Expression of CDK4 or CDK2 in Mouse Oral Cavity Is Retained in Adult Pituitary with Distinct Effects on Tumorigenesis

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Abstract

The keratin 5 (K5) promoter drives transgenic expression to the basal cell layer of stratified epithelia. Surprisingly, analysis of K5CDK4 and K5CDK2 transgenic mouse embryos showed CDK4 and CDK2 expression not only in the expected tissues, but also in the adenohypophysis. This organ is derived from an upwards growth of the primitive oropharynx, a K5-expressing tissue. We show that transgenic expression of CDKs in the embryonic oral ectoderm is specifically retained in undifferentiated cells from the pars intermedia of the adenohypophysis. Interestingly, we found that K5CDK4 mice show a decreased number of pituitary stem cells, even though CDK4 is not expressed in the stem cells but in transit-amplifying (TA)–like cells. Interestingly, CDK4-expressing cells, but not CDK2-expressing cells, strongly synergize with lack of p27Kip1 to generate pituitary carcinomas that appear with shortened latency and are drastically more aggressive than those arising in p27/−/− mice. Thus, we show that deregulation of CDK expression in the primitive oral epithelium plays a unique function, providing a selective advantage that gives rise to transgene-positive TA-like pituitary cells. Furthermore, retention of CDK4 in these TA-like pituitary cells synergizes with loss of p27Kip1 to induce pituitary adenocarcinomas. This model suggests that forced expression of CDK4 sensitizes cells and synergizes with a second change resulting in tumor development. [Cancer Res 2008;68(1):162–71]

Introduction

Progress through the G1 phase of the mammalian cell cycle is mediated by the D-type cyclins (D1, D2, and D3), which associate and activate the closely related CDK4 and CDK6 kinases, resulting in their catalytic activation and substrate recognition (1–3). A key substrate for cyclin/CDK complexes is the Rb family of proteins, pRb, p107, and p130, which negatively regulate the passage from G1 to S phase (3). The activities of CDKs are subject to additional levels of regulation, which include their association with inhibitory molecules such as the INK4 and CIP/KIP family of proteins (4, 5). Disruption of the pRb pathway in genetically engineered mouse models leads to deregulated growth in the pituitary gland. Mice heterozygous for pRb and nullizygous for p18Kip1 or p27Kip1 develop spontaneous tumors in the pars intermedia (6–9), whereas knock-in mice for CDK4R24C, a mutant insensitive to p16Ink4a inhibition, develop pituitary adenomas of the anterior lobe (10, 11).

The pituitary gland is divided into two morphologically distinct compartments, the adenohypophysis and the neurohypophysis. The adenohypophysis is composed of the anterior and intermediate lobes (pars intermedia), and is derived from an upwards growth of the primitive oropharynx, forming Rathke’s pouch. Pituitary murine ontogenesis is carried out by three successive steps: (a) the formation of a rudimentary pouch near E8.5 from an upward involution of the presumptive oral cavity, (b) the formation of a definitive pouch, and (c) complete detachment of the pouch from the oral cavity at E12.5 followed by the sequential emergence of hormone-secreting cells (12).

Previously, we reported the effects of transgenic expression of CDK4 and D-type cyclins in epithelial tissues driven by the keratin 5 (K5) promoter (13–15). Expression of reporter genes under the K5 promoter readily show expression in the basal cell layer of the epidermis, thymic reticulum, digestive tract, and the oral mucosa (16). Although the pituitary gland is derived from the oral ectoderm, it does not express K5; thus, the transgenes under the K5 promoter are not detected in this organ (16). In this article, we describe the retention of human-CDK4 (hCDK4) and human-CDK2 (hCDK2) transgene expression under the K5 promoter in adult mice pituitary glands. We determined that the sustained expression of hCDKs is transgene-specific, as no other K5 transgenic mice analyzed show pituitary gland transgene expression. K5D-type cyclin transgenic mice do not show expression of the respective D-type cyclin in the pituitary. Expression of hCDKs in pituitary cells is maintained in an undifferentiated epithelial-like cell population with characteristics of progenitor transit-amplifying (TA) cells. We observed strong synergistic effects between the expression of hCDK4 and lack of p27Kip1 resulting in aggressive pituitary carcinomas. On the other hand, expression of hCDK2 does not synergize with p27Kip1 loss. We conclude that expression of hCDK4 and hCDK2 are specifically retained in the pituitary pars intermedia, likely through a mechanism that favors the selection of cells with elevated CDK activity. The expression of hCDK4 in a TA-like cell population affects the total number of stem cells and remain in the adult organ as nontumorigenic cells, but collaborate with loss of p27Kip1 in tumor development. These tumors are initiated by the inactivation of the Kip/Cip inhibitory pathways (6–8), suggesting that CDK4 expression synergizes with p27Kip1 loss by accelerating tumor growth and malignant tumor progression.

