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

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Abbreviations: DMBA, 7,12-dimethylbenz(a)anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; K5D3, keratin 5-cyclin D3 transgenic mouse; SCC, squamous cell carcinoma; CDK, cyclin-dependent kinase

We have previously demonstrated that *ras*-mediated skin tumorigenesis depends on signaling pathways that act preferentially through cyclin D1 and D2. Interestingly, the expression of cyclin D3 inhibits skin tumor development, an observation that conflicts with the oncogenic role of D-type cyclins in the mouse epidermis. Here, we show that simultaneous up and downregulation of particular members of the D-type cyclin family is a valuable approach to reduce skin tumorigenesis. We developed the K5D3/cyclin D1$^{-/-}$ compound mouse, which overexpresses cyclin D3 but lacks expression of cyclin D1 in the skin. Similar to K5D3 transgenic mice, keratinocytes from K5D3/cyclin D1$^{-/-}$ compound mice show a significant reduction of cyclin D2 levels. Therefore, this model allows us to determine the effect of cyclin D3 expression when combined with reduced or absent expression of the remaining two members of the D-type cyclin family in mouse epidermis. Our data show that induced expression of cyclin D3 compensates for the reduced level of cyclin D1 and D2, resulting in normal keratinocyte proliferation. However, simultaneous ablation of cyclin D1 and downregulation of cyclin D2 via cyclin D3 expression resulted in a robust reduction in ras-mediated skin tumorigenesis. We conclude that modulation of the levels of particular members of the D-type cyclin family could be useful to inhibit tumor development and, in particular, ras-mediated tumorigenesis.

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.[1-4] 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.[5,6] 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.[7-9] Similarly, abnormalities in the cyclin D2 gene have also been observed in testicular tumors[10,11] and B-cell malignancies.[12] Moreover, overexpression of cyclin D3 has been found in several human cancers,[13-16] such as malignancies of the thymus.[17]

In addition to its growth-promoting functions, cyclin D3 plays a unique, nonredundant and tissue-specific role in muscle differentiation[18,19] as well as during advanced stages of differentiation in the epithelia of the stomach, intestine and gallbladder.[20]

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.[21-25] These studies established that cyclin D1 and D2 are upregulated in mouse skin papillomas and squamous cell carcinomas (SCC), whereas cyclin D3 protein levels remain constant in both normal epidermis and tumors.[21] 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.[21-23,26-29] In contrast, the forced expression of cyclin D3 inhibits skin tumorigenesis, an effect that is mediated by the simultaneous downregulation of cyclin D2.[29]

In this paper, we tested the hypothesis that the simultaneous up- and downregulation of individual D-type cyclins is a valuable approach to inhibit skin tumorigenesis. We used the

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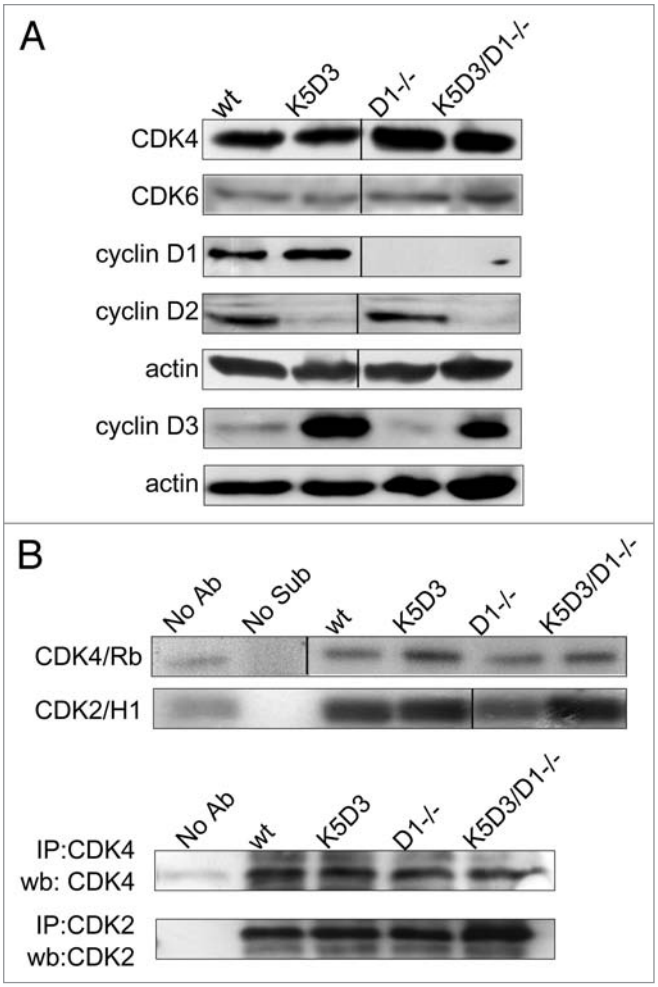

