ABSTRACT

WANG, XIAN. The Role of CDK6 and Cyclin D3 in Keratinocyte Proliferation and Mouse Skin Carcinogenesis. (Under the direction of Dr. Marcelo Rodriguez-Puebla).

CDKs (cyclin-dependent kinases) are a family of serine/threonine protein kinases, which play a critical role in regulating cell cycle. After binding and being activated by their regulatory subunit protein, D-type cyclins, CDK4/6 phosphorylate and inactivate their substrate, pRb, to permit the transcription of genes needed for cell cycle transit from G1 phase to S phase. Biochemical analysis of several human and experimental tumors shows deregulated expression and activating mutations in CDK4 and CDK6 activity. It has been widely assumed that CDK6 and CDK4 plays redundant roles since they share 71% amino acid identity, and they are regulated by the same regulatory subunit (D-type cyclins) during the G1 phase of the cell cycle. Although, recently data support the hypothesis that CDK4 and CDK6 also play non-redundant roles. Therefore, the general purpose of our studies has been to determine the role of CDK6, as well as its main regulatory partner, cyclin D3, in normal and neoplastic proliferation.

In order to investigate the role of CDK6 in tumorigenesis, we generated a transgenic mouse model of elevated CDK6 kinase activity (K5CDK6 transgenic mice). Similar to K5CDK4, overexpressed CDK6 triggered epidermal proliferation. Unexpectedly, CDK6 overexpression results in decreased skin tumor development compared with wild-type siblings. The inhibition in skin tumorigenesis was comparable to that previously reported in K5-cyclin D3 mice. These studies provide in vivo evidence that CDK4 and CDK6 play a related role as a
mediator of keratinocyte proliferation but differ in apoptosis activation and skin tumor development.

We also provided insights into the compensatory mechanism between D-type cyclins. Our data show that simultaneous ablation of cyclin D1 and down-regulation of cyclin D2 resulted in robust reduction of ras-mediated skin tumorigenesis.

Interestingly, increased formation of CDK6/cyclin D3 complexes were noticed in transgenic mice overexpressing CDK6 or cyclin D3, associated with reduced tumor development. Using a K5CDK6/cyclin D3−/− compound mouse model with elevated CDK6 protein level in absence of cyclin D3, we found that the tumor inhibition effect of CDK6 is independent of cyclin D3 expression. On the other hand, cyclin D3 is necessary for CDK6 mediated tumor growth.
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The Role of CDK6 and Cyclin D3 in Keratinocyte Proliferation and Mouse Skin Carcinogenesis

by
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DEDICATION

I would like to dedicate the works presented here to my family.

To My grandmother Dai Qiuning, you taught me how to get along with people and how to face adversity. Thank you for understanding my absence as a necessary sacrifice and not a measurement of my love of you.

To my mother Zhang Guojun and father Wang Zhi, thank you for giving me the opportunity to pursue life ideals.

To my Uncle Wang Xin's family and Uncle Wang Dujiang's family, your countless supports are invaluable.

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At last but not least, to my lovely girlfriend Yin Yuanyuan, I am just speechless at this moment, all I can say is thank you for everything you have done for me.
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Chapter I

Introduction
1.1 Cell Cycle Regulation

Cell division consists of an organized series of events in which a cell duplicates its contents and then divides into two daughter cells, a process known as the cell cycle. The cell cycle can be defined as an ordered set of processes leading to DNA duplication, segregation of the chromosomes and further cell division. Cell cycle regulation is crucial to the survival of a cell. It does not only control the growth and the development of cells but also plays an important role in detection and repair of genetic damage as well as the prevention of uncontrolled cell division. Historically the cell-cycle was divided into two fundamental parts: interphase, which occupies the majority of the cell cycle, and mitosis, which lasts about 30 minutes in typical mammalian cells. However, a detailed analysis of the cellular and biochemical events occurring during the cell division divides the cell cycle into four distinct phases: G1 phase (first Gap), S phase (synthesis), G2 phase (second Gap) and M phase (mitosis) (Figure 1). Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G0 phase. As the first phase of interphase, G1 phase is also called growth phase during which biosynthetic activities of the cell raises to a high rate comparing to M phase. This phase is marked by synthesis of various enzymes that are required in S phase, mainly those needed for DNA replication. During the S phase, the amount of DNA in the cell has effectively duplicated, though the ploidy of the cell remains the same. This progression usually takes 10-12 hours, which is approximately
half the time of a typical mammalian cell cycle. During the G2 phase, a significant biosynthesis also occurs, mainly involving the production of microtubules, which are required during the process of mitosis. Inhibition of protein synthesis during G2 phase prevents the cell from undergoing mitosis. Mitosis occurs exclusively in eukaryotic cell, one significant event of which is the segregation of chromosomes in the cell nucleus into two identical sets. The process of mitosis is complicated and highly regulated. The series of events is sequentially divided into phases, which include prophase, prometaphase, metaphase, anaphase and telophase, corresponding to the completion of one set of activities and the start of the next. During the process of mitosis, the pairs of chromosomes condense and attach to microtubule fibers that pull the sister chromatids to opposite sides of the cell. The last phase of the cell-cycle, called cytokinesis, leads to the division of the nuclei, cytoplasm, organelles and cell membrane into two cells containing roughly equal shares of these cellular components. Therefore, mitosis and cytokinesis together define the mitotic (M) phase of the cell cycle - the division of the mother cell into two daughter cells (Figure 1).

It is widely accepted that the fate of individual cell is dictated by the extracellular signals from the surrounding environment, such as growth factors and cytokines, which mostly play their roles during the G1 phase. It is known that D-type cyclins and cyclin-dependent kinases (CDKs) play a central function in integrating extracellular signals and the initiation of a cell cycle in G1 phase.
Cyclins were firstly discovered by R. Timothy Hunt in 1983 while studying the cell cycle of fertilized sea urchin eggs (Evans, Rosenthal et al. 1983) and were originally named in view of their concentration varies in a cyclical fashion during the cell cycle. The oscillations of the cyclins reflect the fluctuations in cyclin gene expression and destruction by proteolysis at specific points of the cell cycle (Felix, Labbe et al. 1990) (Figure 2). In addition, these oscillations of cyclins will induce fluctuations of CDK activity throughout the cell cycle which finally drives the cell cycle. The first two cyclins discovered by Hunt were named cyclin A and cyclin B respectively. After forming a complex with CDK1 (original named Cdc2 or p34), cyclin A/B form a maturation-promoting factor (MPFs) which activates other proteins through phosphorylation. These phosphorylated proteins, in turn, mediate the specific events during cycle division such as microtubule formation and chromatin remodeling (Hoffmann, Clarke et al. 1993). By themselves, these cyclins do not have enzymatic activity but have binding sites for some CDK substrates and guide the CDKs to specific sub-cellular locations (Maridor, Gallant et al. 1993). It is worth mentioning that cyclins are now classified according to their conserved cyclin box structure, a stretch of 150 amino acid residues, and some of these cyclins do not necessarily alter their level through the cell cycle. To date, at least 29 cyclins have been identified from human genome and clustered into 15 families (Malumbres and Barbacid 2005). These cyclins are also separated into two main groups according to their working time point during the cell cycle. The first group is called G1/S cyclins, which are essential for the control of the cell cycle at the G1/S transition. For instance, D-type cyclins (cyclin D1, D2, D3) cooperate with CDK4/6 to mediated the
Figure 1. The Cell Cycle and CDKs. The cell cycle is divided into four phases, first gap phase (G1), synthesis phase (S phase), second gap phase (G2) and mitosis (M phase). Cyclin-dependent kinase activity fluctuates throughout the cell cycle and regulates the process of the cell cycle. Extracellular signals are integrated in G1 phase by D-type cyclins and cyclin-dependant kinases (CDKs), which are negatively regulated by CKIs (Ink4 and Cip/Kip families). The restriction point is a stage after which the cell cycle can progress independent of mitogenic stimuli.
Figure 2. The oscillations of the cyclins through the cell cycle
initiation of G1/S phase transition, Cyclin E associates with CDK2 to control the progression and completion of G1/S phase transition (Malumbres and Barbacid 2005). The second group is called G2/M cyclins and as the name suggest, they are essential for the control of the G2/M phase transition. G2/M cyclins accumulate steadily during G2 and are suddenly destroyed at the end of the M-phase where cells exit mitosis. For example, Cyclin B/CDK1 regulates progression from G2 to M phase (Malumbres and Barbacid 2005). Similar to cyclins, there are multiple CDKs playing essential roles in different cell cycle phases via forming complex with corresponding cyclins. At least 11 CDKs have been identified in human genome, and most of the known CDKs are involved in cell cycle regulation in a direct or indirect way (Malumbres and Barbacid 2005). For instance, CDK1, 2, 4 have been proven to regulate the cell cycle directly. CDK5 and CDK7 are involved indirectly as activators of other CDKs. Usually Cyclin-CDK complexes in earlier cell-cycle phase help activate cyclin-CDK complexes in later phases.

1.2 G1 phase regulation and pRb pathway

To start a new round of cell division, the cells have to overcome an obstacle in G1 phase, which is called the restriction point or R point (Pardee 1974). Once the cells pass this point, generally they may continue to divide independent of extracellular mitogenic stimuli (Pardee 1974; Pardee 1989). It has been demonstrated that the R-point is guarded by a family of proteins, which is called the retinoblastoma (Rb) family (Yao, Lee et al. 2008). All
three members of the Rb family, pRb, p107 and p130 participate in the control of the mechanisms which are critical for maintaining normal levels of proliferating cells and meanwhile avoiding abnormal hyperplastic and neoplastic growth (Murphree and Benedict 1984). pRb works as the master switch of the cell cycle and is one of the three structurally related proteins that together are called "pocket" proteins (Munger and Howley 2002; Korenjak and Brehm 2005). The three pocket proteins bind different subsets of a transcription factor family called E2Fs. For example, in quiescent cells, active pRb binds to the E2F-DP dimer and inhibits its activity. The role of E2F transcription factor activity in controlling the transition from G1 to S phase has become clear from a large number of studies that have identified multiple E2F-regulated genes (Dyson 1998). E2F transcription factor family guard the transcription of various critical genes required by S or later phases, including cyclin E, cyclin A, cyclin B, DNA polymerase, thymidine kinase, etc (Malumbres and Barbacid 2001). Therefore, pRb acts as a growth suppressor and prevents progression through the cell cycle. Histone Deacetylase (HDAC) is also attracted to chromatin by the pRb-E2F-DP complex which can further block DNA synthesis (Wang, Fu et al. 2001). As long as E2F-DP is inactivated, the cell remains stalled in the G1 phase. Along with the transcriptional activation targets of the pRb/E2F pathway, the important role of the pathway in the control of cell proliferation is also illustrated by several key observations. For example, the deregulation of the pathway is the primary function of DNA tumor virus oncoprotein that promotes cellular proliferation (Nevins 1998).
Given the clearly important role played by the pRb/E2F pathway in controlling cell proliferation, it was speculated that the mutations of pRb favor tumor development. Supporting this hypothesis, the analysis of human tumors revealed a wide spectrum of mutations that alter the Rb/E2F pathway (Weinberg 1995; Sherr 1996; Hunter 1997). Rb mutations have been identified in osteosarcomas, small cell lung carcinomas, breast carcinomas and others (Bookstein, Lee et al. 1989; Toguchida, Ishizaki et al. 1989; Horowitz, Park et al. 1990; Mori, Yokota et al. 1990).

Upon an extracellular mitogenic stimulation, quiescent cells exit from G0 into G1 phase and initiate the synthesis of D-type cyclins, followed by binding and activation of their catalytic partners CDK4 and CDK6 (Sherr 1993). The cyclin D-CDK4/6 complexes then initially phosphorylate pRb, and inhibit its activity. Phosphorylation of pRb allows E2F-DP to dissociate from pRb and become partially activated. The initial activation of E2F permits the transcription of cyclin E, which results in formation and activation of CDK2/cyclin E complexes. These complexes will further phosphorylate pRb (hyper-phosphorylated) which allow the thorough release of the E2F transcription factors. pRb remains phosphorylated throughout S, G2 and M phases. During the M-to-G1 transition, pRb is progressively dephosphorylated by PP1, returning to its growth-suppressive hypophosphorylated state (Nelson, Krucher et al. 1997).

Early studies have demonstrated that some cyclins and CDKs play unique roles in the progression of DNA synthesis. For example, CDK2/cyclin E participates in the initiation of
DNA synthesis and the proper completion of S phase via a few of its substrates such as Cdc6, Cdt1 and Mini Chromosome Maintenance 2 (MCM2) (Yan and Newport 1995; Findeisen, El-Denary et al. 1999). Cdc6 is an essential regulator of DNA replication and plays important roles in the activation and maintenance of the checkpoint mechanisms in the cell cycle that coordinate S phase and mitosis (Coleman, Carpenter et al. 1996). Together with Cdt1, Cdc6 assembles into ORC (origin recognition complex), and this is required for loading Mini Chromosome Maintenance (MCM) proteins onto the DNA, an essential step in the initiation of DNA synthesis (Speck, Chen et al. 2005). It is suggested that sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase which is essential for initiation and elongation of the replication fork (Tercero, Labib et al. 2000; Randell, Bowers et al. 2006). MCM2 is phosphorylated by CyclinE/CDK2, which further activates its helicase activity. Importantly, at the onset of S phase, Cdc6p is phosphorylated by Cdk1/cyclin A, which leads to its export from the nucleus, and then targets it for degradation by the ubiquitinylation/proteosome pathway. Thus, Cdk1 assures that DNA replication is performed only once per cell cycle through controlling the accumulation and degradation of Cdc6.

As DNA replication continues, Cyclin E is destroyed and the level of mitotic cyclins begins to rise (in G2 phase). Cyclin B/CDK1 is the main cyclin/CDK complex playing an essential role in M phase, which initiates the assembly of the mitotic spindle and phosphorylates various proteins involved in nuclear envelope breakdown, centrosome
separation, and condensation of chromosomes (John, Mews et al. 2001; Mishima, Pavicic et al. 2004; Crasta and Surana 2006; Abe, Nagasaka et al. 2011).

1.3 Regulation of cyclins and cyclin-dependent kinases

1.3.1 Cyclin regulation

Even though CDK4/6 are the main driving forces of G1/S progression, their activities are highly dependent on the availability of their regulatory subunits D-type cyclins, which are the rate limiting parameter (Quelle, Ashmun et al. 1993). Studies from several groups have consistently demonstrated that cyclin D serves as a key sensor and integrator of extracellular signals of cells in early mid-G1 phase via binding CDKs and histone deacetylases to modulate local chromatin structure of the genes that are involved in regulation of cell proliferation and differentiation (Fu, Wang et al. 2004). The abundance of cyclin D is regulated by multiple growth factors in a cell-type-dependent pattern (Song, Rana et al. 2003; Pradeep, Sharma et al. 2004). In this regard, it has been shown that several transcription factors such as CREB, STAT, Egr-1 and Tcf/Lef, are able to activate cyclin D1 promoter (Coqueret 2002). The three D-type cyclins (D1, D2 and D3) were originally isolated from mouse macrophages and characterized as early delayed growth factor inducible genes, which are differentially and combinatorially expressed (Matsushime, Roussel et al. 1991; Matsushime, Ewen et al. 1992). On the meantime, deregulation of cyclin D was shown to be associated with a variety of human malignance (Schuuring,
Verhoeven et al. 1992). Many oncogenic signaling pathways are involved in the induction of cyclin D1 expression, which including: Ras/MAP kinase (Albanese, Johnson et al. 1995); PI3K/Akt pathway (Liang and Slingerland 2003); Src (Lee, Albanese et al. 1999); ErbB2 (Lee, Albanese et al. 2000); β-catenin tcf/Lef pathway (Shtutman, Zhurinsky et al. 1999; Lin, Zang et al. 2000); and simian virus 40 small T antigen, a particularly crucial oncogene in transformation of human cells (Watanabe, Howe et al. 1996). Mitogen-induction of cyclin D1 is mostly carried out via Ras-Raf-MAPK/ERK pathway. The MAP kinase ERK activates the downstream transcription factors Myc and AP-1, which in turn activate the transcription of the Cdk4, Cdk6 and Cyclin D genes, and increase ribosome biogenesis (Kerkhoff, Houben et al. 1998; Mateyak, Obaya et al. 1999; van Riggelen, Yetil et al. 2010).

The cyclin D1 protein has been shown to be unstable with a short half-life (~24 minutes), its levels begin to rise early in G1 phase and continue to accumulate until the G1/S-phase boundary when levels are rapidly declined (Diehl, Cheng et al. 1998). Cyclin D1 is degraded mainly via the 26S proteasome in an ubiquitin-dependent manner (Diehl, Zindy et al. 1997). GSK3β-dependent phosphorylation of cyclin D1 on T286 mediates its nuclear export and rapid degradation within the cytoplasm (Diehl, Zindy et al. 1997; Diehl, Cheng et al. 1998). Another GSK3β-independent degradation has also been identified, and this pathway serves to regulate the cellular levels of free cyclin D1. Once synthesized, free-cyclin D1 associates to SCF ubiquitin E3 ligase complex and is quickly degraded (Yu, Gervais et al. 1998). In addition to ubiquitin-dependent degradation, Feng demonstrated the existence of a ubiquitin-independent pathway, such as those mediated by antizyme, may
thus play a crucial role in regulating cellular cyclin D1 levels, which suggested that the N-terminal end of cyclin D1 plays an important role in regulating its stability (Feng, Sekula et al. 2007).

As I mentioned above, in response to the activation of cyclin D-CDK4/6 complex, pRb is partially phosphorylated. The hypophosphorylated pRb dissociates from the E2F/DP1/pRb complex and stops blocking E2F activity. Stimulation of E2F results in transcription of various other cyclins needed for later cell cycle phases including cyclin E and cyclin A. Unlike the mitogen-inducible D-type cyclins, synthesis of cyclin E and cyclin A is highly dependent on E2F activation. Cyclin E binds and activates CDK2, which push the cell from G1 to S phase (G1/S transition) and serves as a rate-limiting component of the machinery that controls entry into S phase and centrosome duplication. The abundance of cyclin E is also post-translational regulated, in particular, by the ubiquitin-proteasome pathway. Both CDK2-associated and free cyclin E appear to be targets for ubiquitination and rapid degradation (Clurman, Sheaff et al. 1996). Once the cells enter S phase, cyclin E binds to and undergoes poly-ubiquitination by the ubiquitin ligase, known as SCF (Skp2) (Nakayama, Nagahama et al. 2000). Like cyclin E, cyclin A starts to accumulate during S phase and is abruptly destroyed before metaphase (Hunt, Luca et al. 1992; Erlandsson, Linnman et al. 2000). Removal of cyclin A is carried out by ubiquitin-mediated proteolysis. A unique function of cyclin A is that it can activate two different cyclin-dependent kinases (CDKs) and functions in both S phase and mitosis. In mitosis, the precise role of cyclin A is still obscure, but it may contribute to the control of cyclin B stability. Cyclin B along with cdc2 (CDK1) forms the
cyclin B/CDK1 complex, which initiates the G2/M transition. Activating cyclin B1/CDK1 immediately triggers its rapid accumulation in the nucleus via an unknown mechanism (Gavet and Pines 2010; Gavet and Pines 2010). The amount of CDK1-associated cyclin B and the activity of the cyclin B/CDK1 complex rise through the cell cycle until mitosis, where they fall abruptly due to degradation of cyclin B. Activation of cyclin B/CDK1 complex results in the breakdown of nuclear envelope and initiation of prophase, and subsequently, its deactivation causes the cell to exit mitosis. In addition, CyclinB/CDK1 complex activates the anaphase-promoting complex (APC/C) which allows the sister chromatids at the metaphase plate to separate and move to the poles and completes mitosis. Conversely, APC/C destroys B type cyclins by attaching them to ubiquitin, which targets them for proteasome dependent destruction, and turns on synthesis of G1 cyclins (D type cyclins) for the next round of a cell cycle. In activation of M phase cyclin and CDK (cyclin B/CDK1) is required for the degradation of geminin which keeps the synthesized DNA from being re-replicated before mitosis (Ma and Poon 2010).

1.3.2 Cyclin-CDK complex formation and regulation

Usually newly synthesized D-type cyclins quickly form complexes with CDKs and are translocated into the nucleus of proliferating cells. Unlike the cyclins, the expression levels of CDKs are relatively consistent throughout the cell cycle. In vitro assays demonstrated that purified recombinant cyclin D1 and CDK4 proteins bind with a very low affinity (Kato,
Matsuoka et al. 1994). The assembly of cyclin D/CDK4 complexes and the appearance of their kinase activities depends upon serum stimulation, indicating that upstream regulators must govern the formation of the active enzymes (Matsushime, Quelle et al. 1994). Some chaperone proteins have been proposed to play a role in the complex assembly. For example, Hsc70 associates with newly synthesized cyclin D1 to promote its stabilization, thereby increasing its availability for assembly with CDK4 (Diehl, Yang et al. 2003). In addition, Hsc70 remains bound to cyclin D1 following its assembly with CDK4 and Cip/Kip proteins, where it ensures the formation of an active catalytic complex (Diehl, Yang et al. 2003). Hsp90 and CDC37 serve to stabilize CDK4 via their capacity to direct proper CDK4 folding and compete with CDK inhibitors to bind and facilitate the assembly of cyclin/CDK complex (Stepanova, Leng et al. 1996). Moreover, association of a growth factor sensor p34 (SEI-1) to cyclin D1-CDK4 renders the complex resistant to inhibitor binding and ectopic expression of p34 (SEI-1) enables fibroblasts to proliferate even in low serum concentrations (Sugimoto, Nakamura et al. 1999).