Materials and Methods

Mouse experiments. The generation of K5CDK4, K5D3, K5D2, and K5-flxGFP-M2SMO (K5-EGFP) transgenic mice was previously reported (13, 14). CdK4-null mice were developed by Tsutsui et al. (17) using gene-targeting.
disruption. Mice nullizygous for p27Kip1 are commercially available (strain B6.129S4-\text{Cdkn1btm1Mlf}; The Jackson Laboratory). K5-CDK4D158N and K5-CDK2 mice were developed by cloning the corresponding cDNAs into the vector pBK5 (14). The transgene was excised from the plasmid vector with BssHII and microinjected into the FVB strain at Science Park Transgenic Mouse Facility, M.D. Anderson Cancer Center, Houston, TX.

Pathologic analysis and immunostaining. Formalin-fixed paraffin-embedded pituitary samples were stained with H&E. Immunohistochemistry was performed with antibodies against adrenocorticotropic hormone (ACTH), α-MSH (Dako North America, Inc.), prolactin and growth hormone (National Hormone and Peptide Program, University of California at Los Angeles).

E13.5 and E17.5 tissue samples were fixed in 4% paraformaldehyde and OCT frozen with liquid nitrogen or embedded in paraffin wax. Tissue cross-sections of developing and adult pituitary were immunostained with antibodies for CDK4 (H-22), CDK1 (C-22), CDK2 (M-20), cyclin D3 (C-16), cyclin D2 (M-20), and proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnologies). For 3,3′-diaminobenzidine chromogen visualization, samples were incubated with biotin-conjugated secondary antibodies (Vector Laboratories) or Alexa Fluor secondary antibodies for immunofluorescence (Molecular Probes). Frozen cross-sections of K5-EGFP transgenic embryos were counterstained with 4′,6-diamidino-2-phenylindole and visualized under fluorescence microscope using a 465 to 495 nm filter.

Western blots, immunoprecipitation, and kinase assays. Pituitary glands and epidermal lysates were homogenized in lysis buffer as we previously described (18). Fresh protein preparations were immunoprecipitated for 2 h at 4° C with protein A–agarose beads and polyclonal antibodies. Immunoprecipitates were electrophoresed and transferred onto nitrocellulose membranes for Western blot analysis with peroxidase-conjugated antibody (Amersham Corp.), followed by chemiluminescence (ECL kit; Amersham Corp.). The antibodies used were anti-mouse CDK4 (C22), anti-human CDK4 (H22), cyclin D2 (M20), cyclin D3 (C16), and CDK2 (M-20; Santa Cruz Biotechnology Inc.).

Figure 1. hCDK4 expression in embryonic and adult mouse pituitary glands. A, immunofluorescence analysis was carried out on pituitary primordia from OCT-embedded samples from 13.5 p.c. K5CDK4 embryos (a and b) using an antibody against human CDK4. Frozen cross-sections from OCT-embedded samples from 13.5 p.c. K5-EGFP embryos (d and e) were mounted and visualized directly using a 465 to 495 nm filter (green). b and e, ×20 magnifications of marked areas on a and d, respectively. B, immunofluorescence analysis for human-CDK4 (a and c) and human-CDK2 (b) expression (green) in pituitary glands from K5CDK4 (a), K5CDK2 (b), and wild-type (c) littermates. Primary antibodies omitted in blank control (d). Original magnifications, ×20. DAPI was used as nuclear counterstain (A, c and f; B, e–h). AL, anterior lobe; IL, pars intermedia or intermediate lobe; N, neurohypophysis; OC, oral cavity; RP, Rathke’s pouch.
For kinase assays, 250 μg of protein lysates were immunoprecipitated with 2.5 μg of antibody against CDK2 or CDK4. Precoated antibody beads (30 μL) were incubated with the lysates for 1 h at 4°C. The beads were washed twice with IGEPAL and kinase buffer as previously described (14). Kinase buffer (30 μL), 0.5 μg of pRb substrate (Santa Cruz Biotechnology, Inc.), or H1 (Upstate Biotechnology), 5 μCi γ-32P-ATP (6,000 Ci/mmol), and 5 μmol/L of ATP was added and incubated for 30 min at 30°C. SDS sample buffer was added, and electrophoresed through acrylamide gel.

