCTGACACCAAT and reverse 5' CTCCTCACAGACCTCCAGCATCCA; *Ccnd2* (64°C) forward 5' ACACCGCACACATAGGCTTCTC and reverse 5' TAAGCATGCCGCAGCTGTTGAC; *Ccne1* (64°C) forward 5' GGCTCCGACCTTTCAGTCCGC and reverse 5' TGGGTCTTGCAAAAACACGGCC; *Myc* (64°C) forward 5' TGACCTAACTCGAGGAGGAGCTGGAATC and reverse 5' AAGTTTGAGGCAGTTAAAATTATGGCTGAAGC; *Cdkn1a* (64°C) forward 5' TTGGAGTCAGGCGCAGATCCACA and reverse 5' CGCCATGAGCGCATCGCAATC; *Cdkn1b* (64°C) forward 5' TTCGGCCCGGTCAATCATGAAG and reverse 5' GCGCTGACTCGCTTCTTCCATATC; *Cdkn1c* (60°C) forward 5' TCCATCACCAATCAGCCAGCAGAA and reverse 5' ATCGCTGGAGGCCAAGCGTTC; 5-8 separate pituitaries were tested for each genotype and run on the BioRad iQ5 Multicolor Real-Time PCR Detection System Machine. Data was collected utilizing the BioRad iQ5 Optical System Software Version 2.0 and analyzed in Microsoft Excel. The analysis was carried out using the change in cycle threshold value method as described by (Goldberg, et al. 2011). A 2 tailed T-Test, conducted in Microsoft Excel, was utilized to determine statistical significance.

4.6 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 (Karl and Capel, 1998). Statistical signficance was querried with a 2 tailed Fisher's exact test conducted in Graphpad Prism. All somite stages, and ss18 and 19 grouped, were compared between wildtype and p21 mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Timely separation of the developing pituitary from the oral ectoderm relies on p21 P27 and P21 do not act synergistically to restrain pituitary proliferation P21 is necessary to maintain basal levels of cell cycle machinery components

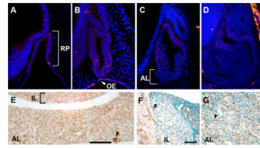

Figure 1.
p21 is expressed in the developing pituitary. Embryos and pituitaries were sectioned sagittally (e10.5, e11.5, e12.5, and e14.5), coronally (e16.5), or transversely (pd21) and immunohistochemically stained for p21 (red [e10.5-e14.5] or brown [e16.5, pd21]) and counterstained with DAPI (blue, e10.5-e14.5) or methyl green (e16.5, pd21). Cells positive for p21 are present within Rathke's pouch (RP) in the embryonic pituitary at e10.5 (A), e11.5 (B), and e12.5 (C). Cells positive for p21 are sparsely scattered at e14.5 (D) and e16.5 (E). At postnatal day 21 in the intermediate lobe (F) and anterior lobe (G) of the pituitary, p21 positive cells are present. N≥3. Magnification: 200x (A-D, F-G), 100x (E). Scale bars = 50 microns.

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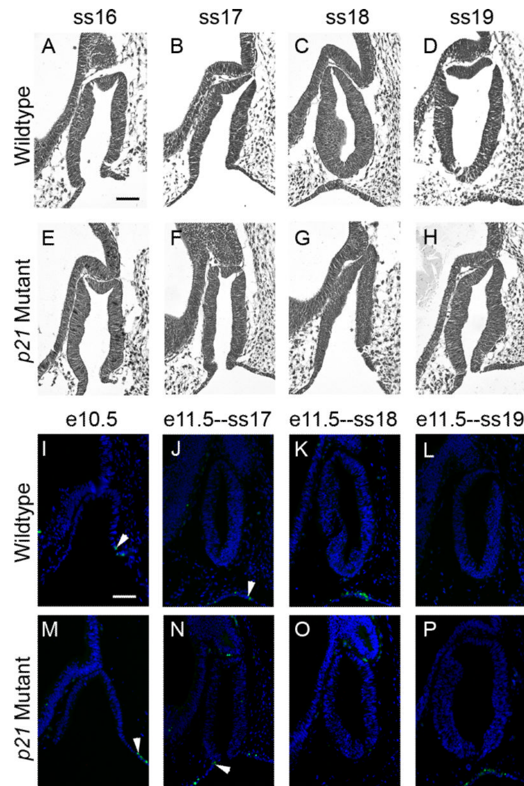

Figure 2.