As long as the cyclin/CDK complex formed, a class of proteins called cyclin-dependent kinase inhibitors (CKIs) is standing by to interact with the complex and inhibit its activity. The CKIs consist of two families: INK4 (Inhibitors for CDK4) and Cip/Kip family. The Cip/Kip family consists of three family members: p21^{Cip1}, p27^{Kip1} and p57^{Kip1}, all of which function as broad-spectrum inhibitors by binding to both cyclin D-CDK4/6 kinases and cyclin E/A-CDK2 kinases (Ekholm and Reed 2000) whereas the INK4 family (p15^{ink4b}, p16^{ink4a}, p18^{ink4c} and p19^{ink4d}) function as narrow-spectrum inhibitors by specifically binding CDK4 and CDK6.
The binding of INK4 proteins to CDK4/6 prevents the binding of D-type cyclins to CDKs thus blocks the formation of cyclin D-CDK4/6 complexes. In addition, the binding of INK4 and CDK4/6 also blocks the binding of CDK4/6 to Cip/Kip family and cause a redistribution of Cip/Kip family members to cyclin-E/CDK2, as a result, these complexes are inhibited (Sherr and Roberts 1999). An intriguing but controversial phenomenon is that cyclin D-CDK4/6 complex assembly is facilitated by the binding of Cip/Kip inhibitor family. Loss-of-function assay proved that knock out of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in mouse embryonic fibroblasts disrupts the assembly of cyclin D-CDK complexes, meanwhile the expression level of cyclin D is dramatically decreased and fails to translocate to the nucleus (Cheng, Olivier et al. 1999). It is also suggested that binding of p21<sup>Cip1</sup> and p27<sup>Kip1</sup> helps the stabilization of cyclin D/CDK4 complexes (Bagui, Mohapatra et al. 2003).

The physical binding to cyclins and CKIs can sufficiently but not fully activate or inhibit CDKs. CDKs are also regulated by post-translational modifications. Full kinase activity requires an activating phosphorylation on certain threonines (T172 for CDK4, T160/161 for CDK1 and CDK2) next to the CDK active site, which is usually blocked by two helices. Upon cyclin binding, rearrangement of the two alpha helices results in unblocking the active site by changing the position of these key amino acid residues. Then the exposed active site is phosphorylated by CDK activating kinase (CAK) which is composed of CDK7-cyclin H and an assembly protein Mat1 (Fisher and Morgan 1994; Kato, Matsuoka et al. 1994; Morgan 1997). Importantly, the phosphorylation of Cyclin D-CDK4/6 by CAK effectively increases the complex activity 80-300 fold (Coqueret 2002). In addition to activating phosphorylation,
CDK inhibitory phosphorylation is vital for regulation of the cell cycle. Two specific kinases, Wee1 and MYT1, phosphorylate CDK4 and CDK2 on Thr14 and Tyr15 respectively (Russell and Nurse 1987; Mueller, Coleman et al. 1995), which inhibits CDKs activities. These cyclin-CDK complexes remain inactivated until dephosphorylation of these sites by the Cdc25 phosphatase family Cdc25A, Cdc25B and Cdc25C (Russell and Nurse 1986; Morgan 1997).

1.4 Deregulation of Cyclin-CDKs in Human and Experimental Tumors

In order to avoid uncontrolled cell division, the cell cycle regulators have to be precisely regulated. Deregulation of many cell cycle components might lead to tumor formation. For instance, the cell cycle inhibitors safeguard the appropriate cell cycle, and mutation of any of these proteins may cause the cells to multiply uncontrollably favoring tumor development. As a tumor suppressor protein and the key regulator of G1/S phase transition, pRb has been found dysfunctional in a majority of human cancers (Murphree and Benedict 1984). The dysfunction of pRb could be either direct mutation of itself or false inactivation of pRb caused by changes of the upstream components of the pRb pathway such as over-activated cyclin/CDKs complex or impaired CKI inhibition (Murakami and Sekiya 1998; Burkhart and Sage 2008). The classical cyclin D–E2F pathway model I discussed above emphasises the link between cyclin D1 overexpression and abnormal inactivation of pRb and further increased proliferation. A large number of publications have implicated the abnormal levels of D-type cyclins in the development of various types of human tumors.
(Bartkova, Lukas et al. 1995; Russell, Thompson et al. 1999; Sutherland and Musgrove 2004; Mermelshtein, Gerson et al. 2005).

Interestingly, it has recently been shown that cyclin D1 interacts with more than 100 proteins in human cancer cell lines (Jirawatnotai, Hu et al. 2011). Even though most interactors among these proteins are involved in cell cycle control and transcriptional regulation, proteins that are involved in DNA repair, RNA metabolism, protein folding, cell structure and cell organization have also been described. Therefore, it is important to keep in mind that the deregulation of cyclin D1 will not only promote mitogen-independent proliferation via Rb pathway, but may also affect other cellular processes, both directly and indirectly, in ways that have potentially oncogenic consequences including angiogenesis (Yasui, Yamamoto et al. 2006), centrosome duplication (Nelsen, Kuriyama et al. 2005) and the DNA damage response. These observations lead us to ask which molecular function of cyclin D1 is crucial during oncogenesis. The rearrangement of cyclin D1 has been reported in a wide range of human cancers (Gillett, Fantl et al. 1994; Kurzrock, Ku et al. 1995). Evidence suggests that some mutations of cyclin D1 could impair its nuclear trafficking and proteolysis that lead to constitutive nuclear localization of cyclin D1, which increases the oncogenicity of cyclin D1 (Kim and Diehl 2009). Meanwhile, other studies have shown that cyclin D1 is exclusively cytoplasmic in some cancers, such as lung, prostate and ovarian cancer (Dhar, Branigan et al. 1999; Comstock, Revelo et al. 2007; Gautschi, Ratschiller et al. 2007). In this regard, it is speculated that the cytoplasmic cyclin D1 inhibits apoptosis following low-level DNA damage and favors the survival of cancer cells (Ahmed, Fan et al.
The amplification and overexpression of the cyclin D1 gene has been reported in breast carcinomas, lung cancer, melanoma and squamous cell carcinomas (Dickson, Fantl et al. 1995; Fujii, Ishiguro et al. 2001; Santarius, Shipley et al. 2010) via various mechanisms. In mantle cell lymphoma (MCL), a translocation juxtaposes the cyclin D1 gene (CCND1) with the immunoglobulin heavy chain locus (IGH) which leading to cyclin D1 overexpression. Amplification of cyclin D2 and cyclin D3 (CCND2 and CCND3) has also been reported in MCL but at a relatively lower rate (Wlodarska, Dierickx et al. 2008). Mutations that increase the stability of either CCND1 mRNA or cyclin D1 protein contribute to the aberrant expression of cyclin D1, too. Another more common event that leads to cyclin D1 overexpression is oncogenic activation of mitogenic signalling pathways such as RAS–MEK–ERK and PI3K pathways (Films, Robles et al. 1994; Diehl, Cheng et al. 1998). Overexpression of cyclin D1 through these pathways also results in deregulation of mitogenic signaling as RAS-induced centrosome amplification, which is CDK4-dependent. In lung and breast cancer cyclin D1 overexpression is associated with shorter patient survival and is often associated with increased metastasis (Thomas, Nadiminti et al. 2005; Jares, Colomer et al. 2007), which is consistent with the ability of cyclin D1 to enhance migration and invasion.

Similarly, abnormalities in the cyclin D2 gene have also been observed in testicular tumors (Sicinski, Donaher et al. 1996; Houldsworth, Reuter et al. 1997), B-cell malignancies (Delmer, Ajchenbaum-Cymbalista et al. 1995). Moreover, overexpression of cyclin D3 has been found in several human cancers, such as pancreatic adenocarcinoma (Ito, Takeda et al.
laryngeal squamous cell carcinoma (Pruneri, Pignataro et al. 2005); invasive ductal carcinoma of the breast (Wong, Chan et al. 2001); renal cell carcinoma (Hedberg, Roos et al. 2002), and malignancies of the thymus (Filipits, Jaeger et al. 2002).

In order to further study the relationship between aberrant expression of D-type cyclins and tumorigenesis, a number of investigators have utilized the mouse model. It has been established that cyclin D1 and D2 are up-regulated in benign mouse skin tumors (papillomas) and squamous cell carcinomas (SCC), whereas cyclin D3 protein levels remain constant in both normal epidermis and tumors (Rodriguez-Puebla, LaCava et al. 1998). Supporting these observations, forced expression of either cyclin D1 or cyclin D2 in mouse epidermis synergizes with Ha-ras activation to increase the number of skin papillomas and SCC, whereas genetic ablation of cyclin D1 and cyclin D2 reduces tumor development (Robles, Rodriguez-Puebla et al. 1998; Rodriguez-Puebla, LaCava et al. 1998; Rodriguez-Puebla, LaCava et al. 1999; Rodriguez-Puebla, Miliani de Marval et al. 2002). In contrast, the forced expression of cyclin D3 inhibits skin tumorigenesis in a mouse model, an effect that is mediated by the simultaneous downregulation of cyclin D2 (Rojas, Cadenas et al. 2007). This observation pointed to a potential role of cyclin D3 as a tumor suppressor in mouse keratinocytes rather than as an oncogene, even though further inspections are needed to prove this speculation.

As the catalytic subunit of cyclin D/CDK complex, CDK4/6 are also found amplified or overexpressed in many human cancers. For example, CDK4 is found overexpressed in sporadic breast carcinomas, lopomatous tumors and sarcomas (Reifenberger, Reifenberger
et al. 1994; An, Beckmann et al. 1999; Dei Tos, Doglioni et al. 2000). CDK4 and CDK6 are both overexpressed in human gliomas (Ichimura, Schmidt et al. 1996). On the other hand, CDK4, but not CDK6, is specifically targeted in familial melanomas, due to germline mutations in the p16\textsuperscript{INK4a} binding domain of CDK4 (Zuo, Weger et al. 1996). CDK6 activity has been found elevated in squamous cell carcinomas (SCCs) (Timmermann, Hinds et al. 1997; Piboonniyom, Timmermann et al. 2002) and neuroblastomas (Easton, Wei et al. 1998) without alteration of CDK4 activity. However, it is difficult to clarify the causal role of these alterations in tumors; for example, Cdk4 is co-amplified with \textit{MdM2} in most of these tumors (Malumbres and Barbacid 2005). As I mentioned above, misregulation of D-type cyclins and INK4 inhibitors is a common feature of most tumor types, suggesting that hyperactivation of CDK4 and CDK6 plays a preponderant role in human carcinogenesis. Interestingly, CDK2 has not been found mutated, overexpressed or amplified in human cancer; although, its natural partner, cyclin E, is often found overexpressed. Moreover, the expression of CDK2 inhibitory proteins \textit{p21}\textsuperscript{Cip1} and \textit{p27}\textsuperscript{Kip1} is frequently repressed in human tumors (Akama, Yasui et al. 1995; Catzavelos, Bhattacharya et al. 1997; Datta, Renshaw et al. 2000; Malumbres and Barbacid 2001; Muller-Tidow, Metzger et al. 2001). Therefore, CDK2 is also potentially involved in human cancer.

Experimental mouse models from our laboratory as well as others have made evident that deregulation of CDK4 plays a major role in tumor development. For instance, a knock-in mouse strain carrying a constitutive activation of CDK4 (Arg24Cys) found in melanoma patients exhibits endocrine neoplasias, epithelial hyperplasias and sarcomas (Sotillo, Dubus
et al. 2001; Rane, Cosenza et al. 2002). Primary CDK4-null keratinocytes are refractory to oncogenic transformation (Zou, Ray et al. 2002). In a similar fashion, CDK4 knockout mice are resistant to Ras-dependent two-stage chemical carcinogenesis (Rodriguez-Puebla, Miliani de Marval et al. 2002). Our lab has also shown that transgenic mice overexpressing CDK4 under the control of a Keratin 5 promoter exhibit increased frequency of skin squamous cell carcinomas than wild-type siblings (Miliani de Marval, Macias et al. 2004). Molecular analysis of K5CDK4 skin tumors shows that forced expression of CDK4 results in increased binding to p27 and p21, which released CDK2 from p27/p21 mediated inhibition and indirectly activated CDK2 kinase activity (Miliani de Marval, Macias et al. 2004).

Therefore, D-type cyclins are causally related to cancer development and tumor cells often become dependent on them for proliferation and survival (Weinstein and Joe 2006), which make them attractive therapeutic targets. However, due to the difficulty of targeting D-type cyclins directly as they lack intrinsic enzymatic activity, a more feasible pharmacological approach targeting G1 CDKs has been developed. However, cyclin D1 has both catalytic and non-catalytic roles that are important in both normal and neoplastic cells (Jirawatnotai, Hu et al. 2011), with the implication that targeting CDK4/CDK6 activity alone may only be partially effective in cyclin D1-dependent cancers.
1.5 Ras Signaling Pathway

The ras proteins (an abbreviation of RA at Sarcoma) belong to a large protein family of small GTPases (Ras superfamily) that are involved in cellular signal transduction. By cycling between two conformational states: one when bound to GTP, the active state, and another one when bound to GDP, the inactive state, Ras proteins function as a binary molecular switch that communicates signals from outside the cell to the nucleus. Multiple signaling pathways involved in cell proliferation, growth, differentiation, apoptosis, migration, adhesion, actin cytoskeleton integrity, senescence and survival are under the control of Ras (Chang, Steelman et al. 2003). The ras genes were firstly identified as transforming oncogenes which is responsible for the carcinogenic activities carried out by Harvey and Kirsten sarcoma viruses (Chang, Furth et al. 1982). It was demonstrated that S phase entry was blocked through inactivation of Ras via specific antibodies or dominant negative mutations (Mulcahy, Smith et al. 1985; Stacey, Roudebush et al. 1991). On the other hand, inducing Ras into quiescent cells promotes S phase entry independent of mitogenic stimuli (Feramisco, Gross et al. 1984). It has been documented that the G1-S transition mediated by Ras involved inactivation of pRb, and cells lacking pRb expression proliferate independent of Ras activity (Mittnacht, Paterson et al. 1997; Peeper, Upton et al. 1997). Physiological and oncogenic activation of Ras stimulates a wide range of downstream signaling pathways. The first identified and also most well studied Ras effector pathway was the Raf1-Mek-Erk/MAPK pathway (Moodie, Willumsen et al. 1993; Vojtek, Hollenberg et al. 1993; Zhang,
Settleman et al. 1993). Cyclin D1 is one important downstream target of this pathway. By inducing cyclin D1 synthesis and its consequent complex formation with CDK4/6, Ras inactivates the pRb and thus drive cell cycle progression (Rodriguez-Puebla, Robles et al. 1999). Supporting this model, our laboratory has demonstrated that ablation of cyclin D1 gene leads to reduced keratinocyte proliferation and repressed tumor development in a Ras-dependent carcinogenesis protocol (Robles, Rodriguez-Puebla et al. 1998). A second important downstream effector of Ras pathway involved in cyclin D1 regulation is the PI3 kinase pathway. By activating protein kinase B (Akt), Ras inactivates GSK-3β. GSK-3β mediates cyclin D1 proteosomal degradation by phosphorylation (Diehl, Zindy et al. 1997; Diehl, Cheng et al. 1998). Therefore, Ras promotes G1 phase progression by both increasing cyclin D1 expression and stability. In addition, Ras promotes cell cycle progression by altering the expression levels of p27Kip1 and p21Cip1 (Liu, Martindale et al. 1996; Medema, Kops et al. 2000).

Mutations in Ras family of proto-oncogenes are very common, ~20-30% of all human tumors and up to 90% in specific cancers such as pancreatic carcinomas (Bos 1989). Two of the most important ras mutations are located at residue G12 and the catalytic residue Q61. The glycine to valine mutation at residue 12 renders the GTPase domain of Ras resistant to GAP catalyzed GTP hydrolysis and thus locking Ras in a permanently "on" state. Mutation of Q61 to K reduces the rate of intrinsic Ras GTP hydrolysis to physiologically meaningless levels. All together substitution at these positions results in constitutive activation of Ras (Scheffzek, Ahmadian et al. 1997). Existence of active Ras protein in human cancers makes it
a therapeutically valuable target and currently treatment based on reovirus is in clinical trials (Thirukkumaran and Morris 2009).

1.6 Mouse skin model in Carcinogenic Research

Animal models have been widely used to investigate mechanisms of human disease and provided substantial applicable information. Particularly, mouse skin model of carcinogenesis has revealed the multistage nature of carcinogenesis. Generally, the adult skin is comprised of three major compartments: in the order of outer to inner, are named epidermis, dermis and appendage respectively (Aldaz and Conti 1989). The mouse epidermis consists of four layers of epithelial cells or keratinocytes. The basal layer comprises resting keratinocytes with high proliferative capacity, proliferating keratinocytes, and unrelated cells such as melanocytes, langerhan's and Merkel cells. Epidermal homeostasis consists of proliferation of basal keratinocyte periodically and detach from basal lamina, move outwards in a columnar fashion in a differentiation process to form the prickle cell layer (stratum spinosum), the granular cell layer (stratum granulosum) and finally, the horny cell layer (stratum corneum). As the cells migrate vertically, they become terminally differentiated, including losing their proliferative potential, possessing expansive cytoskeleton and so thus enforces cell-cell junction. When terminal differentiation completed, the squames exist as a sandwich of dead cells and lipids, which provide the barrier capacity of skin to the environment. Squames are eventually shed from the skin.
surface and replaced by differentiating cells from below (Candi, Schmidt et al. 2005; Blanpain and Fuchs 2006; Koster and Roop 2007). It has been demonstrated that multiple signaling pathways are essential for proper epidermal stratification and the acquisition of the skin barrier function. These pathways involve Notch, MAPK, NF-kB, p63, etc., most of which are also well-known in regulating proliferation, differentiation, apoptosis and carcinogenesis.

There are similarities between human and mouse non-melanoma skin carcinogenesis including the development of malignant squamous cell carcinoma from pre-existing benign lesions. In addition, it is relatively easy to perform tissue specific activation of oncogenes in the epidermis which provide an opportunity to study the role of certain molecular changes in vivo. Thus, the mouse became one of the first animal models to produce experimental skin cancer and has provided numerous valuable data to understand the nature of carcinogenesis (Fujiki, Suganuma et al. 1989; Hennings, Glick et al. 1993; French, Libbus et al. 1994). It is known that tumorigenesis comprises a multi-step mechanism, which includes initiation, promotion and malignant progression. In order to mimic the nature of tumorigenesis, the first multi-stage carcinogenesis model came out in 1950s (Nordling 1953; Armitage and Doll 1954). After decades of modification, nowadays a cutaneous two-stage chemical carcinogenesis protocol is frequently used in laboratories to help in the identification of important molecular pathways involved in cutaneous malignancy. This protocol consists of two-stage application of chemicals to the skin for the initiation and promotion of skin tumors. In the initiation stage, a single subcarcinogenic dose of chemical
initiator mutagen 7,12-dimethybenz[α]anthracene (DMBA) is painted on the back of the mice. DMBA is metabolized by cytochrome p450 and becomes activated in form of DMBA-3,4-diol,1,2-epoxide which induces an irreversible and specific mutation in codon 61 (A-to-T transversion on the second nucleotide) of the protooncogene Ha-ras (Fujiki, Suganuma et al. 1989). The cells carrying ras mutation are able to be promoted even if the promotion is applied one year after initiation. The promotion stage consists of multiple applications of a non-carcinogenic chemical. Multiple chemical agents have been used as tumor promoters, including indole alkaloids, benzo(e)prene and benzoyle peroxide, the most widely used promoter is the phorbol ester TPA (12-o-tetradecanoylphorbol 13-acetate, also known as phorbol 12-myristate13-acetate[PMA]). Unlike initiators, promoters are not mutagenic; they contribute to tumorigenesis by inducing epigenetic changes such as inflammation and epidermal hyperplasia, and these events are initially reversible. The appropriate application of two-stage protocol depends on several parameters. Firstly, the mouse strain susceptibility- different mouse strain reacts very differently to the two-stage chemical carcinogenesis. For example, the FVB strain is very susceptible while C57BL/6 is relatively resistant to tumor formation (Woodworth, Michael et al. 2004). Secondly, the presence of a genetic mutation or transgene expression may exhibit altered susceptibility to tumor formation (Girardi, Oppenheim et al. 2001; Girardi, Glusac et al. 2003). Last but not least, appropriate doses- varying doses of the chemical initiator or promoter can alter the effect of the protocol (Girardi, Glusac et al. 2003). Skin tumors are evaluated by visual inspection and generally, the first clinically apparent papilloma (endophytic, extending outward from
the skin; well-demarcated, symmetrical, pedunculated or dome-shaped papules) appear 6-8 weeks after the first dorsal application of the tumor promoter. Typically 5-10% of these benign tumors may progress to squamous cell carcinomas (endophytic, poorly demarcated, asymmetrical, and non-pedunculated lesion) after 25-30 weeks of promotion.

1.7 Research Focus

As I discussed above, D-type cyclins-CDK4/6 complexes play a central role in cell cycle regulation. Activated CDKs phosphorylate and inhibit pRb, leading to release of E2F transcription factors and the transcription of genes necessary for the G1/S transition. Either direct mutation of pRb or indirect inactivation due to elevated CDK activity results in the disruption of the pRb pathway, which renders cells insensitive to growth signals and leads to deregulated cell proliferation.

A large number of studies during the last decade have shed light on the roles of G1 phase CDKs in tumorigenesis. Our lab has used the mouse model to define the role of CDK4 in epidermal tumorigenesis (Miliani de Marval, Macias et al. 2004; Miliani de Marval, Macias et al. 2004). Importantly, our laboratory has shown that the effect of CDK4 overexpression is in part reduced upon ablation of CDK2 (K5CDK4/CDK2-null compound mouse), which suggests that CDK2 activity is necessary at the initiation and progression stages of Ras-mediated tumorigenesis (Macias, Kim et al. 2007). Although, a great body of evidence has involved CDK4 in human and experimental tumorigenesis, the role of CDK6 in tumor
development is poorly understood. CDK6 is believed to play a redundant function with CDK4 since they share similar expression patterns and share 71% amino acid identity (Meyerson and Harlow 1994). However, how exactly does CDK6 participate in normal and neoplastic proliferation in epidermis has not been established. Thus, part of our studies were directed to understand the effect of CDK6 expression in tumor development. Therefore, we generated a novel transgenic mouse model in which overexpression of human CDK6 was directed to epidermis and this model was utilized to answer the following questions: Does elevated CDK6 kinase activity alter mouse skin homeostasis? Will elevated CDK6 kinase activity contribute to tumor development?

As the regulatory subunit of CDK4/6, D-type cyclins are the rate-limiting factors of CDK4/6 activation and found frequently amplified or overexpressed in human tumors. Previous data from our lab has established the role of D-type cyclins in mouse skin tumorigenesis, which makes them attractive therapeutic targets to cure cancer. However, due to the complicate compensation mechanism existing among D-type cyclins, the therapy strategy of targeting individual D-type cyclin is not sufficient to eliminate tumor formation. One intriguing observation about D-type cyclins is that we found cyclin D1 and D2 positively correlate with mouse skin tumorigenesis while cyclin D3 plays an opposite role. What is more interesting is that the overexpression of cyclin D3 in mouse keratinocytes is associated with decreased cyclin D2 expression (Rojas, Cadenas et al. 2007). Thus, we hypothesized that reduction of both cyclin D1 and cyclin D2 will result in a stronger inhibition of tumor
development. To this point, we developed a mouse line with ablated cyclin D1, overexpressed cyclin D3 and decreased cyclin D2.