**Identification of side population cells in mouse pituitary.** We used a method developed by Goodell et al. for the identification of a side population (SP; cell population enriched in stem cells) cells of mouse pituitary glands (19). Mice were euthanized using carbon dioxide asphyxiation and perfused with PBS to rid the pituitary of any blood. Pituitary glands were washed twice with IGEPAL and kinase buffer as previously described (14). Kinase buffer (30 μL), 0.5 μg of pRb substrate (Santa Cruz Biotechnology, Inc.), or H1 (Upstate Biotechnology), 5 μCi γ-32P-ATP (6,000 Ci/mmol), and 5 μmol/L of ATP was added and incubated for 30 min at 30°C. SDS sample buffer was added, and electrophoresed through acrylamide gel.

**Reverse transcription-PCR of Hoechst dye–sorted SP and main population pituitary cells.** SP and main population (MP) cells were sorted into single cells by incubating in 0.25% trypsin for 15 to 30 min at 37°C. Cells were resuspended in DMEM/Ham’s F12 1/1 serum-free media as previously described (20). Cells were incubated with Hoechst 33342 at 2.5 μg/mL with a 100 μmol/L concentration of verapamil in control samples for 90 min at 37°C. Cells were centrifuged, washed, and resuspended in PBS/2% fetal bovine serum and 2 μg/mL of propidium iodide. Flow cytometric analysis was conducted using a DAKO Cytomation MoFlo. Hoechst dye was excited with a UV laser set at 350 nm and its fluorescence measured using a 450/20 BP filter and a 670 filter. Cells were analyzed and sorted within a living (propidium iodide–negative) population.

**Results**

**K5 promoter drives the expression of CDK4 and CDK2 in mouse pituitary gland.** The K5 promoter has been widely used to study keratinocyte proliferation, differentiation, and skin carcinogenesis (13–15, 21–23). We have previously described the phenotypical consequences of forced expression of hCDK4 under the K5 promoter (K5CDK4 mice; ref. 13). Similarly, overexpression of hCDK2 under the K5 promoter also drives the expression to stratified epithelium (unpublished results). Unexpectedly, a detailed histochemical analysis of K5CDK4 and K5CDK2 mice revealed the expression of the transgene in the developing and adult pituitary gland. The pituitary gland develops from the ectodermal ventriculum that grows upwards from the roof of the primitive oral cavity, known as Rathke’s pouch. Close analysis of K5CDK4 embryos at E13.5 shows the expression of transgenic hCDK4 in the primitive oral epithelium, additionally, expression in the Rathke’s pouch was also observed in a dorsal-ventral gradient (Fig. 1A). The primitive oral epithelium expresses K5, suggesting that the observed expression of hCDK4 in the embryonic pituitary is retained in the developing pituitary (Fig. 1A). Further examination of adult K5CDK4 and K5CDK2 pituitary glands revealed the expression of the transgene in the developing and adult pituitary gland. The pituitary gland develops from the ectodermal ventriculum that grows upwards from the roof of the primitive oral cavity, known as Rathke’s pouch. Close analysis of K5CDK4 embryos at E13.5 shows the expression of transgenic hCDK4 in the primitive oral epithelium, additionally, expression in the Rathke’s pouch was also observed in a dorsal-ventral gradient (Fig. 1A). The primitive oral epithelium expresses K5, suggesting that the observed expression of hCDK4 in the embryonic pituitary is retained in the developing pituitary (Fig. 1A). Further examination of adult K5CDK4 and K5CDK2 pituitary glands.
confirmed positive staining for hCDKs in the pars intermedia (Fig. 1B). Analysis of two independent transgenic lines, K5CDK4 and K5CDK2, showed similar results ruling out the possibility that site insertions are responsible for sustained CDK expression in the pituitary gland. Western blot analysis of adult pituitary glands showed 7- and 12-fold increased expression of CDK4 and CDK2 in K5CDK4 and K5CDK2 mice, respectively, when compared with wild-type siblings (Fig. 2A and B). In order to confirm that the transgenic hCDK4 is expressed in the pituitary gland, we immunoprecipitated pituitary lysates from adult K5CDK4 mice using a polyclonal antibody specific for human CDK4. Figure 2B shows that hCDK4 is expressed in both skin and pituitary glands of K5CDK4 transgenic mice. To corroborate that the pattern of CDK4 expression is only due to overexpression of the transgene, we developed K5CDK4/CDK4−/− compound mice. Western blot and immunofluorescence analysis of pituitary glands from these compound mice also show expression of hCDK4 (Fig. 2C; Supplemental Fig. S1).