Figure 1. Protein gel blot analysis of cell cycle proteins from mouse epidermis. (A) Protein lysates of epidermis samples from wild-type (wt), K5D3, cyclin D1$^{-/-}$ (D1$^{-/-}$) and K5D3/D1$^{-/-}$ siblings were separated using sodium dodecyl sulfate-PAGE and blotted onto a nitrocellulose membrane. 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 (A and B). (B) 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. Protein gel 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 of (A and B) to denote rearrangements of the lines in order to be consistent between the parts.

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.

Results

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.[30] 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.[29] We also established that cyclin D1$^{-/-}$ mice display a 75% reduction in the number of skin papillomas.[31] 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$^{-/-}$ compound mouse. Similar to cyclin D1$^{-/-}$ mice, K5D3/cyclin D1$^{-/-}$ mice show growth retardation and a neurological abnormality evidenced by limb retraction when lifted by their tails that is most pronounced by three weeks.[32,33] To determine whether forced expression of cyclin D3 also results in cyclin D2 downregulation in the cyclin D1$^{-/-}$ background, we performed a biochemical analysis of epidermal extracts from K5D3, cyclin D1$^{-/-}$, K5D3/cyclin D1$^{-/-}$ and wild-type mice. As expected, cyclin D3 protein levels increased 6- to 8-fold in the K5D3 and K5D3/cyclinD1$^{-/-}$ epidermis compared with their wild-type siblings (**Fig. 1A**). Similar to K5D3 mouse, K5D3/cyclin D1$^{-/-}$ epidermis showed 30-fold reduction in cyclin D2 protein levels compared with wild-type mice (**Fig. 1A**). Notably, cyclin D1-null epidermis does not show compensatory changes in cyclin D2 and cyclin D3 protein levels (**Fig. 1A**). Additionally, the D-type cyclin partners CDK4 and CDK6 show no changes in 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$^{-/-}$ and K5D3/cyclin D1$^{-/-}$ mice. The overexpression of cyclin D3 in the epidermis showed 2-fold