Rathke's pouch closure occurs in the absence of *p21*. Embryos were somite staged, sectioned sagittally, and stained either for morphology with hematoxylin and eosin or for cell death with TUNEL (green) and counterstained with DAPI (blue). Somite stage 18 (ss18) is considered equivalent to e11.5. In the wildtype, the pouch typically remains open at ss16 (A) and ss17 (B) but is usually closed by ss18 (C) and ss19 (D). The *p21* mutant also has an open pouch at ss16 (E) and ss17 (F) but unlike the wildtype continues to be open at ss18 (G) and ss19 (H). Cell death analysis reveals TUNEL positive cells in Rathke's pouch near oral ectoderm at e10.5 in the wildtype (I, arrowhead) and *p21* mutant pituitary (M, arrowhead). By e11.5 (ss17-19) TUNEL positive cells are virtually absent from the pituitary but remain present in the underlying oral ectoderm (J-L, N-P, arrowheads). N=≥3. Magnification=200x. Scale bars = 50 microns. p= 0.119 for ss18 and 19.

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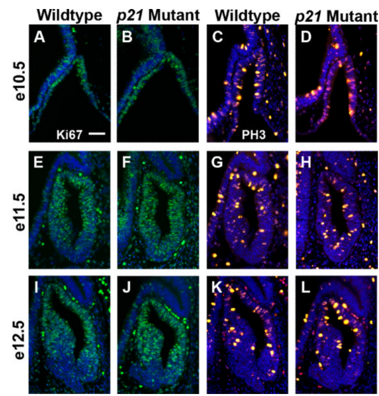

Figure 3.

Loss of *p21* does not affect proliferation in the developing pituitary. Embryonic pituitaries in the sagittal plane were stained with Ki67 in wildtype and *p21* mutant mice at e10.5 (A,B), e11.5 (E,F), and e12.5 (I,J). PH3, a marker of mitosis, was also examined in the wildtype and *p21* mutant pituitaries at e10.5 (C,D), e11.5 (G, H), and e12.5 (K,L). N≥3. Magnification=200X. Scale bar = 50 microns.

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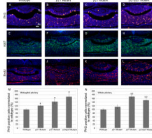

Figure 4.

Proliferation is increased at e16.5 in *p21*, *p27*, and *p21/p27* mutants. Coronal sections of e16.5 pituitaries were immunohistochemically stained for three different markers of proliferation: PH3, Ki67, and BrdU. In the wildtype (A), *p21* mutant (B), *p27* mutant (C), and *p21/p27* mutant (D), PH3 positive cells indicating M phase are seen scattered throughout the intermediate and anterior lobes of the pituitary. Similarly, the general marker Ki67 is present in wildtype (E), *p21* mutant (F), *p27* mutant (G), and *p21/p27* mutant (H) pituitaries. Additionally, BrdU marking S phase is present in wildtype (I), *p21* mutant (J), *p27* mutant (K), and *p21/p27* mutant (L) pituitaries. PH3 positive cells were counted on midsagittal sections as well as sections throughout the whole pituitary for each of the genotypes. *p21* (p=0.05), *p27* (p=0.05), and *p21/p27* (p=0.02) mutant pituitaries all had significantly more PH3 positive cells in midsagittal sections when compared to wildtype pituitaries (M). However, when sections throughout the entire pituitary were counted, *p27* (p=0.0001) and *p21/27* (p=0.004) mutant pituitaries contained significantly more PH3 positive cells than wildtype pituitaries, but *p21* (p=0.25) mutants did not (N). N≥3 Magnification=200x. Scale bar = 50 microns. a = p≤0.05 compared to wildtype, b = p≤0.05 compared to *p21* mutant.

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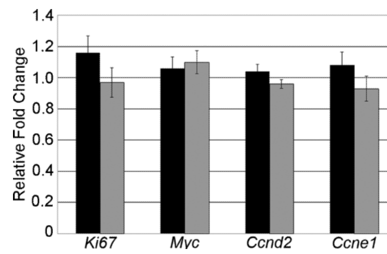

Figure 5.

Cell cycle components are unaltered in *p21* mutants at e16.5. Quantitative Real-Time PCR was performed on mRNA from e16.5 pituitaries. Data are calculated using the ΔΔCT method to compare the fold change in *p21* mutants (gray bars) to wildtype (black bars), with *Gapdh* measured as the reference control. Loss of *p21* did not change *Ki67* (p=0.20), *Myc* (p=0.73), *Ccnd2*(p=0.16) *or Ccne1* (p=0.21) levels compared to wildtype. N=9-10.