Two possible explanations for the continued expression of K5 transgenes in the pituitary gland are that (a) there is a sustained ectopic expression by the endogenous and/or transgenic K5 promoter (leaky promoter) or (b) there is an inherent function of the transgene being expressed which allows this cell population to survive during pituitary organogenesis.

To formally test the first hypothesis, we evaluated the transgenic expression of EGFP under the K5 promoter (K5-EGFP transgenic mice) and the expression of endogenous K5 in embryonic and adult pituitary gland. Analysis of K5-EGFP embryos at E13.5 showed no expression of EGFP in the developing pituitary, although positive staining for the oral cavity was clearly observed (Fig. 1A). Likewise, no positive staining was found in adult pituitary glands from K5-EGFP using immunofluorescence, Western blot,
or fluorescence-activated cell sorting (FACS) analysis (data not shown). We did not detect positive signals from the endogenous K5 in the pituitary glands (data not shown). We also analyzed transgenic expression of CDK4-binding partners, cyclin D2 and cyclin D3, in K5-cyclin D2 and K5-cyclin D3 transgenic mice (14). In this case, increased expression of cyclin D2 and D3 was observed in the skin, but not in the pituitary gland, in which endogenous cyclin D2 and D3 are highly expressed (Fig. 2A).

These results indicate that sustained expression of CDK4 and CDK2 in the pituitary gland is not a result of ectopic K5 promoter activity. Thus, expression of CDK4 and CDK2 seems to provide a selective advantage to pituitary progenitor cells.

We have previously determined that overexpression of hCDK4 or the kinase-dead mutant (CDK4D158N) in mouse epidermis leads to indirect activation of CDK2 through a mechanism that involved the sequestering of p27Kip1 and p21Cip1 (refs. 13, 24; unpublished results). To determine if CDK kinase activity is directly implicated in pituitary transgene expression, we analyzed the pituitary glands of K5CDK4 mice. Analysis of pituitary gland lysates from K5CDK4 mice showed no expression of the transgene, indicating that CDK4 kinase activity is required to maintain its expression in the pituitary pars intermedia (Fig. 2B).

Next, we asked whether the cell population expressing transgenic CDK4 differentiated into pars intermedia hormone-expressing cells (corticotropes) or remained as undifferentiated cells. The pars intermedia of the anterior pituitary gland contains cell lineages expressing α-MSH, β-endorphin, and ACTH products derived from processing of the same precursor, pro-opiomelanocortin (POMC). Thus, we analyzed CDK4 and ACTH expression in the pituitary of K5CDK4 E13.5 embryos. Double immunofluorescence studies revealed that cells expressing hCDK4 do not express ACTH, although both cell populations seem to be adjacent to each other (Fig. 3A). Similarly, hCDK4-expressing cells do not coexpress α-MSH (data not shown).