As the least studied D-type cyclin, cyclin D3 is expressed in nearly all proliferating cells, and it shows the most broad expression pattern of all three D-type cyclins (Bartkova, Lukas et al. 1998). Cyclin D3 is overexpressed in some particular human cancer such as human gliomas, renal cell carcinoma and pancreatic adenocarcinoma (Buschges, Weber et al. 1999; Ito, Takeda et al. 2001; Hedberg, Roos et al. 2002). However, cyclin D3 expression has also been associated with cellular differentiation. In particular, cyclin D3 expression has been found expressed in most terminally differentiated lymphoid tissues (Doglioni, Chiarelli et al. 1998). Similarly, cyclin D3 was detected in termially differentiating spermatids (Zhang, Wang et al. 1999). Supporting these observations, Wang et al demonstrated that cyclin D3 plays a growth inhibitory role in differentiated liver cells and adipocytes by stabilizing C/EBPalpha-CDK2 and C/EBPalpha-Brm complexes (Wang, Shi et al. 2006). Taken together, these observations suggested that cyclin D3 plays dual roles in proliferation and differentiation. We demonstrated that the forced expression of cyclin D3 in mouse skin leads to a refractory effect in the standard two-stage carcinogenesis protocol, presumably through the strong inhibition of cyclin D2 (Rojas, Cadenas et al. 2007). Meanwhile, we noticed an increased CDK6/cyclin D3 complex formation in transgenic mice overexpressing D3. Thus, we want to ask whether cyclin D3 plays an important role in tumor inhibition through the CDK6/cyclin D3 complexes formation.
Chapter II

Unexpected reduction of skin tumorigenesis on expression

of Cyclin-Dependent Kinase 6 in mouse epidermis
2.1 Introduction

Normal cell growth and differentiation requires precise control of the mechanisms that govern the entry into, passage through, and exit from the cell cycle. Progress through the G1 phase of the mammalian cell cycle is mediated by D-type cyclins, which associate and activate CDK4 and CDK6 kinases (Sherr 1995; Weinberg 1995). The pRb family of proteins, pRb, p107 and p130, are key substrates for G1 CDK/cyclin complexes, and negatively regulate the passage of cells from G1 to S phase (Weinberg 1995). Therefore, CDK4 and CDK6 act as master integrators in the G1 phase, coupling with the cell cycle mitogenic signals as well as with their oncogenic properties in cancer cells (Blain, Montalvo et al. 1997; Sherr and Roberts 1999; Sherr and McCormick 2002). CDK4 and CDK6, share 71% amino acid identity and both are expressed ubiquitously (Meyerson and Harlow 1994). As a result, it was assumed that both play a redundant function in G1 phase and tumorigenesis. However, in the last few years, relevant differences were determined between the functional properties of these G1 kinases. Initial experiments identified CDK6 activity in T cells, suggesting that cell-type specific expression might explain the need for two G1 kinases (Lucas, Szepesi et al. 1995). Supporting this hypothesis, CDK6-deficient mice showed a reduced number of red blood cells and lymphocytes, and pronounced thymic atrophy due to decreased proliferation and block of differentiation (Malumbres, Sotillo et al. 2004; Hu, Deshpande et al. 2009). Recently, Bockstaele et al reported difference in CDK4 and CDK6 regulation. They showed that CDK6, but not CDK4, is regulated by CDK-Activating Kinase
(CAK) and suggested a proline-directed kinase to be the main regulator of CDK4 (Bockstaele, Bisteau et al. 2009). Novel functions of CDK6 have recently been reported, for instance, residue selectivity of these kinases on the retinoblastoma protein (Takaki, Fukasawa et al. 2005), different subcellular localizations (Grossel, Baker et al. 1999; Ericson, Krull et al. 2003), and a specific role of CDK6 during the differentiation of a variety of cell types (Ericson, Krull et al. 2003; Matushansky, Radparvar et al. 2003; Ogasawara, Katagiri et al. 2004; Ogasawara, Kawaguchi et al. 2004). Moreover, CDK6 plays a role in halting inappropriate cellular proliferation through a mechanism involving the accumulation of p53 and p130 growth suppressing proteins (Nagasawa, Gelfand et al. 2001), and the activation of CDK6 precedes CDK4 activation in T cells (Lucas, Terada et al. 1992; Lucas, Szepesi et al. 1995; Lucas, Szepesi et al. 1995). Data from tumor studies also suggested similarity and difference between these kinases. For instance, both CDK4 and CDK6 have been found overexpressed in human Gliomas (Schmidt, Ichimura et al. 1994; Ichimura, Schmidt et al. 1996; Costello, Plass et al. 1997; Lam, Di Tomaso et al. 2000). On the other hand, CDK4, but not CDK6, is specifically targeted in melanomas (Wolfel, Hauer et al. 1995; Wölfel, Hauer et al. 1995; Zuo, Weger et al. 1996), whereas CDK6 activity has been found elevated in squamous cell carcinomas (SCCs) (Timmermann, Hinds et al. 1997; Piboonniyom, Timmermann et al. 2002) and neuroblastomas (Easton, Wei et al. 1998) without alteration of CDK4 activity.

The two-stage mouse skin carcinogenesis is a well-suited model for understanding the multistage nature of tumor progression. In this model, tumor initiation is accomplished
through a single topical application of a carcinogen, typically 7,12-
dimethylbenz(a)anthracene (DMBA). This produces a genetic inheritable mutation in the
Ha-ras oncogene. Tumor promotion takes place when the initiated cells are expanded due
to multiple applications of a tumor promoter, usually 12-O-tetradecanoylphorbol-13-
acetate (TPA). This stimulus induces hyperproliferation that promotes the generation of
benign tumors, so-called papillomas. Finally, although papilloma regression is a common
event, in some cases malignant progression occurs and papillomas evolve to squamous cell
carcinomas (SCC).

In the last few years, we and others have used the mouse skin model to study the role
of positive and negative regulators of cell cycle in normal and neoplastic proliferation
(Zhang, Liu et al. 1997; Zhang, Liu et al. 1997; Rodriguez-Puebla, LaCava et al. 1998; Rodriguez-Puebla, LaCava et al. 1999; Rodriguez-Puebla, LaCava et al. 2000; Miliani de
Marval, Gimenez-Conti et al. 2001; Rodriguez-Puebla, Miliani de Marval et al. 2002; Miliani
de Marval, Macias et al. 2004; Miliani de Marval, Macias et al. 2004; Macias, Kim et al.
2007; Rojas, Cadenas et al. 2007; Macias, Miliani de Marval et al. 2008; Rojas, Benavides et
al. 2009). Work from our group and other laboratories have shown that CDK4 is
mechanistically involved in the development of human and experimental epidermal tumors
(Rodriguez-Puebla, Miliani de Marval et al. 2002; Zou, Ray et al. 2002; Miliani de Marval,
Macias et al. 2004; Miliani de Marval, Macias et al. 2004; Yu, Sicinska et al. 2006; Macias,
Kim et al. 2007). These studies showed that forced expression of CDK4 in epidermis results
in increased malignant progression to SCCs (Miliani de Marval, Macias et al. 2004), whereas

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CDK4 ablation completely inhibit skin tumor development (Rodriguez-Puebla, Miliani de Marval et al. 2002). On the other hand, the overexpression of CDK2 or the indirect activation of CDK2 in mouse epidermis induced keratinocyte proliferation but does not affect skin tumor development (Macias, Kim et al. 2007; Macias, Miliani de Marval et al. 2008). Despite the evidence that G1-CDKs are involved in proliferation and tumorigenesis, the actual role of CDK6 in epidermis has not been established. Therefore, to study the role of CDK6 in epithelial growth, differentiation, and tumorigenesis, we generated transgenic mice carrying the CDK6 gene under the control of the keratin 5 promoter (K5CDK6). As expected, transgenic mice showed expression of CDK6 in the epidermal basal cell layer. Analogous to K5CDK4 mice, epidermal proliferation increased substantially in K5CDK6 mice, although no hyperplasia was observed. Interestingly, the overexpression of CDK6 also results in augmented apoptosis in inter follicular epidermis and hair follicle. Biochemical analysis of K5CDK6 epidermal tissues showed increased CDK6 kinase activity with no effect on CDK4 and CDK2 kinase activities, suggesting that sequestration of p27^Kip1 and indirect activation of CDK2 is not a relevant mechanism in K5CDK6 epidermis. We have also studied the susceptibility of K5CDK6 mice to the two-stage chemical carcinogenesis protocol. Surprisingly, we found that forced expression of CDK6 leads to decreased skin tumor development. Moreover, skin tumors from K5CDK6 mice show no progression to squamous cell carcinomas, as we previously observed in K5CDK4 mice. These results mimic the effect of cyclin D3 overexpression in K5-Cyclin D3 mouse epidermis, (Rojas, Cadenas et al. 2007), which also showed reduced tumorigenesis, suggesting that CDK6/cyclin D3 complexes might
play an important role in tumor inhibition. Supporting this hypothesis, biochemical analysis of the K5CDK6 epidermis showed preferential formation of CDK6/cyclin D3 complexes. Overall, we have established that the development of ras-induced skin tumors is diminished by overexpression of CDK6, which result in a surprisingly opposite effect to that observed in K5CDK4 mice. Thus, this model provides in vivo evidence that CDK4 and CDK6 play a similar role as mediator of keratinocyte proliferation, but differ in the activated mechanisms, leading to an opposite effect in tumor development. As a result, we hypothesize that particular CDK/cyclin D complexes play differential roles in epidermis homeostasis and tumor development.

2.2 Materials and methods

2.2.1 Generation of Transgenic Mice

K5Cdk6 transgenic mice were developed by cloning human-Cdk6 cDNA into the vector pBK5, which contains a 5.2-kb bovine keratin 5 regulatory sequences, β-globin intron 2, and the 3’ SV40-polyadenylation sequences. These construct was designated as pK5-CDK6. The transgene was excised from the plasmid vector by digestion with BssHII and microinjected into C57BL/6 x DBA2 hybrid embryos at the Animal Model Core, University of North Carolina School of Medicine. Several founders for K5Cdk6 were obtained from the transgenic facility. Positive founders were genotyped by polymerase chain reaction (PCR) using specific primers for the human transgenes Cdk6 and β-globin intron sequence. Mice used in this
study were generated by mating transgenic and wild type animals for five to seven generations.

2.2.2 Transgene-Specific PCR

Genomic DNA was extracted from mouse tail clips and used for PCR detection of the transgene. We used an upstream primer CTGACCAGCAGTACGAATG and a downstream primer GAGTCCAATCAGTCCAAG specific for the β-globin intron sequence or upstream CTGACCAGCAGTACGAATG and downstream TTTCTTTGCACCTTTCCAGG primers for human CDK6. With this process, we screened all of the transgenic mice lines. The DNA amplification renders a 450-bp PCR product with β-globin primers or an 850-bp band with human-CDK6 primers. PCR was performed by denaturation at 95°C for 1 minute, followed by 32 cycles of amplification as follows: denaturation at 95°C for 30 seconds, annealing at 60°C for 40 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 10 minutes.

2.2.3 Western Blotting

The dorsal sides of the mice were shaved. After they were sacrificed, the dorsal skins were treated with a depilatory agent for 1 minute and then washed. The epidermal tissue was scraped off with a razor blade, placed into homogenization buffer [50mmol/L HEPES, pH7.5, 150mmol/L NaCl, 2.5mmol/L EGTA, 1mmol/L EDTA acid, 0.1% Tween-20, 1 mmol/L dithiothreitol, 0.1 mmol/L phennylmethyl sulfonyl fluoride (PMSF), 0.2 U/ml aprotinin, 10 mmol/L b-glycerophosphate, 0.1 mmol/L sodium vanadate, and 1mmol/NaF], and
homogenized using a manual homogenizer. The epidermal homogenate was centrifuged at 14000 x g at 4°C to collect the supernatant, which was used directly for western blotting analysis or stored at -80°C. The protein concentration was measured with the Bio-Rad protein assay system (Bio-Rad laboratories, Richmond, CA). Protein lysates (25µg from each sample) were electrophoresed through 12% acrylamide gels and electrophoretically transferred onto nitrocellulose membranes. After being blocked with 5% nonfat powdered milk in Dulbecco PBS, the membranes were incubated with 1 µg/ml of specific antibodies. The following antibodies were used: polyclonal antibodies against cyclin D2 (M20), CDK4 (C22), CDK2 (M2), CDK6 (C21), pRb (M153), p107 (C18) (Santa Cruz Biotech., Santa Cruz, CA), p53 (1C12) (Cell Signaling tech, Inc., Boston, MA), and monoclonal antibodies against cyclin D1 (DCS-6), CDK6 (DCS-83) (Santa Cruz Biotech). Secondary antibodies followed by enhanced chemiluminescence (ECL detection kit, GE Healthcare., Piscataway, NJ) were used for immunobloting detection.

2.2.4 Co-Immunoprecipitations and Kinase assays

To study CDK/D-type cyclin complex formations and kinase activities, we used polyclonal antibodies against CDK4 (C-22), CDK6 (C-21) (Santa Cruz Biotech, Santa Cruz, CA), and a monoclonal antibody against cyclin D3 (Ab-1) (NeoMarkers, Fremont, CA) conjugated with protein A-sepharose beads (Thermo Scientific, Inc., Rockford, IL) or Dynabeads® Protein G (Invitrogen, Carlsbad, CA). Fresh protein lysates from epidermal tissue (500 µg) were immunoprecipitated for 1 hour at 4°C with constant rotation. After washing three
times with extraction buffer, proteins that co-immunoprecipitated were analyzed by Western blot as described previously. Protein lysate (50 µg) was loaded as control input. The immunoprecipitation was repeated three times using 250, 500 or 1,000 mg of protein lysate with identical results.

To study the kinase activities, 500 µg of fresh protein extracted and immunoprecipitated in NP-40 lysis buffer (Tris [pH 7.5], 150 mmol/L NaCl, 0.5% NP-40, 50 mmol/L NaF, 1 mmol/L Na₂VO₄, 1 mmol/L DTT, 1 mmol/L PMSF) with precoated antibodies against CDK2, CDK4, and CDK6 for 2 hours at 4°C. Beads were washed twice each with NP-40 buffer and once with kinase buffer (50 mmol/L HEPES [pH 7], 10 mmol/L MgCl₂, 5 mmol/L MnCl₂). Then, 30 µl of kinase buffer, 1 µg of pRb or histone H1 (Upstate Biotechnology Inc., Charlottesville, VA) substrate, 5 µCi of [γ-³²P]ATP (6,000 Ci/mmol), 1 mmol/L DTT, and 5 µmol/L ATP was added to the bead pellet and incubated for 30 minutes at 30°C. SDS sample buffer was added, and each sample was boiled for 3 minutes to stop reaction and electrophoresed through polyacrylamide gels. Western blot and kinase assay bands were quantified using UN-SCANT IT gel software for Windows.

2.2.5 Immunostaining

Epithelial cell proliferation was measured by intraperitoneal injection of 60 µg/g of 5-bromodeoxyuridine (BrdU) 30 minutes before the mice were sacrificed by CO₂ asphyxiation. BrdU incorporation was detected by immunohistochemical staining of paraffin-embedded skin sections with a mouse anti-BrdU (ab-2) monoclonal antibody (Calbiochem, EMB
Biosciences, San Diego, CA), biotin-conjugated anti-mouse antibody (Vector Laboratories, Inc., Burlingame, CA), and avidin-biotin Vectastain Elite peroxidase kit (Vector Laboratories, Inc.) with diaminobenzidine as a chromogen. Apoptotic cells were determined by terminal deoxynucleotidyl transferase mediated dUTP nick-end labelling assays (TUNEL assays) with the FragEL DNA Fragmentation Detection Kit, Colorimetric-TdT enzyme (Calbiochem, EMB Biosciences Inc.) following the manufacture instruction. Briefly, the terminal deoxynucleotidyl transferase (TdT enzyme) binds to exposed 3-OH ends of DNA fragment generated in apoptosis progression and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotides were detected using a streptavidin-horseradish peroxidase conjugate. Counterstaining with methyl green allows for quantification of normal and apoptotic cells. The number of apoptotic cells in the tumors were determined in sections of 250 μm² with a reticule grid. Apoptotic keratinocytes in inter follicular and follicular epidermis were quantified in 2cm sections. To determine the incidence of follicular apoptosis, hair follicles carrying at least 1 apoptotic cell in the bulge were counted as positive hair follicle. In all cases, 12 fields were counted per section on a total of 10 paraffin-embedded sections, representing 5 mice per genotype.

2.2.6 Mouse experiments

Two K5-CDK6 transgenic lines were used for the two-stage carcinogenesis protocol. Mouse experiments were performed with sibling animals to reduce the influence of the genetic background. Eight mice for each transgenic and wild-type groups were used
[K5CDK6(H), K5CDK6(L), Wild type(H) and Wild type(L)] for a total of 16 transgenic and 16 wild-type siblings. Three-week-old K5CDK6 and wild-type mice were initiated with 200 nmol DMBA in 200 µl of acetone on the dorsal surface of the mice. Two weeks later, mice were dosed topically twice weekly with 4.19 µg of TPA in 200 µl of acetone for 25 weeks. Papilloma development was tracked weekly for 25 weeks. Papillomas were counted if 1 mm or larger. Multiplicity and incidence of tumor-bearing animals were compared between K5CDK6 and wild-type mice using Fisher’s exact test.

2.2.7 Statistical analysis

Statistical analysis was done using GraphPad Prism 4 Software (GraphPad Software Inc., San Diego, CA).

2.3 Results

2.3.1 Generation of Transgenic Mice Expressing CDK6

To study the role of CDK6 in squamous epithelial tissues, we generated transgenic mice overexpressing CDK6 driven by the regulatory sequence of the keratin 5 promoter (K5CDK6 mice). The K5CDK6 construct was made by subcloning the human CDK6 cDNA into pBK5 vector containing the 5.2-kb fragment of the bovine keratin 5 promoter (K5), the rabbit β-globin intron 2, and the SV40 3’ polyadenylation signal (Figure 3A). The K5 promoter fragment directs transgene expression to the basal cell compartment of stratified squamous
epithelia, which was shown previously (Ramirez, Bravo et al. 1994). All of the transgenic mice were generated on the hybrid genetic background C57BL/6xDBA2. The genotypes of transgenic mice were characterized by PCR analysis using primers specific for the β-globin and human CDK6 sequences. Four integration-positive mice were selected as founders based on those results and crossed with wild-type siblings to generate four transgenic lines (Figure 3B). A second screening to verify transgene expression was performed by Western Blot analysis of epidermal preparations as described (Rodriguez-Puebla, Robles et al. 1998) (Figure 4A). We observed 5.7-, 4.7-, 2.7- and 5.4-fold increase in CDK6 expression in the transgenic lines A through D, respectively (Figure 4A). Therefore, the transgenic lines A and C, with high and low CDK6 expression, were renamed as K5CDK6(H) and K5CDK6(L) respectively and were used in all of the experiments presented in this chapter (Figure 4A, lines A and C). In addition, we performed immunofluorescence analysis of CDK6 expression in mouse epidermis from K5CDK6(H) and K5CDK6(L) mice. As expected, we observed that CDK6 expression was driven to the basal cell layer of inter follicular epidermis and hair follicle (Ramirez, Bravo et al. 1994) (Figure 4B).

2.3.2 CDK6 Overexpression induces Epidermal Hyperproliferation but not Hyperplasia

The newborn K5CDK6 transgenic mice did not display any obvious developmental abnormalities, and there was no differences in size and weight compared to wild-type
**Figure 3.** pK5-CDK6 construct and PCR screening. (A) Diagram of the K5CDK6 construct. (B) PCR amplification of DNA extracted from mouse tails. CDK6 transgene was amplified resulting in 850-bp product.
Figure 4. Western blot analysis and immuno-localization of transgene expression. (A) Expression and quantification of CDK6 in epidermis from each of the four mouse lines (A-D) and wild-type littermates shows elevated level of CDK6 protein. (B) CDK6 expression was detected by immunofluorescence analysis in paraffin cross sections of wild type (a) and K5CDK6 (b) mice.
littermates. To determine whether the expression of CDK6 transgene influenced the rate of keratinocyte proliferation and/or the architecture of mouse skin, we performed a histopathological analysis of the epidermis of transgenic and wild-type siblings. Analysis of hematoxylin and eosin (H&E) staining on paraffin-embedded sections showed no obvious modifications in the morphology of follicular and inter follicular epidermis from transgenic mice compared with wild-type littermates (Figure 5A). However, the proliferation status of keratinocytes, determined by BrdU incorporation, showed a two-fold increase in the number of proliferating cells in the epidermis of transgenic mice compared with wild-type mice in both K5CDK6(H) (Figure 5) and K5CDK6(L) (data no shown) transgenic lines (p=0.0005; t-test). Thus, we hypothesize that another mechanism compensates for the increase keratinocyte proliferation observed in the K5CDK6 epidermis, resulting in no change in the epidermis structure. Quantification of the number of apoptotic cells showed a two-fold raise in inter follicular epidermis from K5CDK6 compared to wild type siblings (p=0.004; t-test) (Figure 5B). Importantly, the apoptotic cells were localized in the basal cell layer of inter follicular epidermis, suggesting that apoptosis can compensate for the increase number of proliferative basal keratinocytes of K5CDK6 epidermis. It is worth mentioning that hair follicles do not contribute to homeostasis of mouse epidermis; however hair follicle stem cells localize in bulge area seem to participate in epidermis homeostasis in hyperproliferative conditions, such as wound healing (Ito, Liu et al. 2005). The fact that K5CDK6 mice showed altered keratinocyte proliferation leads us to study whether apoptosis in hair follicle can also contribute as a compensatory mechanism.
Therefore, hair follicles carrying at least one apoptotic cell in the bulge were counted to determine the incidence of apoptosis in hair follicles. We observed a two-fold increase in the number of hair follicles carrying apoptotic cells in K5CDK6 mice compared with wild-type siblings (p<0.0001, t test) (Figure 5B). Therefore, increased apoptosis in both interfollicular epidermis and hair follicles can play an important role in K5CDK6 epidermis homeostasis.