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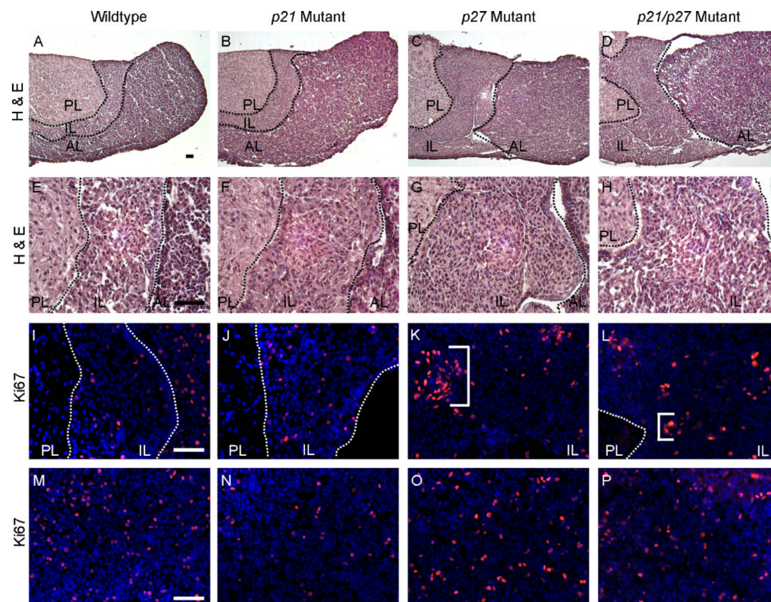

Figure 6.

Cell density and proliferation are altered in the *p27* and *p21/p27* mutants at postnatal day 21. Transverse sections through pituitaries were stained either for morphology with hematoxylin and eosin or for proliferation with Ki67 (red) and counterstained with DAPI (blue). Compared to wildtype (A, E) and *p21* mutant (B, F) intermediate lobes, the *p27* (C, G) and *p21/p27* mutant (D, H) intermediate lobes are larger and very densely packed with cells. Ki67 expression in the intermediate lobe (I-L) and anterior lobe (M-P) reveals positive cells scattered throughout lobes of the wildtype (I, M) and *p21* mutant (J, N) pituitaries. The intermediate lobes of the *p27* (K) and *p21/p27* mutants (L) contain large clusters of Ki67 positive cells, denoted with brackets, unlike the wildtype and *p21* mutants. The anterior lobe also contains positive cells in the *p27* (O) and *p21/p27* mutants (P). N≥3. Magnification=100X (A-D). Magnification=400x (E-P). Scale bar = 50 microns.

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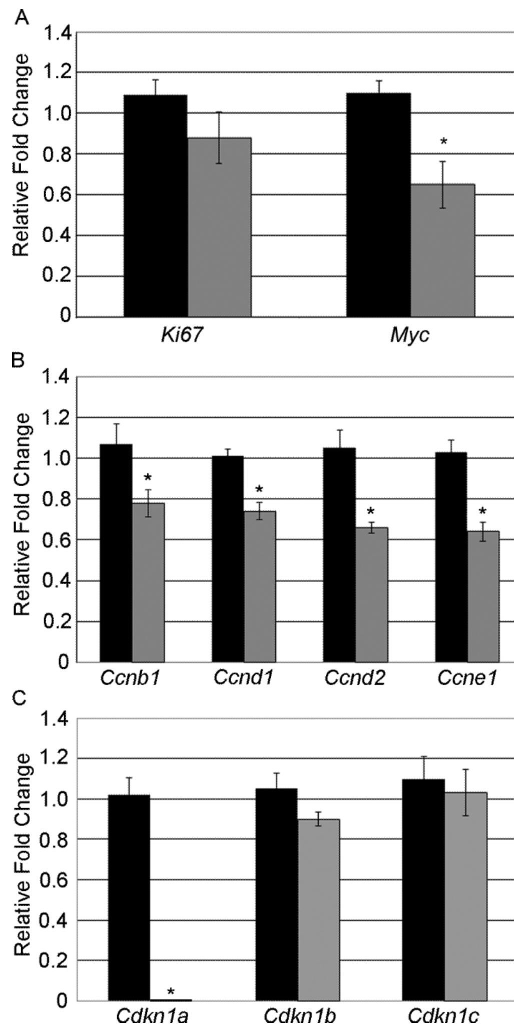

Figure 7.

Quantitative PCR reveals that loss of *p21* results in significant changes in mRNA of cell cycle regulators and proliferation markers at postnatal day 21. Data are calculated using the ΔΔCT method to compare the fold change in *p21* mutants (gray bars) to wildtype (black bars), with *Gapdh* measured as the control. Loss of *p21* resulted in a significant decrease of *Myc* (p=0.005) but no change in *Ki67* (p=0.19) compared to the wildtype (A). *Ccnb1* (p=0.04), *Ccnd1* (p=0.0003), *Ccnd2* (p=0.002), and *Ccne1* (p=0.0002) were all significantly decreased in the *p21* mutant pituitaries compared to wildtype (B). In the *p21* mutant *p21* mRNA was absent and there was no difference in *p27* (p=0.12) or *p57* (p=0.69) mRNA levels compared to wildtype (C). N=7-8. * = p≤0.05.