Collectively, these data indicate that sustained expression of hCDK4 in the pituitary glands of K5CDK4 mice is due to an inherent function of this kinase. The expression of CDK4 could provide a selective advantage to the progenitor cells of the oral cavity during pituitary organogenesis which is retained in the adult pituitary gland.

**hCDK4 overexpression reduces the number of pituitary stem cells.** The fact that hCDK4-retaining cells are not differentiated cells (hormone-expressing cells) suggests that primitive oral epithelium cells are retained in the pituitary of K5CDK4 mice as stem or TA cells which ensure the continuous cell self-renewal required in different organs. Thus, we investigated whether transgenic expression of CDK4 affects the pituitary stem cell population. Two methodologies have proved valuable for the identification of adult stem cells; one is based on dye efflux capacity mediated by the G2 subtype member of the ATP-binding cassette transporter. The exclusion of a vital dye is a characteristic of the SP displayed by multiple adult stem cell types (25). Cells that

![Figure 4](image-url)
efficiently exclude the vital dye Hoechst 33342 have been identified in the bone marrow and are visualized as SP cells enriched in hematopoietic stem cells (19). The mouse adult pituitary also contains a SP displaying verapamil-sensitive Hoechst dye efflux capacity, a stem cell–like characteristic (20).

FACS analysis of pituicytes incubated with Hoechst dye showed that K5CDK4 mice pituitary glands contain a 25% reduction in SP cells in comparison to age-matched wild-type littermates (Fig. 4). Four independent replications of this experiment confirm the reduction of the SP population in K5CDK4 mice (P < 0.01). In order to determine whether hCDK4 is expressed in the SP cells or in other pituitary cell populations (MP), we performed RT-PCR analysis on the total RNA isolated from SP and MP cells. Figure 4D shows that hCDK4 is expressed in the MP, but not in the SP or stem cells. Also, prolactin, a marker for differentiated cells, is only expressed in the MP (Fig. 4D). Considering the latter in conjunction with the fact that hCDK4-expressing cells do not express POMC-derived hormones, we hypothesized that expression of hCDK4 induces the proliferation of pituitary progenitor/stem cells causing an imbalance towards TA-like cells. Thus, disruption of the balance between self-renewal and proliferation causes a decrease in the number of stem cells in favor of TA cells. In contrast to stem cells, which are defined as a slow-proliferating cell population, TA cells are rapid-proliferating cells that give rise to differentiated cell lineages. Double immunofluorescence analysis shows hCDK4 expression in bromodeoxyuridine (BrdUrd)-positive cells at E17.5 in developing pituitary glands, on the other hand, no ACTH-expressing cells were detected in the BrdUrd-positive cell population (Fig. 3B). Thus, the hCDK4-retaining cells exhibit the main characteristic of TA or undifferentiated cells, that is, a high proliferative capacity and do not express POMC-derived hormones such as ACTH (Fig. 3B).

**CDK4 accelerates pituitary tumor formation in p27kip1 nullizygous mice.** Deregression of the pRb pathway results in hyperplasia and pituitary tumor development that arise in the pars intermedia, i.e., p27-/- and p18-/- mice develop pars intermedia tumors with similar origin to those in pRb-/- mice (8, 26, 27).

We have shown that overexpression of hCDK4 in the pars intermedia did not result in hyperplasia or pituitary tumor development, but the fact that hCDK4 is specifically retained in a TA-like cell population led us to hypothesize that CDK4 could contribute to pituitary tumor development synergizing with the loss of p27kip1. In order to rigorously test this hypothesis, we generated K5CDK4/p27-/- compound mice. The morbidity of
these mice was noticeably increased in K5CDK4/p27<sup>−/−</sup> mice as they develop very aggressive pituitary tumors with decreased latency compared with p27<sup>−/−</sup> mice. p27<sup>−/−</sup> single mutants have a decreased survival due to the presence of pituitary tumors and 40% died by 30 weeks (Fig. 5C). In contrast, the survival of K5CDK4/p27<sup>−/−</sup> mice drops dramatically at 13 weeks of age (50% survival), falling to 0% at 28 weeks (Fig. 5C). Changes in the shape of the heads of K5CDK4/p27<sup>−/−</sup> mice, due to intracranial compression, were clearly notable at 12 weeks of age (Fig. 5A). Necropsy of these mice showed the presence of large tumors which expanded dorsally and impinged on the overlying brain (Fig. 5B). These tumors appear hemorrhagic, poorly differentiated (Fig. 5B), and were classified as pars intermedia chromophobe carcinomas, which is relatively rare in mice.