Moreover, we studied whether overexpression of CDK6 affects the pattern of epidermal differentiation by using keratin 5 and keratin 1 immunostaining. During epidermal differentiation there are sequential changes in the expression of the keratins. Keratin 5 and keratin 14 are the major products of basal epidermal cells, the proliferative compartment of the epidermis, whereas keratin 1 and 10 are associated with the commitment to differentiation and migration into the spinous layer (Woodcock-Mitchell, Eichner et al. 1982; Roop, Hawley-Nelson et al. 1983). Figure 6 shows no differences in the pattern of expression of keratin 5 and 1 between K5CDK6 and wild-type siblings. The expression of keratin 5 was confined to the basal layer of interfollicular epidermis and hair follicles, whereas keratin 1 was restricted to terminally differentiated cells.

Therefore, these results suggest that forced expression of CDK6 in epidermis does not affect the morphology of epidermis or epidermal differentiation as a result of the counteraction between both increased proliferation and elevated apoptosis.
**Figure 5.** Skin phenotype of K5CDK6 transgenic mice. (A) Representative paraffin-sections of skin from high expression transgenic K5CDK6(H) (b) and low expression transgenic K5CDK6(L) (d) and the respective normal siblings (a, c) were staining with H&E. BrdU immunostaining of K5CDK6(H) (f) and wild type sibling (e). (B) Number of nucleated cells (a), Quantification of BrdU label index (b), and apoptosis (c) in inter follicular epidermis and percentage of hair follicles with at least one apoptotic cell in the bulge area (d). Shaded bars, K5CDK6(H) transgenic mice; open bars, normal siblings (wt).
**Figure 6.** Keratin expression in K5CDK6 transgenic and normal sibling mice. Expression of keratin 1 (K1) and keratin 5 (K5) was determined on representative paraffin sections of skin from K5CDK6 transgenic and wild type sibling. Specific antibodies against keratin 5 and keratin 1. Right lane, merge of CDK6 expression (green) and DAPI (blue) of the respective image on the left.
2.3.3 Biochemical Analysis of K5CDK6 Mouse Epidermis

To study whether the expression of CDK6 affects associated cell-cycle regulators, we assessed the protein levels of CDKs, cyclin-dependent kinase inhibitors, and cyclins. CDK4 and CDK6 have common functional and biochemical properties; thus, we analyzed whether CDK4 expression is affected as a compensatory mechanism for the increased expression of CDK6. However, Western blot analysis showed no changes in protein expression for CDK4 and CDK2 (Figure 7A). As regulatory subunits of CDK4 and CDK6, D-type cyclins are rate-limiting controllers of G1 phase progression, but again no changes in protein levels of cyclin D1, cyclin D2 and cyclin D3 were observed (Figure 7A). The pRb family members are negative regulators that act in the G1 phase and are the main substrates of CDK4,6/D-type cyclins complexes; however, we did not detect changes in protein levels or mobility consistent with phosphorylation (Figure 7A).

Modification of the protein level of D-type cyclins or CDKs change the kinetics of complex formation in mouse epidermis. In fact, we have previously demonstrated that CDK4 binds preferentially to cyclin D1, whereas CDK6 binds to both cyclin D1 and D3 in transgenic mice overexpressing cyclin D3 (Rodriguez-Puebla, Robles et al. 1998). In addition, epidermis from K5-Cyclin D3 transgenic mice showed elevated CDK6 and CDK4 kinase activity, mainly associated with CDK4,6/Cyclin D1 and CDK6/Cyclin D3 complexes (Rojas, Cadenas et al. 2007). Therefore, we analyzed D-type cyclin/CDKs complex formation and the in vitro associated kinase activities. Epidermal lysates from K5CDK6 and wild-type mice were immunoprecipitated with antibodies against CDK6 and CDK4 followed by Western Blot
analysis to determine association with D-type cyclins. We found that forced expression of CDK6 resulted in elevated CDK6/Cyclin D3 complex formation with minimum binding to cyclin D1 and D2 (Figure 8). We quantified the expression level of CDK6, cyclin D1, cyclin D2 and cyclin D3 in the protein lysates (input) and in immunoprecipitated samples to determine the ratio of input to immunoprecipitation (Figure 8). Therefore, we established that most of the cyclin D3 proteins bind to CDK6, whereas 2% to 4% of the cyclin D1 and cyclin D2 bind to CDK6 in K5CDK6 epidermis. The fact that CDK6 is not expressed to high levels in wild-type epidermis does not allow verification of the ratio of D-type cyclin/CDK6 complex formation in wild-type mice.

We also established that the overexpression of CDK6 does not modify CDK4/D-type cyclin or CDK4/p27^Kip1 complex formation, both of which are similar between K5CDK6 and wild-type littermates (Figure 8). The preferential binding of CDK6 and cyclin D3 was confirmed by reverse co-immunoprecipitation assay in which cyclin D3 immunoprecipitation was follow by Western blot analysis to detect CDK6 (data non-shown). To study whether the overexpression of CDK6 resulted in functional changes in the CDKs, we performed an in vitro analysis of the CDK6, CDK4 and CDK2 kinase activities in epidermis lysates from transgenic and wild-type mice using pRb and histone H1 (H1) as substrates. As expected, CDK6 activity increased 2.5-fold in K5CDK6 transgenic mice compared with wild type siblings, whereas no modification in the level of CDK4 and CDK2 activities were observed (Figure 7B). We conclude that forced expression of CDK6 does not lead to changes in other
Figure 7. Biochemical analysis of cell-cycle regulators in epidermis of K5CDK6(H) transgenic and wild type (wt) mice. (A) Epidermal lysates were separated by SDS-PAGE and blocked onto nitrocellulose membrane. Primary antibodies against CDK2, CDK4, CDK6, cyclin D1, D2, D3, pRb and p107 were used for immunoblot analysis. Actin used as loading control. (B) Kinase activity of CDK6, CDK4 and CDK2 from K5CDK6(H) and wild type mice. Fresh epidermal lysates were immunoprecipitated with specific antibodies against CDKs and in vitro kinase assays were performed with pRb or Histone H1 (H1) peptides as substrates.
**Figure 8.** Co-immunoprecipitation assay of cyclin D/CDK4,6 complexes in epidermis of K5CDK6(H) transgenic and wild type (wt) mice. Epidermis lysates from K5CDK6(H) and wild type (wt) siblings were immunoprecipitated (IP) with CDK6 or CDK4 antibodies and blotted with antibodies against cyclins D1, D2, D3, CDK6, CDK4 and p27Kip1. Protein lysates from wt and K5CDK6 epidermis were load as input. Bands of CDK6, cyclin D1, cyclin D2 and cyclin D3 were quantified in the input and IP and the ratio for each individual protein was calculated as IP/input.
G1-phase CDKs or their regulatory subunits. Therefore, the effect of CDK6 expression in keratinocyte proliferation and apoptosis is not due to modification on protein levels or kinase activities of G1 phase other than CDK6.

2.3.4 CDK6 overexpression reduces skin tumor development

According to the current model of cell proliferation, aberrant levels of a positive regulator of cell-cycle provides a growth advantage that can result in increased tumor development. Supporting this model, we showed that forced expression of CDK4 results in increase malignant progression to skin SCCs in a two-stage carcinogenesis protocol (Miliani de Marval, Macias et al. 2004). This protocol induces skin papillomas development by a single application of a carcinogen followed by biweekly treatment with a tumor promoter causing a selection of cells bearing Ha-ras mutations. To evaluate the role of CDK6 in skin tumorigenesis, we assessed the response of K5CDK6(H) and K5CDK6(L) transgenic mice to the two-stage carcinogenesis protocol.

The dorsal skin of K5CDK6 transgenic mice and wild-type littermates were topically treated with a subcarcinogenic dose of the genotoxic carcinogen DMBA and later promoted with TPA for 25 weeks. The incidence and multiplicity of papillomas were scored in each group for 25 weeks. Papilloma development was delayed 2 to 3 weeks in both K5CDK6 transgenic lines compared with the respective wild type siblings (Figure 9A). The incidence of papilloma formation reached a plateau of 100% at ~17-19 weeks in wild-type siblings. In contrast, K5CDK6(L) mice reached 90% of papilloma incidence and only 60% of K5CDK5(H)
mice developed papillomas, showing that high level of CDK6 expression correlates with increase inhibition of tumor development (Figure 9A). Tumor multiplicity (mean number of tumors per mouse) clearly shows a reduced number of tumors in both K5CDK6 transgenic lines compared with their respective control littermates throughout the experiment. On an interesting note, we established an inverse correlation between CDK6 expression and number of skin tumors. Although the high expression transgenic line (K5CDK6[H]) developed fewer skin tumors (a 75% reduction compared with wild-type siblings), the low expression transgenic line (K5CDK6[L]) showed 50% decrease in the number of papillomas per mouse (Figure 9A).

Biochemical analysis of skin tumors indicates that overexpression of CDK6 is maintained during tumorigenesis (Figure 10). Although certain heterogeneity was observed in CDKs protein levels, no effect on CDK4 and CDK2 expression was observed in transgenic versus wild type tumors. It has been shown that CDK6 plays a role halting cellular proliferation through accumulation of the growth suppressors p53 and p130 (Nagasawa, Gelfand et al. 2001). Nevertheless, immunoblot analysis of p53 and p130 in epidermis and skin papillomas did not show differences between K5-CDK6 and wild type siblings (data non-shown). These datas are consistent with the observations of Ruggeri et al showing that the p53 gene appeared normal in all papillomas and early well-differentiated carcinomas, whereas p53 alterations were observed in squamous cell carcinomas (Ruggeri, Caamano et al. 1991). Histopathological analysis were performed on skin tumors that had undergone 25 weeks of promotion. No differences were observed between K5CDK6 and wild-type tumors,
Figure 9. Kinetics of papilloma formation and biochemical analysis of K5-CDK6 tumors. K5CDK6 transgenic and wild-type sibling mice were initiated with DMBA and promoted with the multiple applications of TPA on dorsal mouse skin. Average number of papillomas per mouse (multiplicity) as function of weeks of study in K5CDK6(H) (A) and K5CDK6(L) (C). Percentage of mice with at least one papilloma as a function of weeks of study (incidence) in K5CDK6(H) (B) and K5CDK6(L) (D).
Figure 10: Immunoblot analysis of wild type (wt) and K5CDK6(H) papilloma lysates developed with antibodies against CDK6, CDK4, CDK2 and actin as loading control.
Figure 11. Increase apoptosis and keratinocyte proliferation in mouse skin tumors. BrdU incorporation in papillomas from wild type (A) and K5CDK6 (B) siblings. Apoptotic keratinocytes in skin papillomas from wild type (C) and K5CDK6 (D) mice. Quantification of BrdU label index (E), and apoptosis (F) in skin papillomas.
and all of them were classified as well-differentiated papillomas with no atypia in basal layers. However, immunostaining analysis showed a two-fold increase in keratinocyte proliferation in papillomas from K5CDK6 mice compared with wild-type littermates (p<0.0001, t-test) (Figure 11). Moreover, we also observed 1.5-fold increase in the number of apoptotic cells in K5CDK6 papillomas compared with wild-type tumors (p=0.001, t-test) (Figure 11). Therefore, forced expression of CDK6 increase keratinocyte proliferation in skin tumors, but similar to normal epidermis CDK6 expression also induces apoptosis.

Collectively, these observations show that, although counterintuitive, overexpression of CDK6 does not result in advantage leading to increased carcinogenesis or increased malignant progression but decrease in papilloma development in ras-dependent tumorigenesis.

2.4 Discussion

For more than two decades, the pRb/p16/Cdk/Cyclin pathway has been implicated in proliferation and tumorigenesis. The fact that both CDK4 and CDK6 have the same substrates, bind to D-type cyclins, and share 71% of amino acid identity leads to the assumption that they play a redundant role in G1 phase. However, in vivo studies partly support a redundant function for these kinases. For instances, CDK4<sup>−/−</sup> mice show growth retardation, reproductive dysfunction associated with defects in seminiferous tubules and corpus luteum, and insulin-deficient diabetes due to a reduction in β-islet pancreatic cells
(Rane, Dubus et al. 1999; Tsutsui, Hesabi et al. 1999). None of these phenotypes were observed in CDK6-deficient mice, which show pronounced thymic atrophy because of the reduction in cell proliferation and the reduced susceptibility to lymphomagenesis (Hu, Deshpande et al. 2009). These results demonstrated that lack of CDK6 or CDK4 affected a different spectrum of tissues and argue against a redundant and compensatory function in those organs. On the other hand, Malumbres et al showed that mice lacking both CDK4 and CDK6 died during embryonic development, supporting the hypothesis of functional compensation between these proteins (Malumbres, Sotillo et al. 2004). Studies performed with experimental and human tumors also suggested similarities and differences between these kinases. For instance, both CDK4 and CDK6 are overexpressed in human gliomas (Schmidt, Ichimura et al. 1994; Ichimura, Schmidt et al. 1996; Costello, Plass et al. 1997; Lam, Di Tomaso et al. 2000). In contrast, CDK4, but not CDK6, is specifically targeted in melanomas (Wölfel, Hauer et al. 1995; Zuo, Weger et al. 1996), whereas CDK6, but not CDK4, activity has been found elevated in SCCs and neuroblastomas (Timmermann, Hinds et al. 1997; Easton, Wei et al. 1998; Piboonniyom, Timmermann et al. 2002).

### 2.4.1 Role of CDK6 in Keratinocyte proliferation, differentiation and apoptosis

Several groundbreaking works in cell culture and in in vivo models have shown that activation of CDK4 and CDK6 is essential for responses to extracellular mitogenic signaling and the progression beyond the restriction point in G1 phase. Our earliest studies have shown that G1-CDKs were differently regulated in mouse epidermis. Whereas CDK4 and
CDK2 remain at constant levels, CDK6 is up-regulated in mouse epidermis upon TPA-induced proliferation, leading to increase CDK6/Cyclin 3 and CDK4/Cyclin D1 complexes (Rodriguez-Puebla, Robles et al. 1998). In this chapter, we show that similar to K5CDK4 transgenic mice, forced expression of CDK6 in mouse epidermis results in increased keratinocyte proliferation, but contrary to K5CDK4, no epidermal hyperplasia was observed. Interestingly, CDK6 expression resulted in an elevated number of apoptotic keratinocytes in inter follicular and follicular epidermis, suggesting that apoptosis behaves as a compensatory mechanism for unrestricted proliferation. This compensatory mechanism was only elicited in K5CDK6 keratinocytes because neither K5CDK4 nor K5CDK2 epidermis showed increase number of apoptotic cells (Miliani de Marval, Gimenez-Conti et al. 2001; Macias, Miliani de Marval et al. 2008). CDK6 overexpression does not result in increased CDK2 activity as was determined in epidermis and papillomas from K5CDK4 mice (Miliani de Marval, Gimenez-Conti et al. 2001; Miliani de Marval, Macias et al. 2004). Therefore, the increased apoptosis observed in the K5CDK6 mouse epidermis seems to be independent of the role of CDK2 mediating apoptosis (Gil-Gomez, Berns et al. 1998; Choi, Eom et al. 1999; Hakem, Sasaki et al. 1999; Maddika, Ande et al. 2008).

Here, we have determined that increased CDK6 activities were associated with elevated CDK6/Cyclin D3 complex formation. Thus, one might speculate that this particular complex plays an important role in epidermis homeostasis by keeping inappropriate proliferation in check. Therefore, we hypothesize that CDK6/Cyclin D3 complex inactivate pRb and/or p107, inducing apoptosis by a similar mechanism exhibited by lack of pRb (Ruiz,
Santos et al. 2005). Although we did not observe changes in pRb or p107 phosphorylation status in epidermal extract from K5CDK6 mice, in vitro kinase assays clearly showed increase CDK6 activity against a pRb peptide. Taken together, these results suggest that forced expression of CDK6 induces apoptosis as a compensatory mechanism for keratinocyte hyperproliferation. The molecular mechanism by which CDK6, but not CDK4, stimulate apoptosis in mouse epidermis remains unclear and is beyond the scope of this study, but suggests that CDK6 may play a role in halting cellular growth when proliferation is inappropriate.

In the last few years, several alternative roles for CDK6 blocking cell differentiation and/or inducing cell proliferation has been described (Grossel and Hinds 2006; Grossel and Hinds 2006; Nerlov 2007). For instance, CDK6 disrupts C/EBP-Runx1 interaction and Runx1 DNA binding, leading to blocked myeloid differentiation (Fujimoto, Anderson et al. 2007). Moreover, Bone Morphogenetic Protein-2 (BMP2)-induced osteoblast differentiation requires down-regulation of CDK6 (Ogasawara, Kawaguchi et al. 2004). The ability of CDK6 to interfere with Runx-C/EBP cooperation and control terminal differentiation might apply to several cell types. Thus, we hypothesized that overexpression of CDK6 can alter the pattern of keratinocyte differentiation. However, immunofluorescence analysis of keratin 5/keratin 1 distribution in basal and suprabasal cell layers of K5CDK6 mice showed no modifications in the pattern of expression, suggesting no alterations in keratinocyte differentiation. Consistent with our data, changes in keratinocyte differentiation or proliferation by ablation of CDK6 in mouse models have not been reported (Malumbres,
Sotillo et al. 2004; Hu, Deshpande et al. 2009). Interestingly, Hoi et al have recently reported that Runx1 directly promotes proliferation of Hair Follicle Stem Cells (HFSCs) and epithelial tumor formation in mouse skin (Hoi, Lee et al. 2010). Therefore, whether CDK6 disrupt the Runx1 role on HFSCs, leading to changes in keratinocyte proliferation and a reduction in tumor formation, warrants further investigation.

2.4.2 CDK6 in Tumor development

Our early studies established that CDK4 and CDK6 remain at constant levels in mouse papillomas and increased expression of D-type cyclin drive Cyclin/CDK complex formation and CDK activity in skin tumorigenesis (Rodriguez-Puebla, LaCava et al. 1998). In vivo studies demonstrated that transgenic expression of CDK4 results in increased epidermal proliferation, epidermal hyperplasia and enhanced malignant progression to SCCs in a two-stage chemical carcinogenesis model (Miliani de Marval, Gimenez-Conti et al. 2001; Miliani de Marval, Macias et al. 2004). We also showed that the role of CDK4 in skin carcinogenesis partly depends on CDK2 activation through sequestration of p27Kip1 and p21Cip1 by CDK4 (Miliani de Marval, Gimenez-Conti et al. 2001; Macias, Kim et al. 2007). An important role of CDK4 in mouse epidermal tumorigenesis was further supported by the fact that ablation of CDK4 leads to reduction of skin tumorigenesis (Rodriguez-Puebla, Miliani de Marval et al. 2002). These results and the convincing evidence showing that disabling the pRb pathway is essential for tumor formation, led us to hypothesize that elevated CDK6 kinase activity would also enhance ras-mediated tumor development. Surprisingly, we found that elevated
CDK6 kinase activity did not increase tumor development or affect malignant progression to SCC. In fact, K5CDK6 transgenic mice developed a lower number of tumors per mouse, and an increased fraction of the K5CDK6 mice remained refractory to tumor development. Importantly, the inhibitory action of CDK6 was dose dependent because the high expression K5CDK6(H) transgenic line showed enhanced resistance to papilloma development compared with K5CDK6(L) transgenic line. We also analyzed the rate of malignant conversion to SCC, but changes were not observed in K5CDK6 mice compared with wild type siblings. These results are clearly opposite of the increased malignant progression observed in K5CDK4 mice under the same protocol (Miliani de Marval, Macias et al. 2004). Similar to K5CDK6 model, ablation of pRb in mouse epidermis results in reduced number of papillomas and increased apoptosis (Ruiz, Santos et al. 2005). The similar effect in skin carcinogenesis noted in K5CDK6 and pRb−/+ mice contrasted with that of K5CDK4 mice. Thus, we hypothesize that CDK6- or CDK4-mediated phosphorylation and inactivation of pRb family members are responsible for the different effects in papilloma development. Therefore, the following hypotheses merit future investigations to understand the different effects of CDK4 and CDK6 expression in mouse epidermis. First, differences in residue selectivity for pRb phosphorylation by CDK6 and CDK4 might induce a different rate of pRb inactivation, leading to increased apoptosis and, consequently, reduction in tumorigenesis in K5CDK6 mice (Takaki, Fukasawa et al. 2005). Second, CDK6 might preferentially phosphorylate pRb, but not p107, which in turn will inhibit tumor development. Remarkably, reduced expression of p107 in pRb−/-;p107+/− compound mice led to partial restoration in
the incidence, number, and size of tumors (Santos, Ruiz et al. 2008). Another alternative hypothesis to be tested should include pRb-independent mechanisms. It is known that chromosome replication is a highly regulated mechanism which seems to be mainly regulated by the assembly of the Mcm2-7 complexes onto replication origins. This mechanism depends on the CDK levels to allow licensing DNA duplication; however, the roles of each member of the CDK family have not been clearly established (Blow and Hodgson 2002; Porter 2008). Importantly, Braden et al have recently shown that CDK4 and CDK6 activities are critical determinants of pre-replication complex assembly by allowing the accumulation of the licensing factors cdc6 and cdt1 (Braden, McClendon et al. 2008). Therefore, determining whether G1-CDKs play a unique or shared role during DNA duplication will allow us to establish whether CDK6 up-regulation affects DNA duplication and further triggers the apoptotic pathway.

It is worth mentioning that overexpression of cyclin D3 also results in reduced number of skin papillomas. Interesting, cyclin D3 preferentially bind to CDK6 in K5-Cyclin D3 mouse epidermis (Rojas, Cadenas et al. 2007). Thus, it is tempting to hypothesize that CDK6/cyclin D3 complexes play a unique role in mouse epidermis blocking tumor development. Supporting a specific role for cyclin D3 complexes in inhibition of cell proliferation, Wang et al have shown that cyclin D3/CDK4,6 maintains growth-inhibitory activity of C/EBPα (Wang, Shi et al. 2006). Whether different CDKs/D-type cyclins complexes play unique role in proliferation and apoptosis, and whether these effect are tissue specific remain to be
determined. However, our results strongly suggest that modulation of the levels of D-type cyclins and/or G1-CDKs should be useful on planning cancer therapies.
Chapter III

Combined effect of cyclin D3 expression and abrogation of cyclin D1 prevent mouse skin tumor development
3.1 Introduction

D-type cyclins are a family of key cell-cycle regulators, as they are the regulatory subunits of cyclin-dependent kinase 4 and 6 (CDK4, CDK6), which phosphorylate the pRb family of proteins that are critical substrates for cell-cycle progression (Meyerson and Harlow 1994; Sherr and Roberts 1999; Farkas, Hansen et al. 2002; Leng, Noble et al. 2002). The highly conserved sequence among these three members of the D-type cyclin family suggests that they have functionally redundant roles, but each member is expressed in a tissue-specific manner (Sherr 1995; Ciemerych, Kenney et al. 2002). Consistent with their role in promoting growth, abnormal levels of D-type cyclins have been implicated in the development of various types of human tumors. The rearrangement and/or amplification of the cyclin D1 gene has been reported in a wide range of human cancers, including carcinoma of the uterine cervix, breast carcinomas, and head and neck squamous cell carcinomas (Dickson, Fantl et al. 1995; Cheung, Yu et al. 2001; Fujii, Ishiguro et al. 2001). Similarly, abnormalities in the cyclin D2 gene have also been observed in testicular tumors (Sicinski, Donaher et al. 1996; Houldsworth, Reuter et al. 1997) and B cell malignancies (Delmer, Ajchenbaum-Cymbalista et al. 1995). Moreover, overexpression of cyclin D3 has been found in several human cancers (Ito, Takeda et al. 2001; Wong, Chan et al. 2001; Hedberg, Roos et al. 2002; Pruner, Pignataro et al. 2005), such as malignancies of the thymus (Filipits, Jaeger et al. 2002). In addition to its growth-promoting functions, cyclin D3 plays a unique, nonredundant, and tissue-specific role in muscle differentiation (Kiess, Gill
et al. 1995; Mariappan and Parnaik 2005) as well as during advanced stages of differentiation in the epithelia of the stomach, intestine and gallbladder (Bartkova, Lukas et al. 1998).

In recent years, our group as well as other investigators have utilized the mouse skin model to investigate the role of D-type cyclins in normal and neoplastic proliferation (Zhang, Liu et al. 1997; Rodriguez-Puebla, LaCava et al. 1998; Rodriguez-Puebla, LaCava et al. 1999; Miliani de Marval, Gimenez-Conti et al. 2001; Rodriguez-Puebla, Miliani de Marval et al. 2002). These studies established that cyclin D1 and D2 are up-regulated in mouse skin papillomas and squamous cell carcinomas (SCC), whereas cyclin D3 protein levels remain constant in both normal epidermis and tumors (Rodriguez-Puebla, LaCava et al. 1998). Supporting these observations, forced expression of either cyclin D1 or cyclin D2 in mouse epidermis induces skin papillomas and SCC, whereas genetic ablation of cyclin D1 and cyclin D2 reduce tumor development (Rodriguez-Puebla, LaCava et al. 1998; Rodriguez-Puebla, Robles et al. 1998; Rodriguez-Puebla, LaCava et al. 1999; Rodriguez-Puebla, Miliani de Marval et al. 2002; Yamamoto, Ochiya et al. 2002; Miliani de Marval, Macias et al. 2004; Rojas, Cadenas et al. 2007). In contrast, the forced expression of cyclin D3 inhibits skin tumorigenesis, an effect that is mediated by the simultaneous downregulation of cyclin D2 (Rojas, Cadenas et al. 2007).

In this chapter, we tested the hypothesis that the simultaneous up- and down-regulation of individual D-type cyclins is a valuable method to inhibit skin tumorigenesis. We used the two-stage mouse skin carcinogenesis protocol, which is a model well-suited for
understanding the multistage nature of tumor progression. In this model, tumor initiation is accomplished through a single topical application of a carcinogen, typically 7,12-dimethylbenz(a)anthracene (DMBA). This produces an inheritable genetic mutation in the Ha-ras oncogene, and tumor promotion occurs when the initiated cells are expanded as a result of multiple applications of a tumor promoter, usually 12-O-tetradecanoylphorbol-13-acetate (TPA). This stimulus induces hyperproliferation that promotes the generation of benign tumors (the so-called “papillomas”).

We generated K5D3/cyclin D1−/− mice, which overexpress cyclin D3 but lack cyclin D1 expression in skin. Similar to K5D3 transgenic mice, biochemical analysis of K5D3/cyclin D1−/− epidermis shows a robust reduction of cyclin D2 levels. Therefore, this compound mouse model allows us to determine the effect of cyclin D3 expression with reduced or absent expression of the remaining two members of the D-type cyclin family. Notably, the overexpression of cyclin D3 and the simultaneous ablation of cyclin D1 led to a robust inhibition of ras-dependent skin tumorigenesis, with no effect in normal keratinocyte proliferation and differentiation. We conclude that expression of cyclin D3 combined with minimal or absent expression of cyclin D1/D2 is sufficient to support epidermis homeostasis but not ras-mediated tumor development. Moreover, our results provide genetic evidence that individual modulation of D-type cyclin levels could be a useful method to inhibit ras-mediated tumorigenesis.
3.2 Materials and Methods

3.2.1 Experimental Animals

The generation of the cyclin D1 knockout (cyclin D1<sup>-/-</sup>) and K5-cyclin D3 (K5D3) transgenic mice has been described in references (Sicinski, Donaher et al. 1995; Rodriguez-Puebla, LaCava et al. 2000). To generate K5D3/cyclin D1<sup>-/-</sup> compound mice, we crossed cyclin D1<sup>+/+</sup> and K5D3 mice. Subsequently the K5D3/cyclin D1<sup>-/-</sup> mice were mated with cyclin D1<sup>+/+</sup> siblings. The genotype of each mouse was screened by PCR (Sicinski, Donaher et al. 1995; Rodriguez-Puebla, LaCava et al. 2000).

3.2.2 Two-stage carcinogenesis protocol

Twelve mice of each genotype were utilized for the two-stage carcinogenesis protocol, a number based on power calculation using a 2x2 Chi-squared test. Carcinogenesis was initiated in three-week-old mice with a single topical application of DMBA (200 nMol in 200 µl of acetone) to the dorsal skin. Two weeks after DMBA initiation, TPA (6.8 nMol in 200 µl of acetone) was applied twice a week to the dorsal skin of each mouse for 30 weeks, and papilloma development was tracked weekly. Papillomas 1 mm in diameter or larger were scored once a week. Multiplicity and incidence of tumor-bearing animals were compared among the four genotypes (K5D3, cyclin D1<sup>-/-</sup>, K5D3/cyclin D1<sup>-/-</sup> and wild-type mice) at 24 weeks using the t-test.
3.2.3 Protein gel Blotting and Kinase assays

After the mice were sacrificed, the dorsal skins were treated with a depilatory agent for 1 minute and washed with tap water. The epidermal tissue was scraped off using a razor blade, placed into homogenization buffer [50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 2.5 mmol/L EGTA, 1 mmol/L EDTA acid, 0.1% Tween 20, 1 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.2 U/ml of aprotinin, 10 mmol/L b-glycerophosphate, 0.1 mmol/L sodium vanadate, and 1 mmol/L NaF], and homogenized using a manual homogenizer. The epidermal homogenate was centrifuged at 14,000 x g at 4°C to collect the supernatant, which was used directly for Western blotting analysis or stored at -80°C. Mouse skin and tumors were ground in homogenization buffer [60 mM Tris-HCl, pH 8.6, 5 mM ethylenediaminetetraacetate (EDTA), 5 mM ethylene glycolbis(2-aminoethyl ether)-N,N,N0,N0-tetraacetic acid (EGTA), 300 mM sucrose, anti-protease and anti-phosphatase cocktails]. The homogenates were sonicated and centrifuged at 10,000 x g for 10 min. The supernatants were collected and used directly for Western blot analysis. The protein concentration was measured with the Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). Protein lysates (30 µg from each sample) were electrophoresed through 12% acrylamide gels and electrophoretically transferred onto nitrocellulose membranes. After blocking the membranes with 5% nonfat powdered milk in Dulbecco PBS, they were incubated with 1 µg/ml of specific antibodies. The following antibodies were used: polyclonal antibodies against cyclin D2 (M20), CDK4 (C22), CDK2 (M2), CDK6 (C21), (Santa Cruz Biotech, Santa Cruz, CA), and monoclonal antibodies against cyclin
D1 (DCS-6), cyclin D3 (Ab-1) (NeoMarkers, Fremont, CA), CDK6 (DCS-83) (Santa Cruz Biotech). Incubation with secondary antibodies followed by exposure to enhanced chemiluminescence (ECL detection kit; GE Health Care, Piscataway, NJ) was used for immunoblot detection.

To study the kinase activities, 500 µg of fresh protein was extracted and immunoprecipitated in tween-20 lysis buffer (Hepes [pH 7.5], 150 mmol/L NaCl, 1mmol EDTA, 25 mmol EGTA, 10% Glycerol, 0.1% tween 20, 1 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L DTT, and 1 mmol/L PMSF) with precoated antibodies against CDK2, CDK4, and CDK6 for 2 hours at 4°C. The beads were washed twice with NP-40 buffer and once with kinase buffer (50 mmol/L HEPES [pH 7], 10 mmol/L MgCl2, 5 mmol/L MnCl2). Subsequently, 30 µL of kinase buffer, 1 µg of pRb or histone H1 (Upstate Biotechnology Inc., Charlottesville, VA) substrate, 5 µCi of [γ-32p] ATP (6000 Ci/mmol), 1 mmol/L DTT, and 5 µmol/L ATP were added to the bead pellet and incubated for 30 minutes at 30°C. SDS sample buffer was added, and each sample was boiled for 3 minutes to terminate the reaction, and they were electrophoresed through polyacrylamide gels. The bands obtained by Western blot and the kinase assay were quantified using UNSCAN IT gel software for Windows.

3.2.4 Immunostaining

Epithelial cell proliferation was measured by intraperitoneal injection of 60 µg/g (body weight) of 5-bromodeoxyuridine (BrdU) 30 minutes before the mice were sacrificed by CO2 asphyxiation. BrdU incorporation was detected by immunohistochemical staining of
paraffin-embedded skin sections with a mouse anti-BrdU (ab-2) monoclonal antibody (Calbiochem; EMB Biosciences, San Diego, CA), biotin-conjugated anti-mouse antibody (Vector Laboratories, Burlingame, CA), and the avidinbiotin Vectastain Elite peroxidase kit (Vector Laboratories) with diaminobenzidine as a chromogen. Apoptotic cells were identified by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assays with the FragEL DNA Fragmentation Detection Kit, Colorimetric-TdT enzyme (Calbiochem; EMB Biosciences) according to the manufacturer’s instructions. Counterstaining with methyl green allows for the quantification of normal and apoptotic cells. The numbers of apoptotic cells in the tumors were determined in sections of 250 µm² with a reticule grid.

3.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 Software (GraphPad Software Inc., San Diego, CA).

3.3 Results

3.3.1 Generation and skin characterization of K5D3/cyclin D1⁻/⁻ compound transgenic mice

We have recently reported the generation of a transgenic mouse (K5D3) wherein the constitutive expression of cyclin D3 was targeted to the basal cell layer of the epidermis by the 5’ regulatory sequence of the bovine keratin 5 (K5) gene (Rodriguez-Puebla, LaCava et al. 2000). Notably, the epidermis of the K5D3 transgenic mice revealed a significant decrease
of cyclin D2 expression and concomitant reduction of the number of chemically-induced skin papillomas (Rojas, Cadenas et al. 2007). We also established that cyclin D1<sup>−/−</sup> mice display a 75% reduction in the number of skin papillomas (Robles, Rodriguez-Puebla et al. 1998). Therefore, to study the effect of a simultaneous reduction of both cyclin D1 and cyclin D2 in keratinocyte proliferation and skin tumorigenesis, we generated a K5D3/cyclin D1<sup>−/−</sup> compound mouse. Similar to cyclin D1<sup>−/−</sup> mice, K5D3/cyclin D1<sup>−/−</sup> mice show growth retardation and neurological abnormality evidenced by limb retraction when lifted by their tails that is most pronounced by three weeks (Fantl, Stamp et al. 1995; Sicinski, Donaher et al. 1995). To determine whether forced expression of cyclin D3 also results in cyclin D2 downregulation in the cyclin D1<sup>−/−</sup> background, we performed a biochemical analysis of the epidermal extract from K5D3, cyclin D1<sup>−/−</sup>, K5D3/cyclin D1<sup>−/−</sup> and wild-type mice. As expected, cyclin D3 protein levels increased 6- to 8-fold in the K5D3 and K5D3/cyclin D1<sup>−/−</sup> epidermis compared with their wild-type siblings (Figure 12). Similar to K5D3 mouse, K5D3/cyclin D1<sup>−/−</sup> epidermis showed 30-fold reduction in cyclin D2 protein levels compared with wild-type mice (Figure 12). Notably, cyclin D1-null epidermis does not show compensatory changes in cyclin D2 and cyclin D3 protein levels (Figure 12). Additionally, the D-type cyclin partners CDK4 and CDK6 show no changes in the protein level among the four genotypes analyzed.

To determine whether variation in D-type cyclin levels affect the kinase activity of CDKs in mouse keratinocytes, we evaluated the CDK4 and CDK2 kinase activities in epidermal lysates from wild-type, K5D3, cyclin D1<sup>−/−</sup> and K5D3/cyclin D1<sup>−/−</sup> mice. The overexpression of cyclin D3 in the mouse epidermis showed 2-fold increase in CDK4 kinase
Figure 12. Protein gel blot analysis of cell cycle proteins from mouse epidermis. Protein lysates of epidermis samples from wild-type (wt), K5D3, cyclin D1\(^{-/-}\) (D1\(^{-/-}\)) and K5D3/D1\(^{-/-}\) siblings were separated using sodium dodecyl sulfate-PAGE and blotted onto a nitrocellulose membrane. Primary antibodies against cyclin D1, cyclin D2, cyclin D3, CDK4 and CDK6 were used for immunoblot analysis. β-actin was used as the loading control. A vertical line was depicted to note rearrangements of the lines in order to be consistent between the panels A and B.
Figure 13. Kinase activity of CDK4 and CDK2 from wild-type (wt), K5D3, cyclin D1-/ (D1-/−) and K5D3/D1-/− epidermis. Fresh epidermal lysates were immunoprecipitated with specific antibodies against CDK4 and CDK2 and in vitro kinase assays were performed with pRb or Histone H1 (H1) peptides as substrates. No Ab: immunoprecipitated with normal IgG; No Sub: No substrate were used during the kinase reaction. Western Blot analysis of the immunoprecipitated with antibodies against CDK4 and CDK2 serve as control for the efficiency of the immunoprecipitations. A vertical line was depicted in blots to denote rearrangements of the lines in order to be consistent between the panels.
activity compared with wild-type siblings. These data are consistent with our previous report, which demonstrated that the overexpression of cyclin D3 activates CDK4 and CDK6 to compensate for the reduced levels of cyclin D2 (Rojas, Cadenas et al. 2007) (Figure 13). Cyclin D1 ablation did not affect CDK4 kinase activity in mouse keratinocytes, but it did reduce CDK2 kinase activity by 2-fold compared with wild-type mice (Figure 13). Consistent with these results, our group and others have shown that elevated levels of D-type cyclin/CDK4 complexes bind and sequester p27$^{kip1}$/p21$^{cip1}$ with the consequent activation of CDK2 (Hsieh, Barnett et al. 2000; Macias, Kim et al. 2007). Therefore, we hypothesize that lack of cyclin D1 leads to the release of p27$^{kip1}$/p21$^{cip1}$ from CDK4,6 complexes, which, in turn, bind and inhibit CDK2 activity. Together, these results show that the K5D3/cyclin D1$^{-/-}$ mouse model represents a unique tool to investigate the effects of the simultaneous reduction of cyclin D1 and D2 levels in keratinocyte proliferation and skin tumor development.

We next asked whether the simultaneous ablation of cyclin D1 and the reduction of cyclin D2 affected epidermal homeostasis. To this end, we examined formalin-fixed, paraffin-embedded skin cross-sections of K5D3/cyclin D1$^{-/-}$, cyclin D1$^{+/}$, K5D3 and wild-type siblings. The gross histological appearance of the cyclin D1$^{-/-}$ epidermis revealed a mild hypoplasia, although it was not statistically significant (Figure 14). Hematoxylin and Eosin (H&E)-stained sections showed that both K5D3 and K5D3/cyclin D1$^{-/-}$ epidermis exhibits increased thickness and an elevated number of keratinocytes compared to wild-type littermates (Figure 14) (1.8-fold increase; p=0.0015, t-test). In addition, we did not observe
any obvious alterations in the morphology of the follicular and inter follicular epidermis between K5D3 and K5D3/cyclin D1−/− littermates (Figure 14). Thus, the expression of cyclin D3 appears to be sufficient to compensate for the lack of cyclin D1 and the severe down-regulation of cyclin D2 in the K5D3/cyclin D1−/− mouse epidermis. To determine whether the mild hyperplasia observed in K5D3/cyclin D1−/− is a result of increased keratinocyte proliferation, we analyzed the BrdU incorporation in the inter follicular epidermis. Consistent with previous observations (Rodriguez-Puebla, LaCava et al. 2000), we detected a 1.6-fold increase in the number of proliferative keratinocytes in the basal cell layer of K5D3 and K5D3/cyclin D1−/− compared to wild-type siblings (p<0.0001, t-test) (Figure 15). It is worth noting that significant differences were not observed among the number of apoptotic cells in epidermis of the four genotypes analyzed (data no shown). Collectively, these findings suggest that the sole expression of cyclin D3 in mouse basal keratinocytes is sufficient to maintain epidermal homeostasis, including the inter follicular epidermis and hair follicles.
Figure 14. Skin phenotype of K5D3/cyclin D1<sup>−/−</sup> compound mice. (A) Representative paraffin-sections of skin from wild-type (wt), D1<sup>−/−</sup>, K5D3 and K5D3/D1<sup>−/−</sup> siblings were stained with H&E. (B) Quantification of the total number of epithelial cells in the inter follicular epidermis of the four genotypes analyzed.
Figure 15. Epidermal proliferation in K5D3/D1-/- compound mice. BrdU immunostaining of wild-type (wt), D1<sup>−/−</sup>, K5D3 and K5D3/D1<sup>−/−</sup> paraffin-sections. Arrows indicate BrdU-positive cells. (B) The BrdU label index of the inter follicular epidermis of the four genotypes analyzed.
3.3.2 Reduced sensitivity to ras-dependent skin tumorigenesis upon simultaneous reduction of cyclin D1 and cyclin D2 protein levels

We have previously reported that cyclin D1- and cyclin D2- knockout mice display a significantly reduced sensitivity to the development of papillomas that are induced by chemical carcinogenesis (Robles, Rodriguez-Puebla et al. 1998; Rojas, Cadenas et al. 2007). Similarly, transgenic expression of cyclin D3 also decreases skin tumorigenesis as a result of cyclin D2 downregulation (Rojas, Cadenas et al. 2007). Therefore, we next asked whether simultaneous reduction of cyclin D1 and cyclin D2 would potentiate the tumor inhibition observed in cyclin D1⁻/⁻ and cyclin D2⁻/⁻ mice (Robles, Rodriguez-Puebla et al. 1998; Rojas, Cadenas et al. 2007). To test this idea, K5D3/cyclin D1⁻/⁻ mice and control littermates were subjected to the two-stage carcinogenesis protocol. This protocol induces skin papillomas after a single application of a carcinogen followed by biweekly treatments with a tumor promoter that favors the selection of cells bearing Ha-ras mutations. The dorsal skin of K5D3/cyclin D1⁻/⁻, cyclin D1⁻/⁻, K5D3 and wild-type littermates was topically treated with a subcarcinogenic dose of the genotoxic DMBA, and tumorigenesis was subsequently promoted via biweekly applications of TPA for 25 weeks. The incidence and multiplicity of papillomas were scored in each group for 24-30 weeks. As previously reported (Rojas, Cadenas et al. 2007), after 24 weeks of promotion, K5D3 mice displayed a significantly lower number of tumors per mouse compared to wild-type mice (45% reduction, p=0.03, t-test) (Figure 16A). Consistent with our previous data, papilloma multiplicity in cyclin D1⁻/⁻ animals was 18% of the quantity observed in the wild-type cohort at the same week.
(p=0.0003, t-test). Notably, the average number of tumors per mouse was significantly reduced in the K5D3/cyclin D1−/− group (6.5% compared to wild-type mice and 36% compared to cyclin D1−/− mice) (Figure 16A). Furthermore, when the experiment was extended to 30 weeks, cyclin D1−/− mice reached a total number of 4.5 papillomas per mouse, whereas K5D3/cyclin D1−/− barely showed 1.3 papillomas per mouse (71% reduction; p=0.02, t-test) (Figure 16A). The incidence of papillomas was remarkably varied among the groups. Wild-type and K5D3 mice developed visible tumors within 5-7 weeks of promotion, and they showed a 50% penetrance by 8.5 and 9.5 weeks, respectively. Tumor development in cyclin D1−/− mice began at 10 weeks with a 50% penetrance by 12.5 weeks. Notably, the tumor development of K5D3/cyclin D1−/− littermates was severely delayed, beginning at week 13 and reaching an incidence of 50% by week 16.5 (Figure 16B). The size of the tumors also varied among the genotypes. Wild-type papillomas were the largest, reaching of up to 300 mm³. Conversely, the K5D3/cyclin D1−/− papillomas were much smaller than the other groups, and 63% of the tumors never reached a volume of 30 mm³ (Figure 18A). The tumor size of the cyclin D1−/− and K5D3 animals was between the size of the wild-type and the K5D3/cyclin D1−/− papillomas (Figure 18A). Together, these results indicate that although cyclin D3 can compensate for the lack of cyclin D1 and cyclin D2 in normal epidermis homeostasis, it is not sufficient to drive skin tumor development.