Immunohistochemical staining of K5CDK4/p27<sup>−/−</sup> pituitary tumors showed that the majority of the tumor mass stained positive for pars intermedia hormones ACTH and α-MSH (Supplemental Fig. S2). In comparison, positive staining for prolactin and growth hormone was observed only in the remnants of the anterior lobe (Supplemental Fig. S2). These data corroborate that K5CDK4/p27<sup>−/−</sup> tumors such as previously described p27<sup>−/−</sup> tumors originate in the pars intermedia (7, 8), supporting the hypothesis that CDK4 expression synergizes with the lack of p27<sup>Kip1</sup>.

To determine if elevated levels of CDK2 could also synergize with the lack of p27<sup>Kip1</sup>, we developed K5CDK2/p27<sup>−/−</sup> compound mice. As opposed to K5CDK4/p27<sup>−/−</sup> mice, transgene expression of CDK2 in K5CDK2/p27<sup>−/−</sup> compound mice did not accelerate the development of pituitary tumors (Fig. 5B).

We have established that pituitary cells retaining hCDK4 expression are in the proliferative compartment and do not express ACTH as do corticothroph-differentiated cells (Fig. 3). Therefore, we hypothesize that hCDK4-expressing cells are in a primed proliferative state which can synergize with an additional hit, such as p27<sup>Kip1</sup> loss, and are primary contributors to the phenotype observed in our compound mice. To determine the

Figure 6. Immunohistochemical and biochemical analysis of pituitary tumors. A, PCNA (c and d, green) was used as a marker of proliferation in pituitary tumors from K5CDK4/p27<sup>−/−</sup> compound mice and costained with an antibody for human-CDK4 (e, red) and α-MSH (f, red). g and h, merge images from c and e and d and f, respectively; double-labeled areas (white-yellow). Note that most of the PCNA-positive cells expressed hCDK4 (g), but did not express α-MSH (h). DAPI was used as a nuclear counterstain. B, CDK4 and pRb immunoblots of protein lysates from pituitary glands of K5CDK4/p27<sup>−/−</sup> mice and control littermates. C, CDK4 in vitro kinase assay was conducted with antibodies against endogenous CDK4 (C-22) and human CDK4 (H-22) using a pRb peptide as a substrate. D, kinase activity for CDK2 was done using histone H1 as a substrate.
contribution of hCDK4-expressing cells to accelerated pituitary development in K5CDK4/p27−/− pituitary tumors, we conducted double immunofluorescence experiments. We determined that hCDK4 is coexpressed with the proliferation marker PCNA (Fig. 6A). On the other hand, in most cases, PCNA and intermediate lobe hormone (α-MSH) expression are mutually exclusive (Fig. 6A). We conclude that hCDK4-expressing cells are the primary contributors to pituitary tumorigenesis and progression in K5CDK4/p27−/− compound mice.

Immunoblot analysis corroborates that hCDK4 is expressed in pituitary tumors and lack of p27Kip1 expression seems to cause an expansion of the hCDK4-expressing cell population (Fig. 6B). We further studied the phosphorylation status of pRb in K5CDK4/p27Kip1 mice. In vivo analysis shows increased levels of hyperphosphorylated pRb in K5CDK4/p27−/− pituitary tumors compared with p27−/−, K5CDK4 and wild-type pituitary glands (Fig. 6B).

We also determined CDK4 and CDK2 kinase activity in pituitary and tumor samples from wild-type, p27−/−, K5CDK4, and K5CDK4/p27−/− pituitary lysates. These assays were performed with antibodies that specifically recognized human or mouse CDK4. As expected, an augmentation in the CDK4-kinase activity was observed in K5CDK4/p27−/− compound mice, the more significant increase being in hCDK4 kinase activity (Fig. 6C). We also observed that lack of p27Kip1 increased endogenous CDK4 activity, which synergizes with hCDK4 kinase activity as observed in K5CDK4/p27−/− mice (Fig. 6C, lane 6). We previously established that increased expression of CDK4 results in the activation of CDK2 through a mechanism involving sequestration of p21Cip1 and p27Kip1 in epithelial tissues (13, 24), thus, we also assayed CDK2 kinase activity in pituitary lysates. Pituitary lysates from p27−/− and K5CDK4/p27−/− mice show strong CDK2 kinase activity compared with wild-type and K5CDK4 mice (Fig. 6C). In fact, no quantitative difference was observed in the elevation of CDK2 kinase activity between p27−/− and K5CDK4/p27−/− mice. This result implies that increased CDK2 kinase activity is not due to overexpression of hCDK4, but to the lack of p27Kip1, also suggesting that the synergistic effect of CDK4 expression and lack of p27Kip1 in tumorigenesis is due to an inherent function of CDK4 and not to the indirect activation of CDK2.

Discussion

Transgenic expression of CDKs in the pituitary gland. It is known that the pituitary gland develops from two different sources; one of them is an ectodermal vesicle that grows upwards from the roof of the primitive oral cavity, a K5-expressing epithelium forming the Rathke’s pouch. However, no expression of endogenous K5 or K5-driven transgenes was detected in embryonic or adult pituitary gland (data not shown); suggesting that the K5 promoter is turned off during pituitary organogenesis. Surprisingly, we observed hCDK4 and hCDK2 expression under the K5 promoter, which is maintained in the pars intermedia of embryonic and adult pituitary. Analysis of CDK4 kinase-dead transgenic mice, K5-CDK4ΔD158N, does not show expression of the CDK4ΔD158N in the pituitary gland, suggesting that the kinase activity of CDK4 is necessary to maintain its expression during pituitary organogenesis. This result also indicates that indirect activation of CDK2 by the noncatalytic function of CDK4ΔD158N is not sufficient to retain the primitive epithelial cell phenotype (K5 transgene–positive cells) in the adult mouse pituitary gland. However, overexpression of hCDK2 under the K5 also allows the retention of the primitive epithelial cell phenotype. These contradictory results could be explained by the difference in the level of CDK2 expression. Although expression of CDK4D158N elevates CDK2 kinase activity, CDK2 protein levels remain unchanged. In contrast, elevated CDK2 protein levels in K5CDK2 mice may affect additional pathways required for the retention of the primitive epithelial phenotype.

Immunolocalization of hCDK4 in pituitary embryos at E13.5 shows the distribution of the transgenic protein in the primitive pars anterior and pars intermedia (Figs. 1 and 3A). On the other hand, hCDK4-positive cells localize almost exclusively in the pars intermedia in E17.5 embryos (Fig. 3B). The pars intermedia, which is separated from the anterior lobe by a residual cleft of the original Rathke’s pouch, is lined by cuboidal epithelial cells. These epithelial cells are clearly observed in adult and E17.5 pituitaries as hCDK4-positive cells; however, other sparse cells also express hCDK4 cells (Figs. 1B and 3B). Thus, it is plausible that epithelial-like cells derived from the primitive oral epithelium are retained in the E13.5 pituitaries from K5CDK4, but later, only the progenitors of the pars intermedia maintain an active K5 promoter. Several groups have described epithelial-like cells in pituitary glands from different species; however, an unambiguous characterization of these cells has yet to be established (28–31).

Analyses of embryonic pituitary also show that the epithelial-like cells expressing hCDK4 do not express the major hormones found in the pars intermedia, ACTH and α-MSH. In addition, these cells are BrdUrd- and PCNA-positive cells. This pattern of expression is also observed in the adult pars intermedia and pituitary tumors, suggesting that the hCDK4-expressing cells are proliferative cells, and the expression of hCDK4 is lost during the differentiation process.