Biochemical analysis of D-type cyclin levels in papillomas demonstrated that cyclin D2 remained downregulated in tumors obtained from K5D3 and K5D3/cyclin D1−/− mice (4- and
Figure 16. Kinetics of papilloma formation. Wild-type (wt), K5D3, Cyclin D1-/- (D1-/-) and K5D3/D1-/- siblings were initiated with DMBA and promoted with multiple applications of TPA on dorsal mouse skin. (A) Average number of papillomas per mouse (multiplicity) as a function of weeks of study. (B) Percentage of tumor-free mice as a function of weeks of study (incidence).
5-fold reduction compared with wild-type mice, respectively) (Figure 17). Therefore, to understand how the D-type cyclin levels affect keratinocyte proliferation, we quantified the BrdU incorporation in skin papillomas collected at 24 weeks after the initiation of oncogenic promotion. The number of proliferating BrdU+ cells were significantly reduced in cyclin D1−/− papillomas compared with the wild-type cohort (3-fold reduction, p<0.0001, t-test) (Figure 18B). Notably, overexpression of cyclin D3 appeared to compensate for the lack of cyclin D1, as K5D3/cyclin D1−/− exhibited comparable number of proliferative keratinocytes as shown by wild-type and K5D3 tumors (Figure 18B). Importantly, very few apoptotic cells could be visualized by TUNEL staining, and their numbers were similar among the four genotypes analyzed (data not shown). Typically, DMBA application induces tumor initiation by inducing an activating mutation in codon 61 of the Ha-ras gene (Quintanilla, Brown et al. 1986). To test whether the downregulation of D-type cyclins result in papilloma development by an alternative pathway that was independent of ras, we characterized the spectrum of codon 61 Ha-ras mutation in skin tumors. Mutational analysis of tumors collected at 20 weeks of oncogenic promotion showed the presence of Ha-ras mutation in all tumors of the four genotypes analyzed. Therefore, we concluded that tumor development in cyclin D1−/− and K5D3/cyclin D1−/− animals depends on an Ha-ras mutation as the initiating event.

We evaluated the histopathological features of papillomas at 24 weeks of oncogenic promotion, taking into account dysplastic and anaplastic changes, including disturbed cell polarity, differentiation, abnormal mitosis, nuclear hyperchromatism and the nuclear/cytoplasmic ratio. Fifty percent of the wild-type and K5D3 tumors were classified as
regular papillomas, whereas the other 50% were classified as well-differentiated squamous cell carcinomas (SCC) with expansion of the basal and spinous layers, loss of polarity, and cords of epidermal cells that were contiguous to the basal layer invading the dermis. In contrast, 100% of cyclin D1\(^{-/-}\) and K5D3/cyclin D1\(^{-/-}\) tumors were classified as well differentiated papillomas with basal cell hyperplasia, mild acanthosis and hyperkeratosis, but they showed no invasion of epidermal cells into the dermis (Table 1). These results suggest that ablation of cyclin D1 and simultaneous ablation of cyclin D1/downregulation of cyclin D2 results in the development of benign tumors (well-differentiated papillomas) compared to the malignant progression observed in wild-type tumors.

We conclude that the simultaneous downregulation of cyclin D1 and cyclin D2 enhances the tumor inhibition observed in cyclin D1\(^{-/-}\) and K5D3 mice under conditions where ras is constitutively activated. These data underscore a role for the cyclin D3-cyclin D2 negative feed-back loop as a tumor suppressor mechanism that prevents ras-induced carcinogenesis.
Figure 17. Biochemical analysis of skin tumors. Immunoblot analysis of wild-type (wt), K5D3, D1/- and K5D3/D1/- papilloma lysates developed with antibodies against cyclin D1, cyclin D2, cyclin D3, CDK4 and CDK2. β-actin was used as the loading.
Figure 18. Skin papilloma volume and tumor keratinocyte proliferation (A) Skin papillomas were classified according their volume and depicted as percentage of the total number of tumors for each genotype. (B) BrdU label index of immunostained paraffin-sections of wild-type (wt), K5D3, cyclin D1<sup>−/−</sup>, and K5D3/D1<sup>−/−</sup> papillomas.
Table 1. Histopathological analysis of skin tumor

<table>
<thead>
<tr>
<th>Mice</th>
<th>No of tumors/group</th>
<th>No (%) of tumors classified as:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Papilloma&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SCC&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Wild type</td>
<td>16</td>
<td>8 (50)</td>
<td>8 (50)</td>
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<tr>
<td>K5D3</td>
<td>12</td>
<td>6 (50)</td>
<td>6 (50)</td>
<td></td>
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<tr>
<td>Cyclin D1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9</td>
<td>9 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>K5D3/Cyclin D1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>14</td>
<td>14 (100)</td>
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<sup>a</sup> No atypia in basal layers, basal cell hyperplasia, mild acanthosis and hyperkeratosis, and no invasion of epidermal cells into the dermis. <sup>b</sup> Well-differentiated SCC with expansion of basal and spinous layer, loss of polarity, and cords of epidermal cells contiguous to the basal layer invading the dermis.
3.4 Discussion

We have previously reported that the overexpression and/or ablation of D-type cyclins results in varied sensitivities to chemically-induced mouse skin tumors (Robles, Larcher et al. 1996; Rodriguez-Puebla, LaCava et al. 1999; Yamamoto, Ochiya et al. 2002; Rojas, Cadenas et al. 2007). By using the two-stage carcinogenesis model, we provided genetic evidence that ras-mediated tumorigenesis depends on signaling pathways that act preferentially through cyclin D1 and cyclin D2 (Robles, Rodriguez-Puebla et al. 1998; Rojas, Cadenas et al. 2007). In fact, the ablation of cyclin D1 and cyclin D2 results in a 75% and 45% reduction in the number of skin papillomas, respectively (Robles, Rodriguez-Puebla et al. 1998; Rojas, Cadenas et al. 2007). Moreover, forced expression of cyclin D1 and cyclin D2 leads to an increased number of skin tumors and resistance to Ca^{2+}-induced keratinocyte differentiation (Yamamoto, Ochiya et al. 2002; Rojas, Cadenas et al. 2007). In contrast, the overexpression of cyclin D3 in mouse keratinocytes decreased skin tumorigenesis, which is associated with downregulation of cyclin D2 (Rojas, Cadenas et al. 2007). Therefore, to test whether simultaneous downregulation of cyclin D1 and cyclin D2 could potentiate the inhibition of skin tumorigenesis, we developed a K5D3/cyclin D1^{−/−} compound mouse. Biochemical analysis of mouse epidermis showed that, in accordance with K5D3 mice (Rojas, Cadenas et al. 2007), K5D3/cyclin D1^{−/−} mice exhibit a significant reduction of cyclin D2 protein level. Therefore, we used this model to study the consequences of simultaneous ablation and downregulation of cyclin D1 and cyclin D2 in normal keratinocyte proliferation.
and tumor development. Notably, K5D3/cyclin D1\(^{-/-}\) mice showed a dramatic inhibition of skin tumor development compared to K5D3 and cyclin D1\(^{-/-}\) mice. Even when the experiment was extended to 30 weeks of promotion, the inhibitory effect in the K5D3/cyclin D1\(^{-/-}\) mice was exacerbated. These results support a role of ras signaling acting through both cyclin D1 and cyclin D2, but not through cyclin D3. This is consistent with our previous results establishing that skin tumor development is only restored in K5D3 mice after restitution of cyclin D2 levels in K5D3/K5D2 double transgenic mice (Rojas, Cadenas et al. 2007). Supporting these data, we have previously reported that cyclin D1 and cyclin D2 are overexpressed in skin papillomas of wild-type mice, whereas the levels of cyclin D3 remains constant compared with normal and hyperproliferative skin (Rodriguez-Puebla, LaCava et al. 1998).

Our results established a clear difference between the effect of cyclin D3 overexpression in papilloma development and keratinocyte proliferation. Lack of cyclin D1 reduces keratinocyte proliferation in epidermis and tumors, but overexpression of cyclin D3 (K5D3/cyclin D1\(^{-/-}\)) returns the rate of proliferation to that of wild-type in both epidermis and tumors. These observations suggest that the individual or simultaneous ablation of cyclin D1 and downregulation of cyclin D2 is well tolerated by keratinocytes. However, the reduced number of papillomas observed in the K5D3/cyclin D1\(^{-/-}\) mice indicates that decreased levels of cyclin D1 and D2 affect an early stage of tumor development by disturbing the initiation stage and/or the clonal expansion of Ha-ras-mutated keratinocytes. Although, it is not clear why cyclin D3 overexpression (associated to cyclin D2
downregulation) compensate the absence of cyclin D1 in keratinocyte proliferation, but not in skin tumor development, it is tempting to speculate that the cyclin D1/D2 are essential for proliferation/expansion of the chemically initiated hair follicle stem cells. Therefore, understanding the differences between the regulations of the cell-cycle in keratinocytes stem cells versus normal keratinocytes should permit appreciate how the expansion of initiated stem cells and normal keratinocyte proliferation are differently affected by deregulation of D-type cyclin levels.

The analysis of tumor size shows that tumorigenesis is also affected during the growth stage (tumor promotion). In fact, 63% and 46% of K5D3/cyclin D1⁻/⁻ and K5D3 papillomas are smaller than 30 mm³, and tumors >300 mm³ were not observed in the K5D3/cyclin D1⁻/⁻ compound mice. Conversely, wild-type mice develop a considerable number of tumors >300 mm³. Analysis of apoptosis in skin tumors shows no significant differences among the four genotypes analyzed. Thus, mechanisms other than changes in the rate of proliferation or apoptosis are involved in the reduced size of tumors overexpressing cyclin D3, and this implication warrants further investigation. Histopathological analysis of skin tumors show no malignant progression of the K5D3/cyclin D1⁻/⁻ and cyclin D1⁻/⁻ tumors; instead, they were all classified as benign papillomas (Table 1). Notably, 50% of the K5D3 tumors showed areas of invasion and were classified as well-differentiated squamous cell carcinomas, similar to the wild-type tumors. Thus, it is conceivable that cyclin D3 expression behaves as an oncogene, even when cyclin D2 level is reduced, but the simultaneous downregulation and ablation of cyclin D2 and cyclin D1 abrogate the oncogenic function of cyclin D3. These
results suggest that cyclin D1 play a unique role during the malignant conversion to SCC, since overexpression of each individual D-type cyclin member can induce malignant progression to SCC (Yamamoto, Ochiya et al. 2002; Rojas, Cadenas et al. 2007), but only cyclin D1 seems to be essential to allow malignant progression in wild type and cyclin D3 overexpressing mice (Table 1). Therefore, it is possible that in the context of ras-mediated tumorigenesis, skin tumors become “addictive” to cyclin D1 expression, which is regulated for Ha-ras (Rodriguez-Puebla, Robles et al. 1999; Kim, Gautier et al. 2002). Supporting this idea, early studies of skin tumorigenesis showed that cyclin D1 is expressed in small incipient papillomas, but overexpression was more notable in advance stages of skin tumorigenesis (Bianchi, Fischer et al. 1993; Robles and Conti 1995). Moreover, the kinetics of cyclin D1 overexpression coincides with the expression of the mutated Ha-ras allele (Rodriguez-Puebla, LaCava et al. 1999). These results implicate that functional differences among the three D-type cyclin exist in mouse epidermis, although the mechanisms involved in these variances have not yet been established.

The mechanism by which cyclin D3 expression represses cyclin D2 but not cyclin D1 remains unknown; although transcriptional and post-transductional mechanisms are likely to be involved (Rojas, Cadenas et al. 2007). However, this negative feedback loop is specific for cyclin D3, because modifications in the levels of other D-type cyclins were not observed in K5-cyclin D1 and K5-cyclin D2 transgenic mice (Rodriguez-Puebla, LaCava et al. 2000; Rojas, Cadenas et al. 2007). In addition, the role of cyclin D3 in cell proliferation appears to be tissue specific and controversial. In fact, cyclin D3 expression has been
associated with terminal differentiation in muscle cells and epithelium (Kiess, Gill et al. 1995; Skapek, Rhee et al. 1995; Bartkova, Lukas et al. 1998; Mariappan and Parnaik 2005).

Moreover, D-type cyclins, specifically cyclin D1, participate in pRb-independent pathways that may also be involved in the inhibition of cell proliferation and tumorigenesis. Furthermore, it has been recently reported that the reduction of cyclin D1 levels in human cancer cells impairs the recruitment of RAD51 to damaged DNA, affecting DNA repair (Jirawatnotai, Hu et al. 2011). In addition, a genetic-proteomic screen has revealed that cyclin D1 plays a transcriptional role during development (Bienvenu, Jirawatnotai et al. 2010).

In summary, we developed a new model to study the effect of simultaneous downregulation of cyclin D1 and cyclin D2 in skin tumorigenesis. It is important to note, that cyclin D1/cyclin D2 double knockout mice were developed by the Sicinski group, but these mice died within the first 3 weeks of life (Ciemerych, Kenney et al. 2002). To the best of our knowledge this is the first time that the simultaneous downregulation of two members of the D-type cyclin family has been assessed to determine their potential use in tumor inhibition. We demonstrated that keratinocytes tolerate proliferation with very low levels of cyclin D2 and cyclin D1, but skin tumor development is blocked at an early-stage of tumorigenesis. Therefore, our study presents the potential use of the cyclin D3-cyclin D2 negative feedback loop and/or the simultaneous inhibition of cyclin D1 expression as a possible target for cancer therapies.
Chapter IV

Ablation of cyclin D3 inhibits mouse skin tumor development without affecting CDK6 mediated proliferation
4.1 Introduction

Numerous studies have proposed that the retinoblastoma (Rb) family of proteins, pRb, p107 and p130, regulate the passage through the restriction point (R-point) (Sherr 1994; Weinberg 1995). Additional studies demonstrated that Rb functions, including the ability to interact with E2F, were regulated by sequential phosphorylation. Proper controls of the mechanisms which regulate R-point passage are critical for maintaining normal levels of proliferating cells, avoiding hyperplastic and neoplastic growth. The primary kinase responsible for the regulation of cell cycle is the cyclin-dependent kinases (Lukas, J. and Bartkova, J. et al. 1995; Lukas, J., Parry, D. et al. 1995; Medema, R.H., et al 1995). Cyclin-dependent kinase 2, 4, and 6 belong to a family of serine/threonine kinases whose primary targets are the members of the pRb protein family. The activity of CDK4 and CDK6 is dependent upon the availability and complex formation with their regulatory subunits D-type cyclins (D1, D2 and D3). In addition to cyclin binding, the activity of CDKs is subject to other levels of regulation, which including their association with inhibitory molecules such as the INK4 and CIP/KIP family (Xiong, Hannon et al. 1993; Xiong 1996). A wide spectrum of mutation in pRb pathway has been identified in variety of human tumors. Loss of Rb function contributes to a wide array of human cancers. Another frequent mutation in human cancers disrupting Rb pathway is p16\(^{\text{INK4a}}\) which is responsible for the control of D type cyclin/CDK4 kinase activity. The absence of p16\(^{\text{INK4a}}\) activity results in elevated CDK4 activity, which leads to phosphorylation of Rb and E2F accumulation (He, Olson et al. 1995).
Finally, deregulated expression of D-type cyclin, as well as CDK4, leads to an increased cyclin D/CDK4 complex formation and thus interrupts the pRb pathway, amplification of the CDK4 gene has been seen in sarcomas and gliomas (Khatib, Matsushime et al. 1993; He, Allen et al. 1994; Ragazzini, Gamberi et al. 2004).

As the least studied D-type cyclin, cyclin D3 is expressed in nearly all proliferating cells and shows the most broad expression pattern of all three D-type cyclins (Bartkova, Lukas et al. 1998). However, cyclin D3 expression has also been associated with cellular differentiation. In particular, cyclin D3 expression has been found expressed in most terminally differentiated/quiescent lymphoid tissues (Doglioni, Chiarelli et al. 1998). Similarly, cyclin D3 was detected in termially differentiating spermatids (Zhang, Wang et al. 1999). Supporting these observations, Wang et al demonstrated that cyclin D3 plays a growth inhibitory role in differetiated liver cells and adipocytes by stabilizing C/EBPalpha-CDK2 and C/EBPalpha-Brm comlexes (Wang, Shi et al. 2006). In addition, a recently published paper suggested that cyclin D3 promotes myoblast differentiation by enhancing muscle specific gene expression and cell cycle exit (Gurung and Parnaik 2012). Cyclin D3 is overexpressed in particular human cancers such as human gliomas, renal cell carcinoma and pancreatic adenocarcinoma (Buschges, Weber et al. 1999; Ito, Takeda et al. 2001; Hedberg, Roos et al. 2002). Importantly, cyclin D3-null mice display deficient maturation of granulocytes in the bone marrow and have reduced levels of neutrophil granulocytes in their peripheral blood. In addition, absence of cyclin D3 greatly reduced susceptibility of T-cell malignancies triggered by specific oncogenic pathways (Sicinska, Lee et al. 2006).
together, these observations suggest that cyclin D3 plays dual roles in proliferation and differentiation. However, more investigations are required to fully understand the mechanism under which cyclin D3 plays the unique role to other D-type cyclins.

Our group previously reported the effects of transgenic expression of CDKs and D-type cyclins in epithelial tissues driven by the keratin 5 (K5) promoter (Rodriguez-Puebla, LaCava et al. 2000; Miliani de Marval, Gimenez-Conti et al. 2001; Miliani de Marval, Macias et al. 2004; Rojas, Cadenas et al. 2007; Macias, Miliani de Marval et al. 2008). Transgenes under the K5 promoter readily express in the basal cell layer of the epidermis, thymic reticulum, digestive tract, and the oral mucosa (Ramirez, Bravo et al. 1994). In this regard, we have previously demonstrated that overexpression of cyclin D3 in mouse epidermis results in increased epidermal proliferation associated with a significantly decreased cyclin D2 expression level (Rojas, Cadenas et al. 2007). However, the K5D3 transgenic mouse skin exhibits a strong refractory effect to standard two-stage carcinogenesis protocol, presumably through the strong inhibition of cyclin D2 (Rojas, Cadenas et al. 2007). Similarly, we demonstrated that overexpression of CDK6 in mouse skin (K5CDK6) results in an increased epidermal proliferation associated with elevated CDK6 kinase activity, whereas the susceptibility of K5CDK6 epidermis to chemical carcinogenesis protocol was inhibited, through the induction of elevated apoptosis in hair follicle (Chapter II). We noticed that elevated CDK6 is prone to bind cyclin D3, which consists with previous observation (Rojas, Cadenas et al. 2007). According to these observations, we asked whether the inhibitory role of CDK6 in mouse skin tumorigenesis depends on the formation of cyclin D3/CDK6 complex.
Before addressing this question, we also would like to clarify the effects of cyclin D3- ablation in normal and neoplastic epidermis. To this end, we generated K5CDK6/D3-null compound mice and subject this model, as well as the D3-null mice, to a standard two-stage carcinogenesis protocol as we did before.

4.2 Materials and Methods

4.2.1 Mouse Models

The generation of K5-CDK6 transgenic mice were previously reported (Chapter II). The cyclin D3 knockout mice were generated and kindly provided by Sicinski’s group (Sicinska, Aifantis et al. 2003). K5CDK6/D3−/− compound mice were obtained by interbreeding the K5CDK6 mice with D3+/− mice. K5CDK6/D3+/− mice were then backcrossed with cyclin D3+/− mice in order to obtain K5CDK6/D3−/− mice.

4.2.2 Western Blotsdfs

For immunoblots, protein lysates were collected from epidermal skin scrapes with RIPA lysis buffer [150mmol/L NaCL, 1% IGEPAL, 0.5% DOC, 0.1% SDS, 50mmol/L Tris (pH8.0)]. Papillomas were snap frozen in liquid nitrogen and crushed with a pestle and mortar. Homogenates from epidermal scrapes or papillomas were sonicated and centrifuged at 14,000 r.p.m. at 4 °C. Supernatants were boiled in 2x lameli sample buffer for western blot analysis or stored at -80 °C. The protein concentration was measured with the Bio-Rad
protein assay system (Bio-Rad laboratories, Richmond, CA). Protein lysates (30µg from each sample group) were electrophoresed through 10% or 12% acrylamides gels based on the target protein molecular weight and electrophoretically transferred onto nitrocellulose membranes. After being blocked with 5% nonfat powdered milk in 0.1% Tween20-TBS, the membranes were incubated with 1µg/ml of specific antibodies. The following antibodies were used: polyclonal antibodies against cyclin D2 (M20), CDK4 (C22), CDK2 (M2), CDK6 (C21), pRb (M153), p107 (C18) (Santa Cruz Biotech, Santa Cruz, CA), and p53 (1C12) (Cell Signaling Tech Inc., Boston, MA), and monoclonal antibodies against cyclin D1 (DCS-6), CDK6 (DCS-83) (Santa Cruz Biotech). Secondary antibodies followed by enhanced chemiluminescence (ECL detection kit, GE Health Care, Piscataway, NJ) were used for immunoblotting detection.

4.2.3 Co-Immunoprecipitations and Kinase Assays

To study CDK/D-type cyclin complex formations and kinase activities, we utilized polyclonal antibodies against CDK6 (C21) (Santa Cruz Biotech) conjugated with protein A-sepharose beads (Thermo Scientific Inc., Rockford, IL). Fresh protein lysates from epidermal tissue (500µg) or papillomas (250µg) were immunoprecipitated for one hour at 4°C with constant rotation. After washing three times with extraction buffer, proteins that co-immunoprecipitated were analyzed by Western blot as described previously. Protein lysate (50µg) was loaded as a control input. The immunoprecipitation was repeated 3 times using 250, 500 or 1000 µg of protein lysate with identical results.
To assess the CDK2 kinase activities, 500µg of fresh protein was extracted and immunoprecipitated in NP-40 lysis buffer (Tris [pH 7.5], 150mmol/L NaCl, 0.5% NP-40, 50mmol/L NaF, 1mmol/L Na$_3$VO$_4$, 1mmol/L DTT, and 1mmol/L PMSF) with precoated antibodies against CDK2 for 2 hours at 4°C. Similarly to CDK2, CDK4 and CDK6 were immunoprecipitated from 500µg of fresh protein lysate in a Tween-20 lysis buffer (50mmol/L Hepes [pH 7.5], 150mmol/L NaCl, 1mmol/L EDTA, 25mmol/L EGTA, 10% Glycerol, 0.1% Tween-20, 1mmol/L NaF, 0.1mmol/L Na$_3$VO$_4$, 1mmol/L DTT, and 0.1mmol/L PMSF). Incubated beads were washed twice each with NP-40 or Tween-20 buffer and once with kinase assay buffer (50 mmol/L Hepes [pH 7.5], 10mmol/L MgCl$_2$, 5mmol/L MnCl$_2$). Then 30µl of kinase buffer, 1 µg of pRb or histone H1 (Upstate Biotechnology Inc., Charlottesville, VA) substrate, 5 µCi of [γ-$^{32}$P] ATP (6000 Ci/mmol.), 1mmol/L DTT, and 5µmol/L ATP were added to the bead pellet and incubated for 30 minutes at 30°C. SDS sample buffer was added, and each sample was boiled for 3 minutes to stop the reaction and electrophoresed through polyacrylamide gels. Western blot and kinase assay bands were quantified using UNSCANT IT gel software for windows.