The fact that EGFP and cyclin D2 and D3, are not expressed in the pituitary gland in the respective transgenic mice strongly suggests that the retention of hCDKs is due to an intrinsic function of CDK4 and CDK2. Western blot analysis of cyclin D2 and cyclin D3 in pituitary glands from wild-type mice show that both cyclins are highly expressed in the pituitary gland. Thus, it is feasible that cyclin D2 and cyclin D3 are not the rate-limiting factors for the activation of CDK4 in this organ. Thus, increased expression of these cyclins in the pituitary should not result in overactivation of endogenous CDK4. This would explain why cyclin D2 and/or cyclin D3 do not favor the selection of a cell population in which the K5 promoter remains active. We can hypothesize that early expression of CDK4 or CDK2 in the primitive oral epithelium results in a selective advantage of a cell population that remains as epithelial-like cells.

Pituitary stem cells. Our analysis also showed a reduction in the number of pituitary stem cells and the expression of hCDK4 being limited to TA-like proliferative cell population. Thus, we hypothesized that overexpression of CDK4 enhances proliferation causing an increase of differentiation from stem cells toward TA cells. Therefore, the overexpression of CDK4 disrupts the balance between stem cell self-renewal and proliferation, resulting in the reduction of the overall stem cell population. Consistent with our hypothesis, the self-renewal of hematopoietic stem cell population is affected in p21−/− and p27−/− mice. In such cases, self-renewal was impaired by p21Cip1 ablation leading to stem cell exhaustion, likely through CDK4 and/or CDK2 activation (32), whereas lack of p27Kip1 greatly increases the number of TA cells or the progenitor pool size (33). Also, it has been recently shown that activation of the pRb pathway by depletion of pRb in mouse skin caused a
reduction in the number of epidermal stem cells (34). In such cases, decreased stem cell number is associated with a reduction in the number of benign tumors, however, these benign skin tumors progress rapidly to squamous cell carcinomas. It is worth mentioning that K5CDK4 skin showed a mild reduction of skin papillomas in a two-stage carcinogenesis model, but also exhibited a sharp increase in carcinoma formation (24). Whether disrupting the equilibrium between stem cells and TA cells is a general mechanism of deregulated CDK4 expression which fosters an increased propensity towards malignancy merits further investigation.

**Pituitary tumor development.** p27−/− and p18−/− mice develop pars intermedia tumors of the same origin as those in pRb−/− mice (8, 26, 27). Interestingly, reduced tumor latency was observed in pRb+/−/p27−/− compound mice, suggesting that these genes cooperate to suppress tumor development by integrating different regulatory signals (35). Here, we have established that overexpression of CDK4 in the pars intermedia synergizes with loss of p27Kip1, resulting in the development of aggressive adenocarcinomas that are not observed in p27−/−/p27−/− compound mice. These results support the model in which p27Kip1 and pRb impinge in separate pathways (35), but also support the idea that CDK4 expression induces malignant progression through synergism with a second event. In support, we have previously reported that overexpression of CDK4 synergizes with Ha-ras mutations in mouse skin carcinogenesis, increasing the rate of malignant conversion (24). Thus, it is conceivable that cells overexpressing CDK4 are in a primed state, which can then synergize with an additional hit such as Ha-ras activation or loss of p27Kip1.

We have observed that although CDK2 transgene expression is also retained in the pituitary gland, it does not synergize with p27kip1 loss in tumor development. Thus, it is feasible that CDK4 and p27Kip1 affect alternative pathways and not only act through pRb phosphorylation. For example, it has been shown that CDK4 negatively regulates TGF-β effector proteins, R-Smads, via phosphorylation (36). In turn, it has been shown that Smads 1 and 4 regulate the expression of T-Pit, a transcription factor which controls the differentiation between stem cells and TA cells is a major barrier to the development of pituitary tumors in both the pars anterior and the pars distalis, which are composed of hyperplastic and well-differentiated cells (11). On the other hand, overexpression of hCDK4 in a p27−/− background leads to the development of aggressive adenocarcinomas derived from the pars intermedia with 100% incidence. The distribution of CDK4R24C mutation in human pituitary tumorigenesis has been extensively examined, all these studies failed to find CDK4R24C or CDK4R24H mutations.

In conclusion, we propose that early overexpression of CDK4 in the primitive oral cavity results in a selective advantage that allows these cells to survive and further remain as TA cells which are prone to malignant transformation.

**Acknowledgments**

**References**


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