4.2.4 Pathological Analysis and Immunostaining

Formalin fixed paraffin embedded epidermis samples were stained with H&E. Epithelial cell proliferation was measured by intraperitoneal injection of BrdU 30 minutes before the mice were sacrificed by CO$_2$ asphyxiation. BrdU incorporation was detected by immunohistochemical staining of paraffin-embedded skin sections with a mouse anti-BrdU
(Ab-2) monoclonal antibody (Calbiochem; EMB Biosciences, SanDiego, CA), biotin-conjugated antimouse antibody (Vector Laboratories, Burlingame, CA), and avidin biotin Vectastain Elite peroxidase kit (Vector Laboratories) with diaminobenzidine as chromogen.

Apoptotic cells were determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assays with the FragEL DNA Fragmentation Detection Kit, Colorimetric-TdT enzyme (Calbiochem; EMB Biosciences) following the manufacturer's instructions. Briefly, the terminal deoxynucleotidyl transferase (TdT enzyme) binds to the exposed 3'-OH ends of a DNA fragment generated in apoptosis progression and catalyzes the addition of biotin-labeled and unlabeled deoxynucleotides. Biotinylated nucleotide were detected using a streptavidin-horseradish peroxidase conjugate. Counterstaining with methyl green allows for quantification of normal and apoptotic cells. The numbers of apoptotic cells in the tumors were determined in sections of 250 µm2 with a reticule grid. Apoptotic keratinocytes in inter follicular and follicular epidermis were quantified in 2cm sections. To determine the incidence of follicular apoptosis, hair follicles carrying at least 1 apoptotic cell in the bulge area were counted as a positive hair follicle. In all cases, 12 fields were counted per section on a total of 10 paraffin-embedded sections, representing three mice per genotype.

4.2.5 Two-stage chemical carcinogenesis

For two-stage carcinogenesis, 10-15 mice from each group were initiated with a single topical application of 200 nmol of DMBA in 200ul of acetone on the dorsal mouse skin at 21
days of age. Two weeks after initiation, mice received 4 ug of TPA in 200ul of acetone twice a week for 25 weeks. Skin tumors larger than 1mm in diameter were counted once a week until the end of the experiment at 20 weeks. Multiplicity and incidence of tumor-bearing animals were compared among different groups. Malignant progression to SCC was determined by macroscopic observation and further confirmed by histopathological analysis of paraffin-embedded H&E stained cross sections.

4.3 Results

4.3.1 Generation of K5CDK6/D3−/− mice and biochemical/histological evaluation of mouse epidermis

Cyclin D1 and cyclin D2 have been largely implicated in induction of cell proliferation and contribute to neoplastic development in human and mouse. As the least studied D-type cyclin, cyclin D3 is expressed in nearly all proliferating cells and it shows the most broad expression pattern of all three D-type cyclins (Bartkova et al., 1998). As aforementioned, CDK6 and cyclin D3 transgenic mice showed similarities in skin tumor development and CDK6 seems to preferentially bind to cyclin D3 in mouse keratinocytes. Therefore, to determine whether cyclin D3 is necessary for CDK6-induced epidermal proliferation and suppressed tumor development, we generated K5CDK6/cyclin D3−/− (K5CDK6/D3−/−) compound mice and subject this model to the classic two-stage carcinogenesis protocol. Consistent with previous results, H&E staining of paraffin-embedded epidermis cross
sections showed no obvious modifications in the morphology of follicular and inter follicular epidermis between K5CDK6, cyclin D3\(^{-/-}\), K5CDK6/D3\(^{-/-}\) mice, and their wild-type siblings (Figure 19). Supporting these data, we found no difference in the number of nucleated cells in the inter follicular epidermis among the four genotypes analyzed (Figure 19). The proliferation status of keratinocytes, determined by BrdU incorporation, showed a 3-fold increase in the number of S-phase cells in K5CDK6 mice compared with wild-type mice. On the other hand, cyclin D3\(^{-/-}\) mice do not show a difference in the level of keratinocyte proliferation compared with wild-type siblings. In addition, ablation of cyclin D3 in K5CDK6 background (K5CDK6/D3\(^{-/-}\) mice) did not affect the elevated proliferation triggered by CDK6 overexpression (Figure 20). Thus, we conclude that mouse epidermal keratinocytes proliferate normally in absence of cyclin D3, likely through compensation by other D-type cyclins. Consistent with this idea, lack of cyclin D3 does not affect the proliferation status of K5CDK6 epidermis. We previously showed in chapter II that forced expression of CDK6 triggered follicular and inter follicular apoptosis; therefore, we verified whether the CDK6-induced apoptosis depends on cyclin D3 expression by measuring the number of hair follicles carrying apoptotic cells (incidence of apoptosis in hair follicles). We observed a two-fold increase in the incidence of apoptotic hair follicles in K5CDK6 and cyclin D3\(^{-/-}\) mice compared with wild-type siblings (Figure 21). Interestingly, K5CDK6/D3\(^{-/-}\) compound mice showed cumulative effect and the incidence of apoptosis in the hair follicle is even higher (~3-fold). Supporting our data, it was reported that down regulation of cyclin D3 results in
Figure 19. Skin histology and quantification. (A) Representative paraffin-section of skin from wild-type (wt), cyclin D3−/− (D3−/−), K5CDK6 and K5CDK6/cyclin D3−/− (K5CDK6/D3−/−) siblings were stained with H&E. (B) Number of nucleated cells.
Figure 20. Proliferation in epidermis of K5CDK6/cyclin D3+/− mice. (A) BrdU immunostain of wild-type (wt), D3+/−, K5CDK6 and K5CDK6/D3+/− paraffin-sections. (B) The BrdU label index of the inter follicular epidermis of the four genotypes analyzed.
Figure 21. Quantification of apoptotic labeling index in the hair follicle of wild-type (wt), K5CDK6, cyclin D3+/−(D3+/−) and K5CDK6/cyclin D3+/−(K5CDK6/D3+/−) mice using tunel assay.
increased apoptosis in leukemia cells (Wang, Pavletic et al. 2002). In addition, the altered apoptosis in hair follicles from different groups could explain the differences observed in tumor development between K5CDK6 and wild-type mice (Chapter II). It worth mentioning that the stem cells localized in the bulge region of the hair follicle retain carcinogenes and have been hypothesized as the origin of skin papillomas (Morris, Fischer et al. 1986; Morris, Tryson et al. 2000; Lapouge, Youssef et al. 2011).

Biochemical analysis of mouse epidermis shows that cyclin D3 ablation does not affect transgenic expression of CDK6 in K5CDK6/D3−/− compound mice, which is still much higher than wild-type littermates (Figure 22A). Interestingly, lack of cyclin D3 expression results in a mild increased level of cyclin D2. Moreover, loss of cyclin D3 in K5CDK6 background is associated with a significant increased level of cyclin D2, which suggests compensation among D-type cyclins (Figure 22A). These observations confirm our previous statements that cyclin D2 is regulated by cyclin D3. No differences were observed in the other cell-cycle regulators such as CDK2 and CDK4.

To study whether the absence of cyclin D3 changes the activity of the overexpressed CDK6, we performed an in vitro analysis of CDK6 kinase activities in epidermal lysates from each group. CDK6 activity increased 2.4-fold in K5CDK6 mice compared with wild-type mice, whereas a mild reduction in the cyclin D3−/− mice was observed. Importantly, cyclin D3 ablation in K5CDK6 background reduces the augmented kinase activity, although it remains higher than wild-type (1.6-fold) (Figure 22B). These observations led us to hypothesize that although cyclin D3 plays an important role in the activation of CDK6, other D-type cyclins
could compensate for the absence of cyclin D3. Therefore, we analyzed D-type cyclin/CDKs complex formation. Epidermal lysates from K5CDK6, cyclin D3<sup>−/−</sup>, K5CDK6/D3<sup>−/−</sup>, and wild-type siblings were immunoprecipitated with antibodies against CDK6 followed by Western blot analysis to determine associations with D-type cyclins. However, we did not observe alterations in levels of CDK6/cyclin D2 and CDK6/cyclin D1 among four genotypes analyzed (Figure 22C). Thus, it is clear that lack of cyclin D3 does not result in increased CDK6/cyclin D1 and/or CDK6/cyclin D2 complexes and likely, binding of cyclin D3 to CDK6 stimulates a greater level of CDK6 activity compared to CDK6/cyclin D1 and CDK6/cyclin D2. However, at present the molecular mechanisms that lead to a stronger kinase activity in the CDK6/cyclin D3 complexes is unknown. Moreover, it is unknown the molecular mechanism underlying the inverse correlation between cyclin D2 and cyclin D3 and whether this mechanism is unique for mouse keratinocytes. We conclude that ablation of cyclin D3 reduces the CDK6 kinase activity, but does not affect normal keratinocyte proliferation or CDK6-induced keratinocyte hyperproliferation and apoptosis.

### 4.3.2 Cyclin D3 deficiency reduces skin tumor development and malignant progression

We have previously demonstrated that forced expression of CDK6 results in decreased skin tumor development in a two-stage carcinogenesis protocol. This protocol induces skin papilloma development by a single application of a carcinogen follow by bi-weekly treatment with a tumor promoter agent causing a selection of cells bearing Ha-ras mutations. In order to investigate the role of cyclin D3 in K5CDK6 skin tumorigenesis, we
Figure 22. Biochemical analysis of K5CDK/cyclin D3−/− mouse epidermis. (A) Immunoblot analysis of wild-type (wt), cyclin D3−/− (D3−/−), K5CDK6, K5CDK6/D3−/− epidermal lysates for CDK2,4,6, cyclin D1,D2,D3 and actin as loading control. (B) In vitro kinase assay for CDK6 using pRb peptide as substrate. (C) Epidermis lysates from each group were immunoprecipitated with CDK6 antibody and blotted with antibodies against cyclin D1 and cyclin D2.
challenged the K5CDK6/D3−/− mice and the control mice to the two-stage carcinogenesis protocol. Four groups of mice (K5CDK6, D3−/−, K5CDK6/D3−/− and wild type) were topically treated with a subcarcinogenic dose of the genotoxic carcinogen DMBA and two weeks later promoted bi-weekly applications of TPA for 20 weeks. The incidence and multiplicity of papillomas were scored in each group for 20 weeks. As previously reported in chapter II, after 20 weeks of promotion, K5CDK6 mice displayed a significantly lower number of tumors per mouse compared with wild-type mice (66% reduction, P=0.0015, t-test). Ablation of cyclin D3 also results in decreased number of tumors per mouse (73% reduction, P=0.0001, t-test). K5CDK6/D3−/− mice exhibited a similar tumor multiplicity to cyclin D3−/− mice (72% reduction compared with wild-type mice, P=0.0001, t-test). The incidence of papilloma formation was different among the groups. Wild-type and K5CDK6 mice developed visible tumors within 5 to 7 weeks of promotion (latency), and showed a 50% penetrance by 8.5 weeks and 12 weeks respectively. Papilloma development was significantly delayed in cyclin D3−/− mice, beginning at 10 weeks (Figure 23). Notably, the incidence of papillomas in cyclin D3−/− mice increased rapidly and reached an incidence of 50% by 11 weeks. Importantly, increased CDK6 expression and ablation of cyclin D3 results in increased latency in which the first papillomas were observed at 9 weeks and reduced incidence in which a 50% penetrance was reached at 14 weeks. Upon 20 weeks of promotion, four genotype groups of wild-type, cyclin D3−/−, K5CDK6, and K5CDK6/cyclin D3−/− mice end up with an incidence of 100%, 84%, 79% and 66% respectively (percentage of tumor free mice were 0%, 16%, 21% and 34% respectively). Although K5CDK6/cyclin D3−/−
Figure 23. Kinetic of papilloma formation in K5CDK6/cyclin D3\(^{-}\)/ compound mice. Wild-type(wt), cyclin D3\(^{-}\)/D3\(^{-}\), K5CDK6 and K5CDK6/D1\(^{-}\)/ siblings were initiated with DMBA and promoted with multiple applications of TPA on dorsal mouse skin. (A) Average number of papillomas per mouse as a function of weeks of study (multiplicity). (B) Percentage of tumor-free mice as a function of weeks of study (incidence).
exhibited the most severe delayed tumor development, there is no significant difference among K5CDK6, cyclin D3\(^{-/-}\), and K5CDK6/cyclin D3\(^{-/-}\) mice. As we have observed that K5CDK6, cyclin D3\(^{-/-}\) and K5CDK6/cyclin D3\(^{-/-}\) epidermis has increased hair follicle apoptosis compared with wild-type siblings. Thus we speculated that forced expression of CDK6 and absence of cyclin D3 leads to the decreased papilloma incidence by increasing the number of apoptotic cells in the bulge region of hair follicle. Altogether, our results suggest that increase apoptosis in the hair follicle are responsible for the reduced number of tumors.

Biochemical analysis of skin tumors from the four groups was performed to determine the expression of the main cell cycle regulators during tumor development (Figure 24A). In order to eliminate the influence of the tumor sample quality, we quantified the western blot pictures, and then the digitized bands were normalized to the beta-actin expression (Figure 24B). CDK6 protein levels remain significantly higher in K5CDK6 tumor compared with wild type papillomas regardless the existence of cyclin D3. Differ from what we observed in epidermis, no compensatory expression of cyclin D2 was observed in cyclin D3\(^{-/-}\) tumors or K5CDK6/cyclin D3\(^{-/-}\) tumors. In fact, the expression of cyclin D2 was moderately reduced in both cyclin D3\(^{-/-}\) and K5CDK6/cyclin D3\(^{-/-}\) tumors, even though more samples are required to gain a better statistical conclusion. It is worth mentioning that the papilloma samples have distinct rates of epithelial/stromal/keratinized areas that can potentially mask differences in the level of expression of the keratinocyte fraction. Consistent with our previous reports, in vitro kinase assays show that CDK6 kinase activity is markedly elevated in K5CDK6 papillomas in comparison to wild-type papillomas. Importantly, the CDK6 kinase
activity was not reduced in K5CDK6/cyclin D3−/− tumors compared to K5CDK6 papillomas (Figure 25A). Correspondingly, the proliferation in K5CDK6 and K5CDK6/cyclin D3−/− tumors was both increased 2-fold compared with wild-type tumors (Figure 26). In addition, CDK2 kinase activity is augmented in K5CDK6 tumor, which may contribute to the increased proliferation (Figure 25A). Co-immunoprecipitation demonstrated that this hyperactivation of CDK2 is probably due to the increased CDK6/cyclin D3/p21 complex which releases p21 from CDK2 further increasing its kinase activity (Figure 25B). Therefore, it is tempting to speculate that the non-catalytic role of CDK6 sequestering p21 may play an important role during the promotion stage.

In order to determine if tumor growth is affected by modulating CDK6 and cyclin D3, we measured the size of papillomas from each genotype group. Most of the skin papillomas did not reach more than 50mm³ in the four groups analyzed (~88% of wild-type, 57% of K5CDK6, 87% of cyclin D3−/−, and 95% of K5CDK6/cyclin D3−/− papillomas), but 23.5% of the K5CDK6 tumors are larger than 100mm³ whereas only 8% wild type papillomas reach the size (Figure 27). Moreover, 8% K5CDK6 papillomas are bigger than 300mm³. Thus, we speculate that once the initiated cells overcome the inhibitory barrier at an early stage of tumorigenesis, overexpression of CDK6 leads to faster growth of papillomas compared to wild-type tumors. Cyclin D3−/− mice did not develop any tumor bigger than 100mm³, whereas 8.3% wild-type tumor reach the size of 100mm³, which suggested that the deficiency of cyclin D3 might affect the tumor growth. Supporting this idea, the size distribution of K5CDK6/cyclin D3−/− papillomas resembles the size of wild type tumors, which
Figure 24. Biochemical analysis of K5CDK6/cyclin D3⁻/⁻ tumors. (A) Protein lysates from 20 week papillomas obtained from wild-type (wt), K5CDK6, cyclin D3⁻/⁻(D3⁻/⁻) and K5CDK6/D3⁻/⁻ mice were separated by SDS-PAGE, transferred to nitrocellulose membrane and blotted for CDK2,4,6 and cyclin D1,D2,D3. Actin used as loading control. (B) Quantification of Western blot signals.
Figure 25. In vitro kinase assay and co-immunoprecipitation for CDK6 in tumors. (A) CDK in vitro kinase assays for CDK2 and CDK6 using H1 and pRb peptides as substrates. (B) Lysates from the wt, K5CDK6, D3-/ and K5CDK6/D3-/- tumors were immunoprecipitated with CDK6 antibody and blotted with antibodies against cyclin D1, D3, CDK6, p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1}.
Figure 26. BrdU immunostaining of paraffin-sections of papillomas from wild-type (wt), K5CDK6, cyclin D3⁻/⁻ (D3⁻/⁻) and K5CDK6/D3⁻/⁻ mice (A) and Brdu label index (B).
Figure 27. Skin papillomas were classified according to their volume and depicted as percentage of the total number of tumors for each genotype.
means the ablation of cyclin D3 efficiently impairs the growth advantage induced by CDK6. We conclude that lack of cyclin D3 expression inhibits papilloma development at the initiation or an early stage of tumorigenesis. However, the simultaneous ablation of cyclin D3 and forced expression of CDK6 does not exhibit additive effects in the number of skin papilloma developed. Thus, the inhibitory effect of CDK6 is independent of cyclin D3 expression.

4.4 Discussion

It is widely accepted that D-type cyclins play pivotal roles as proto-oncogenes in a number of human and mouse malignancies via binding and activating CDK4/6. As a catalytic subunit of CDK/cyclin D complex, CDK4,6 are essential regulators of the G1/S phase transition and they have been assumed playing redundant functions. Even so, this concept has been recently challenged by the different phenotypes observed upon ablation of CDK6 and CDK4 in mice (Rane, Dubus et al. 1999; Tsutsui, Hesabi et al. 1999; Hu, Deshpande et al. 2009). We have reported in chapter II that forced expression of CDK6 in mouse skin induces keratinocyte proliferation and apoptosis in hair follicle bulge region, associated with elevated CDK6/Cyclin D3 complex formation. To our surprise, the increased expression of CDK6 significantly inhibits skin tumorigenesis. Similar to our results, forced expression of cyclin D3 in mouse epidermis (K5-cyclin D3 transgenic mice) also exhibit reduced number of papillomas under the same chemically-induced tumor protocol (Rojas, Cadenas et al. 2007). Genetic mouse models have shown that cells proliferate well in the absence of CDKs or
cyclins, which suggests the existence of a redundancy or plasticity in normal cell proliferation by the remaining CDKs and cyclins, possibly through the reassignment of cyclin-CDK complexes or compensation by increased kinase activity (Mataraza, Tumang et al. 2006). It was also demonstrated that the compensation between D-type cyclins exists in cancer cells. As an illustration, cyclin D3 compensates for the loss of cyclin D1 during ErbB2-induced mammary tumor initiation and progression (Zhang Q., Sakamoto K., Liu C.B. et al., 2011). Therefore, we investigated whether ablation of cyclin D3 affects CDK6-induced keratinocyte proliferation and the CDK6-inhibitor role in skin tumorigenesis.

In this chapter, we examined the skin of cyclin D3−/− mice and did not observe any structural and functional abnormality. The total number of proliferating cells in cyclin D3−/− epidermis remains comparable to wild-type mice. Our biochemical analysis of cyclin D3−/− epidermis does not show alteration of cyclin D1 expression, although a moderately increased expression of cyclin D2 was observed. We speculated that the compensatorily elevated cyclin D2 replaces the ablated cyclin D3 and contributes to maintain epidermal homeostasis. In addition, our previous study showed that forced expression of cyclin D3 in mouse skin associated with decreased level of cyclin D2. Likewise, some other groups also reported the increase in cyclin D2 expression in B cell in cyclin D3 deficient mice (Mataraza, Tumang et al. 2006; Peled, Yu et al. 2010). These observations suggest that similar to cyclin D1- and cyclin D2- null mice, ablation of cyclin D3 is well tolerated by keratinocytes. The importance of cyclin D3 to CDK6 functions was evaluated by knocking out cyclin D3 in K5CDK6 background. Lack of cyclin D3 cannot suppress the epidermal hyperproliferation
mediated by CDK6, suggesting that cyclin D3 is dispensable for the CDK6-induced keratinocyte hyperproliferation. In contrast, lack of cyclin D3 results in reduced CDK6 kinase activity in mouse keratinocyte. In addition, the biochemical analysis of K5CDK6/cyclin D3−/− epidermis showed that the ablation of cyclin D3 in K5CDK6 background results in a significant increase of cyclin D2 protein level. We interpret these findings as an indication that cyclin D3 preferentially bind and activate CDK6 and in consequence lack of cyclin D3 reduce the CDK6 kinase activity; however, the D3-independent CDK6 activity (likely mediated by cyclin D2 expression) is enough to support normal keratinocyte proliferation.

We also studied whether cyclin D3 is required for CDK6-mediated inhibition of mouse skin tumorigenesis. Overexpression of CDK6 significantly reduces both the average number of papillomas per mouse (multiplicity) and the number of mice with at least one papilloma (incidence). We found that similar to cyclin D1- and cyclin D2- null mice, lack of cyclin D3 also leads to a significant reduction of papilloma incidence and multiplicity. Similar to our observation, it has been reported that down-regulation of cyclin D3 induces cell cycle arrest and apoptosis (Izumi T, Hiroyuki H and Takashi S, 2005; (Wang, Pavletic et al. 2002; Chen, Glasser et al. 2011). Thus we hypothesized that the elevated apoptosis is responsible for the inhibited papilloma development. K5CDK6/cyclin D3−/− epidermis exhibit similar susceptibility to chemically-induced skin carcinogenesis as K5CDK6 and cyclin D3−/− mice. The fact that forced expression of CDK6 leads to diminished number of tumors independently of the cyclin D3 level suggests the existence of two independent inhibitory mechanisms. Supporting this notion, we showed that CDK6-mediated apoptosis is stronger upon absence
of cyclin D3. Moreover, even though the differences in incidence (and latency) are not statistically significant, the trend shows that simultaneous overexpression of CDK6 and ablation of cyclin D3 result in a robust reduction of tumorigenesis. Therefore, it is tempting to speculate that forced expression of CDK6 led to increased apoptosis and lack of cyclin D3 might result in reduced proliferation leading to none or reduced activation of the hair follicle bulge stem cells. The analysis of tumor size showed that tumorigenesis process is also affected during the promotion stage (tumor growth) in K5CDK6 mice. We noticed that 23.5% of the K5CDK6 tumors are larger than 100mm³ whereas only 8% wild type papillomas reach the size. Moreover, 8% K5CDK6 papillomas are bigger than 300mm³. Thus, we speculated that once the initiated cells overcome the inhibitory barrier at an early stage of tumorigenesis, overexpression of CDK6 leads to faster growth of papillomas compared to wil-type tumors. This is supported by the fact that proliferation in K5CDK6 tumor was increased 2-fold, along with augmented CDK6 and CDK2 kinase activities. Cyclin D3⁻/⁻ mice did not develop any tumor bigger than 100mm³ . In addition, the size of K5CDK6/D3⁻/⁻ papillomas resembles the distribution observed in wild type mice. We suggest that the deficiency of cyclin D3 might also affect the tumor development by inhibiting tumor growth, as well as impairing the growth advantage induced by CDK6.

In summary, our data suggested that CDK6 might play different roles in different stage of tumorigenes- During the early stage, CDK6 exhibits a cyclin D3-independent tumor inhibition, whereas, once the initiated cell overcome the inhibitory barrier, CDK6 favors the tumor growth which is cyclin D3-dependent.
CHAPTER V

General Discussion
5.1 General Discussion

The pRb/p16/cyclin D/CDK4,6 pathway has been implicated in proliferation and tumorigenesis for more than two decades. Whereas a positive role of CDK4 was clearly established in mouse skin tumor (Rodriguez-Puebla, Miliani de Marval et al. 2002; Miliani de Marval, Macias et al. 2004), the question remained whether CDK6 is a mediator of keratinocyte transformation by oncogenic ras. The fact that both CDK4 and CDK6 bind to D-type cyclins, have the same substrates and share 71% amino acid identity leads us to assume that they play redundant roles in G1 phase of the cell cycle. Supporting the assumption of their functional compensation behavior, Malumbres et al showed that mice lacking both CDK4 and CDK6 died during embryonic development. However, this assumption is argued against by the fact that CDK4-null mice exhibit growth retardation, reproductive dysfunction and insulin deficient diabetes, whereas CDK6-deficient mice show pronounced thymic atrophy and reduced susceptibility to lymphomagenesis. Studies based on experimental and human tumors also suggested similarities and differences between these two kinases. Therefore, we proposed to generate a transgenic animal model with forced expression of CDK6 and elevated CDK6-associated kinase activity to investigate the role of CDK6 in both normal epidermis and skin tumor development. Similar to CDK4, forced expression of CDK6 in mouse epidermis induces hyperproliferation in basal epidermal keratinocytes. However, no hyperplasia of epidermis was observed due to an elevated apoptosis in both hair follicle and inter hair follicle keratinocyte, which is unique in K5CDK6
epidermis. By using the two-stage carcinogenesis protocol which consists of the initiation of stem cells localized in the bulge region of the hair follicle, followed by the promotion of initiated cells, we showed that K5CDK6 transgenic mouse has a significantly reduced number of papillomas in comparison to their wild-type siblings, which is in contrast to the K5CDK4 mice. Both CDK4 and CDK6 play positive roles in Rb pathway; however, only K5CDK4 mice exhibit an accelerated rate of malignant progression. We suggest that the forced expression of CDK6 inhibits tumorigenesis through inhibiting the initiation of cells or the clonogenic expansion of the initiated cells.

As the regulatory partners, D-type cyclins are implicated in tumorigenesis and their roles have been widely discussed. It has been suggested that the existence of compensatory mechanisms among the three D-type cyclins. That is why the efficacy of targeting individual D-type cyclin in fighting cancer is usually unsatisfactory. Therefore, the simultaneous regulation of multiple cyclins in tumor cells has been suggested to reduce cancer cell proliferation in vitro and decrease the tumor burden in vivo. Our previous results have established that cyclin D1 and D2 contribute to the development of tumors (Robles, Rodriguez-Puebla et al. 1998; Rodriguez-Puebla, Robles et al. 1999; Rojas, Cadenas et al. 2007). In contrast, the forced expression of cyclin D3 inhibits skin tumorigenesis by downregulating cyclin D2 probably through a negative feedback loop. In chapter 3, we developed the K5D3/cyclin D1−/− mouse model, which allows us to determine the effect of forced expression of cyclin D3 combined with absent or reduced expression of the other two D-type cyclins. No defects in normal keratinocyte proliferation and differentiation was
observed in K5D3/cyclin D1<sup>−/−</sup> mouse, which suggested that expression of cyclin D3 combined with minimal or absent expression of cyclin D1,D2 is sufficient to support epidermal homeostasis. However, after subjected to a two-stage carcinogenesis protocol, K5D3/cyclin D1<sup>−/−</sup> mice significantly lost the susceptibility to skin tumor formation. We concluded that simultaneous ablation of cyclin D1 and downregulation of cyclin D2 via cyclin D3 overexpression could efficiently inhibit ras-mediated tumor development. Moreover, our findings provided the evidence that combined modulation of multiple members of D-type cyclins might be a suitable strategy for tumor prevention and therapy.

In addition to the novel role of CDK6 in mouse epidermis, we also noticed the increased formation of CDK6/cyclin D3 complex in mouse skin overexpressing cyclin D3 or CDK6 (Rojas, Cadenas et al. 2007). Then we would ask if CDK6/cyclin D3 complexes mediate the inhibited tumor formation observed in K5D3 and K5CDK6 transgenic mice. To address this question, we generated K5CDK6/cyclin D3<sup>−/−</sup> compound mice. Similar to cyclin D1- and cyclin D2- null mice, ablation of cyclin D3 is well tolerated by keratinocytes. Moreover, the lack of cyclin D3 cannot suppress the epidermal hyperproliferation mediated by CDK6, suggesting that cyclin D3 is dispensable for the CDK6-induced keratinocyte hyperproliferation. Upon a two-stage carcinogenesis protocol, cyclin D3<sup>−/−</sup> mice developed fewer papillomas then wild-type siblings. In addition, the sizes of cyclin D3<sup>−/−</sup> papillomas are generally smaller than wild-type papillomas. We conclude that similar to cyclin D1 and cyclin D2, cyclin D3 is a mediator of keratinocyte transformation by oncogenic ras. The multiplicity and incidence among cyclin D3<sup>−/−</sup>, K5CDK6 and K5CDK6/cyclin D3<sup>−/−</sup> mice are comparable,
suggesting that CDK6 plays it tumor inhibition role independent of cyclin D3. Interestingly, tumor developed in K5CDK6 mice were bigger than wild type mice, whereas the size of K5CDK6/cyclin D3−/− papillomas resembles that of wild-type, suggesting that ablation of cyclin D3 efficiently impairs the growth advantage induced by CDK6. We conclude that overexpression of CDK6 may play different roles during different carcinogenesis stages: by affecting cell survival upon genotoxic stress (DMBA treatment), CDK6 plays an inhibitory role during the initiation process, which is cyclin D3 independent; on the other hand, once the initiated cells successfully overcome the inhibitory barrier in early stage, overexpressed CDK6 leads to faster papilloma growth in a cyclin D3-dependent manor.

A central part of the inhibitory activity of cyclin D3 seems to be mediated via the regulation of cyclin D2, in fact overexpression of cyclin D3 results in strong reduction of cyclin D2 and cyclin D3-ablation led to a mild increase of cyclin D2 level. The molecular mechanisms mediating this feed-back loop remained unknown, however it is worth mentioning that Cyclin D3 interacts with several transcription factors and modulates their transcriptional activations (Liu, Sun et al. 2004; Jian, Yan et al. 2005). Some of these transcription factors (VDR, ATF, STAT) are indirectly involved in cyclin D2 regulation (White, Shore et al. 2006). Thus, we speculate that some of these transcription factors might be involved in the negative feed-back loop regulating cyclin D3 and cyclin D2.
Bibliography


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Appendix I

Exploration of the molecular mechanisms by which the CDK6/cyclin D3 play a suppressive role in epidermis
Introduction

In the last two decades, the role of CDK6 and CDK4 regulating the pRb pathway has been widely described by multiple groups (Sherr 1995; Sherr 1996; Nevins 2001). However, recently several independent studies have indicated novel roles of CDK6 that are not shared for CDK4 (review in (Grossel and Hinds 2006; Grossel and Hinds 2006)). Our studies on cell cycle deregulation in mouse skin tumorigenesis have shown that CDK4 plays an important role in malignant progression to SCCs (Miliani de Marval, Gimenez-Conti et al. 2001)(Miliani de Marval, Gimenez-Conti et al. 2001; Rodriguez-Puebla, Miliani de Marval et al. 2002; Miliani de Marval, Macias et al. 2004; Miliani de Marval, Macias et al. 2004)(Wang, Sistrunk et al. 2011). Nonetheless, the results generated in the last chapters have revealed a unique role for CDK6 and its partner cyclin D3, which - as oppose to other G1 CDKs - led to inhibition of skin tumor development (Miliani de Marval, Macias et al. 2004; Rojas, Cadenas et al. 2007; Wang, Sistrunk et al. 2011). Using genetically modified animals we have demonstrated that the tumor suppressive activity of CDK6 is associated with increased apoptosis affecting an early stage of tumor development (Wang, Sistrunk et al. 2011). These results are compelling and support earlier observations that suggest that this particular kinase may play additional roles in cell differentiation of a variety of cell types (Grossel, Baker et al. 1999; Grossel and Hinds 2006; Grossel and Hinds 2006; Hu, Deshpande et al. 2009). We have recently reported that the putative tumor suppressor activity of CDK6/cyclin D3 complex is associated with elevated apoptosis in inter follicular epidermis.
and hair follicle keratinocytes stem cells (KSCs). Therefore, our studies led us to hypothesize that CDK6 plays a unique tumor suppressive function in mouse epidermis associated with elevated apoptosis.

**Materials and Method**

**Adult Keratinocyte Preparation and Flow Cytometry**

Dorsal skin from 7 to 8 week old wild type or K5CDK6 transgenic mice were collected, scrap fat pad with scalpel. Cut pelts into small sections (1cm x 1.5cm) and float on 0.25% Trypsine (No EDTA) for 1.5 hour at 37°C. Scrap the epidermis with scalpel, collect in low Ca2+ EMEM with 10% serum, and stir slowly for 30 minutes. Filter the epidermis through nylon meshes, collect filtrate in a 50ml centrifuge tube and centrifuge for 3 to 5 minutes at 1000 r.p.m. with break on. Carefully remove supernatant the resuspend cell pellet and repeat the last two steps. ~1x10^6 isolated adult keratinocytes were incubated with primary anti-CD34 (eBioscience) antibody for 1 hour at room temperature (following the manufacture's instruction). Cells were rinsed, and then incubated with FITC secondary antibody at recommended dilution for 30 minutes at room temperature. Rinse and resuspend cells in 0.5 ml PBS and analyzed on flow cytometer. Propidium iodide was used to indicate live cells and only live cells were proceed for gating. Use 490 to 520nm filter to gate EGFP positive and negative cells. Samples incubated without secondary antibody was set as negative control.
Western Blotting

The tumor samples were homogenized in lysis buffer as we previously described (Chaper II). The homogenate was centrifuged at 14000 x g at 4°C to collect the supernatant, which was used directly for western blotting analysis or stored at -80°C. The protein concentration was measured with the Bio-Rad protein assay system (Bio-Rad laboratories, Richmond, CA). Protein lysates (25µg from each sample) were electrophoresed through 12% acrylamide gels and electrophoretically 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 p53 (1C12; Cell Signalling Inc.), p15(Ab-3; NeoMarker), p16 (M-156), p21 (M-19), TRF1 (H-242) and p130 (C-20; Santa Cruz Biotech Inc.)

Immunostaining

Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin wax. Tissue cross-sections of epidermis and skin papilloma were immunostained with antibodies for CDK6 (C-21), Samples were then incubated with Alexa Fluor secondary antibodies for immunofluorescence (Molecular Probes) and visualized under fluorescence microscope using a 465 to 495 nm filter.
Results

Role of CDK6 in hair follicle stem cells (KSCs)

We have established that forced expression of CDK6 led to reduced number of papillomas per mouse. We also found that K5CDK6 mice display elevated incidence of hair follicles carrying apoptotic cells in the bulge area and elevated number of apoptotic cells per bulge area (Chapter II, IV). In this regard, pioneer investigations showed that label retaining cells (LRCs) in the bulge region of the hair follicle (HF) retain carcinogen-DNA adducts supporting the concept that keratinocytes stem cells (KSCs) of the bulge region are in part responsible for the skin papilloma development (Morris, Fischer et al. 1986; Kangsamaksin, Park et al. 2007). Thus, our findings suggest that the CDK6-mediated apoptosis might decrease the number of initiated cells. Alternatively, CDK6 expression might affect the clonal expansion of initiated cells, with the consequent reduction in the number of papillomas. Thus, we determined whether CDK6 expression in bulge region affect the population of KSCs. We isolated adult keratinocytes from 7-8 week-old K5CDK6 transgenic mice and wild-type siblings, and stained them with antibody against CD34, a cell surface marker expressed in KSCs (Trempus, Morris et al. 2003). Flow cytometry analysis of K5CDK6 keratinocytes showed a 2-fold increase in the proportion of KSC (4.7%) compared with wild-type counterparts (2.5%) (Figure 28). We speculated that modification in CDK6 expression affect asymmetric cell division of KSCs. In such forced expression of CDK6 favors the increase of stem cell pool, and consequently reduce the transit amplifying cell (TA) cell
Figure 28. Flow cytometry analysis of Keratinocyte Stem Cell (KSC) from K5CDK6 and wild-type (wt) littermates.
pool. Although premature, we can speculate that overexpression of CDK6 may impact the bulge region by favoring cell renewal (increased number of bulge stem cell), but reducing the number of TA cells susceptible to be amplified upon TPA promotion. In addition, CDK6-mediated apoptosis can also impair TPA-mediated activation/amplification of the initiated cells. Therefore, this initial set of experiments warrant further investigation of the early mechanisms of skin tumor initiation.

**Molecular mechanisms involved in CDK6-induced apoptosis**

The role of cyclin-dependent kinases inducing apoptosis has been previously considered for CDK2 and CDK6 (Gil-Gomez, Berns et al. 1998; Choi, Eom et al. 1999; Hakem, Sasaki et al. 1999; Ojala, Tiainen et al. 1999; Ojala, Yamamoto et al. 2000; Maddika, Ande et al. 2008). Notably, the v-cyclin-CDK6 complex induces apoptosis through the phosphorylation and inactivation of the anti-apoptotic function of Bcl-2 (Ojala, Yamamoto et al. 2000). Recent reports have demonstrated a new aspect of CDK2-induced apoptosis by AKT activity. PI3K/AKT pathways cause cytoplasmic distribution of CDK2 –through phosphorylation of CDK2-Thr39 – that is essential for the CDK2-induced apoptosis (Maddika, Ande et al. 2008). Similarly, we looked into the subcellular distribution of CDK6 in epidermis and skin tumors. We found cytoplasmic distribution of CDK6 in the skin papillomas and proliferative areas of anagen hair, whereas nuclear localization was observed in interfollicular epidermis (Figure 29). Therefore, we ask whether CDK6-induced apoptosis is correlated with its subcellular distribution.
Figure 29. CDK6 distribution in mouse keratinocytes. CDK6 expression was detected by immunofluorescence analysis in paraffin sections of mouse papillomas at 20 weeks of promotion (A) and untreated mouse skin (B). Nuclear localization of CDK6 was observed in mouse inter follicular epidermis (B, I), whereas cytoplasmic distribution of CDK6 was observed in papillomas (A) and hyperproliferative areas of the hair follicle (B, II).
Global analysis of protein expression in K5CDK6 epidermis

As part of the global characterization of the K5CDK6 mouse model and to investigate whether forced expression of CDK6 alters other pathways, we chose to study the pattern of protein expression by using an Antibody Microarray (Panorama Antibody Microarray-Cell Signaling, Sigma-Aldrich). Briefly, protein lysate from K5CDK6 and wild type epidermis were labeled with Cy™ dye (Cy™3 for K5CDK6 and Cy5 for wild type samples). The data were normalized using actin as reference or by summed fluorescence intensities with Scan Array Express Software (ScanArray Express, Perkin Elmer Life Science) (Table II). CDK6 protein level was found elevated 2.5-fold in transgenic mice; thus, we only reflected as significant the differences equal or higher than 2-fold increase or decrease in the K5CDK6 sample. We found that 15 proteins were elevated in K5CDK6 epidermis (Table II), among them several regulators of cell proliferation (cyclin A, Cdc7 Kinase) and senescence (p16INK4a, p21CIP1, ARF, TRF-1).
Table II: Antibody Microarray.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Area</th>
<th>Fold Increase in K5CDK6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDK6</td>
<td>Cell Cycle</td>
<td>2.53±0.20</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>Cell Cycle</td>
<td>2.20±0.33</td>
</tr>
<tr>
<td>ARF</td>
<td>Cell Cycle</td>
<td>3.38±1.14</td>
</tr>
<tr>
<td>P16^{ink4a}</td>
<td>Cell Cycle</td>
<td>2.10±0.32</td>
</tr>
<tr>
<td>P21^{Cip1}</td>
<td>Cell Cycle</td>
<td>1.92±0.21</td>
</tr>
<tr>
<td>Trf-1</td>
<td>Nuclear</td>
<td>2.25±0.11</td>
</tr>
<tr>
<td>HSP 70</td>
<td>Cell Stress</td>
<td>2.72±0.35</td>
</tr>
<tr>
<td>Cytokeratin 8.12</td>
<td>Cytoskeleton</td>
<td>2.10±0.16</td>
</tr>
<tr>
<td>Cytokeratin 8.13</td>
<td>Cytoskeleton</td>
<td>2.19±0.12</td>
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<tr>
<td>Cytokeratin pep 4</td>
<td>Cytoskeleton</td>
<td>3.06±0.03</td>
</tr>
<tr>
<td>MAP2(2a+2b)</td>
<td>Cytoskeleton</td>
<td>3.05±0.20</td>
</tr>
<tr>
<td>Vinculin</td>
<td>Cytoskeleton</td>
<td>3.05±0.20</td>
</tr>
<tr>
<td>S-100 b</td>
<td>Neurobiology</td>
<td>3.26±0.02</td>
</tr>
<tr>
<td>PKB/AKT</td>
<td>Signal Transduction</td>
<td>2.59±0.29</td>
</tr>
<tr>
<td>Cdc7 kinase</td>
<td>Signal Transduction</td>
<td>2.16±0.02</td>
</tr>
</tbody>
</table>

Fold increase in protein lysates from K5CDK6 epidermis compared to wild type siblings. The fluorescence intensity was normalized by two independent methods with similar results (actin as reference and by summed fluorescence intensity).
Upregulation of senescence markers in K5CDK6 skin tumors

Cellular senescence is a physiological mechanism for thwarting the proliferation of tumor cells. Thus, it has been hypothesized that encouraging cancer-prone cells to senesce might be a way to attack cancer in early stages. Senescence-inducing stressors inhibit G1 CDKs by increasing the expression of the CDK inhibitors, which include p16\textsuperscript{Ink4a}, p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1}(Collado, Blasco et al. 2007; Malumbres and Barbacid 2009). Consistent with this data, it has been shown that inhibition of CDK2 and CDK4 is crucial for establishing senescence (Collado, Blasco et al. 2007). Our results suggest that part of the tumor suppressive activity triggered by CDK6 might be mediated by cellular senescence. Therefore, we evaluated whether K5CDK6 papillomas expressed classical markers of senescence such as the CKIs and TRF-1. We have previously reported that skin papillomas from wild type mice display increased expression of p16\textsuperscript{Ink4a} and p15\textsuperscript{Ink4b} compared to normal skin(Rodriguez-Puebla, LaCava et al. 1998). We found that papillomas from K5CDK6 mice have a high level of p15\textsuperscript{Ink4b}, p16\textsuperscript{Ink4a} and p21\textsuperscript{Cip1}, but not TRF1, compared to wild type tumors (Figure. 30). Analysis of mouse epidermis from transgenic and wild type mice showed almost undetectable levels of p16\textsuperscript{Ink4a} and p15\textsuperscript{Ink4b} (data no shown), suggesting that these markers are normally upregulated during the carcinogenesis process, and that forced expression of CDK6 enhances their expression.
Figure 30: Biochemical analysis of senescence markers in papillomas. (A) Western blot analysis of mouse skin papillomas showing elevated expression of p16\textsuperscript{Ink4a}, p15\textsuperscript{Ink4b} and p21\textsuperscript{Cip1} in K5CDK6 tumors compared with wild type tumors. WT, wild type papillomas; K6, K5CDK6 papillomas. (B) Quantification of the protein levels from two K5CDK6 and two wild type tumors. The values were normalized with actin protein level.
Discussion

Our studies with the K5CDK6 transgenic mouse model showed that CDK6 expression led to increased proliferation, apoptosis and senescence, and thus favoring tumor inhibition. Our results support the hypothesis that CDK6 inhibits an early stage of tumorigenesis leading to reduced number of “initiated” cells or fail in the clonal expansion of initiated cells resulting in reduced number of tumors. Thus, future directions should include analysis of whether CDK6 expression disturbs the initiation stage (characterized by a Ha-Ras mutation in codon 61) and/or the clonal expansion of the initiated cell in the two-stage carcinogenesis protocol (DMBA/TPA regimen) by determing the effect of CDK6 overexpression in the frequency of Ha-ras mutation (mutation carried by 90% of skin papillomas initiated by DMBA). Although this work is in the preliminary stages, we have shown that several links exist between CDK6 expression and other potential pathways that might result in inhibition of an early stage of the skin tumorigenesis, such as initiation or clonogenic expansion of the initiated cells. We expect that upon progress in these directions, we will understand the novel role of CDK6 in skin tumorigenesis to better approach CDK6 as a therapeutic target.