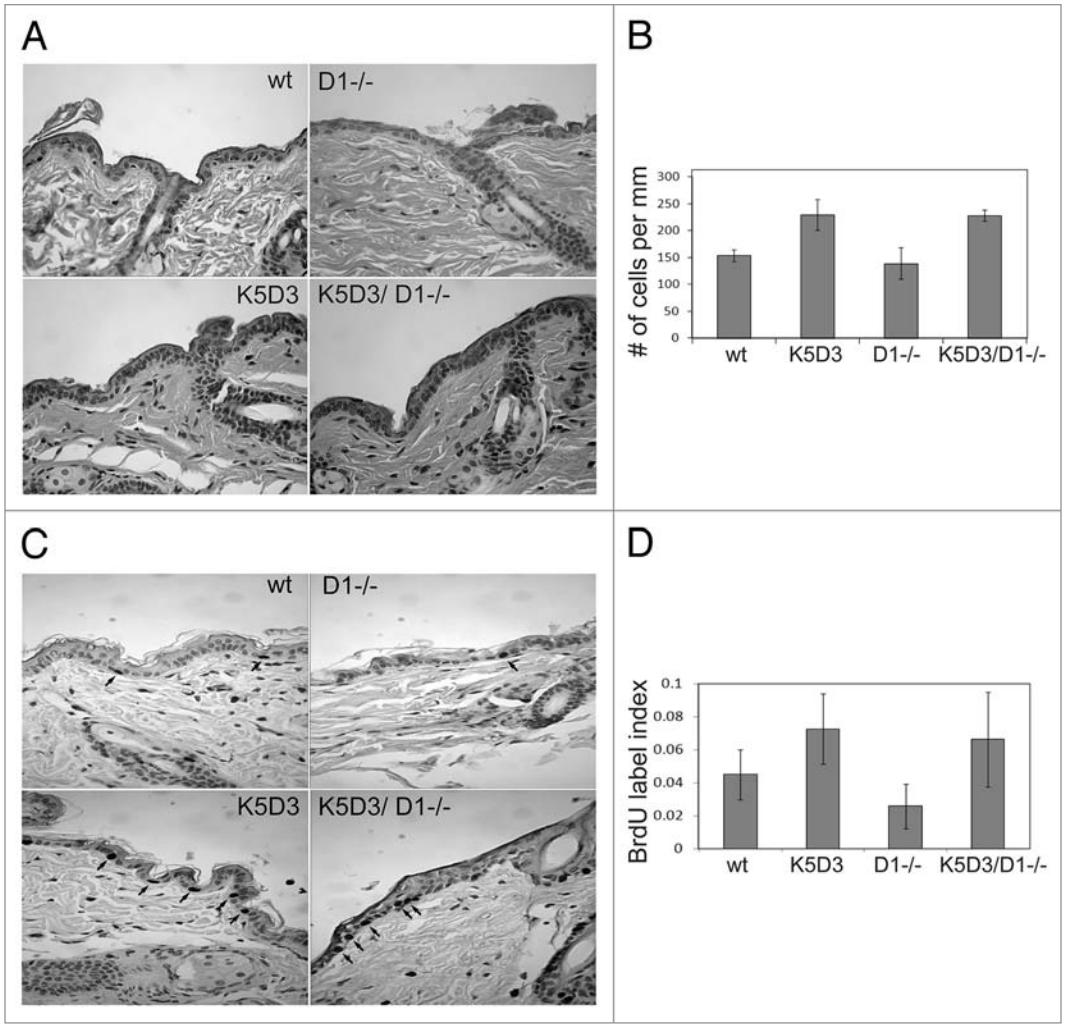

Figure 2. Skin phenotype of K5D3/cyclin D1$^{-/-}$ compound mice. (A) Representative paraffin-sections of skin from wild-type (wt), D1$^{-/-}$, K5D3 and K5D3/D1$^{-/-}$ siblings were stained with H&E. (B) Quantification of the total number of epithelial cells in the interfollicular epidermis of the four genotypes analyzed. (C) BrdU immunostaining of wild-type (wt), D1$^{-/-}$, K5D3 and K5D3/D1$^{-/-}$ paraffin-sections. Arrows indicate BrdU-positive cells. (D) The BrdU label index of the interfollicular epidermis of the four genotypes analyzed.

increase in CDK4 kinase 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 [29] (**Fig. 1B**). 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 (**Fig. 1B**). 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.[34,35] 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 affect 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 (**Fig. 2**). Hematoxylin and Eosin (H&E)-stained sections showed that both K5D3 and K5D3/cyclin D1$^{-/-}$ epidermis exhibit increased thickness associated with an increased number of keratinocytes compared with wild-type samples (**Fig. 2**) (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 interfollicular epidermis between K5D3 and K5D3/cyclin D1$^{-/-}$ littermates (**Fig. 2**). Thus, the expression of cyclin D3 appears to be sufficient to compensate for the lack of cyclin D1 and the severe downregulation 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

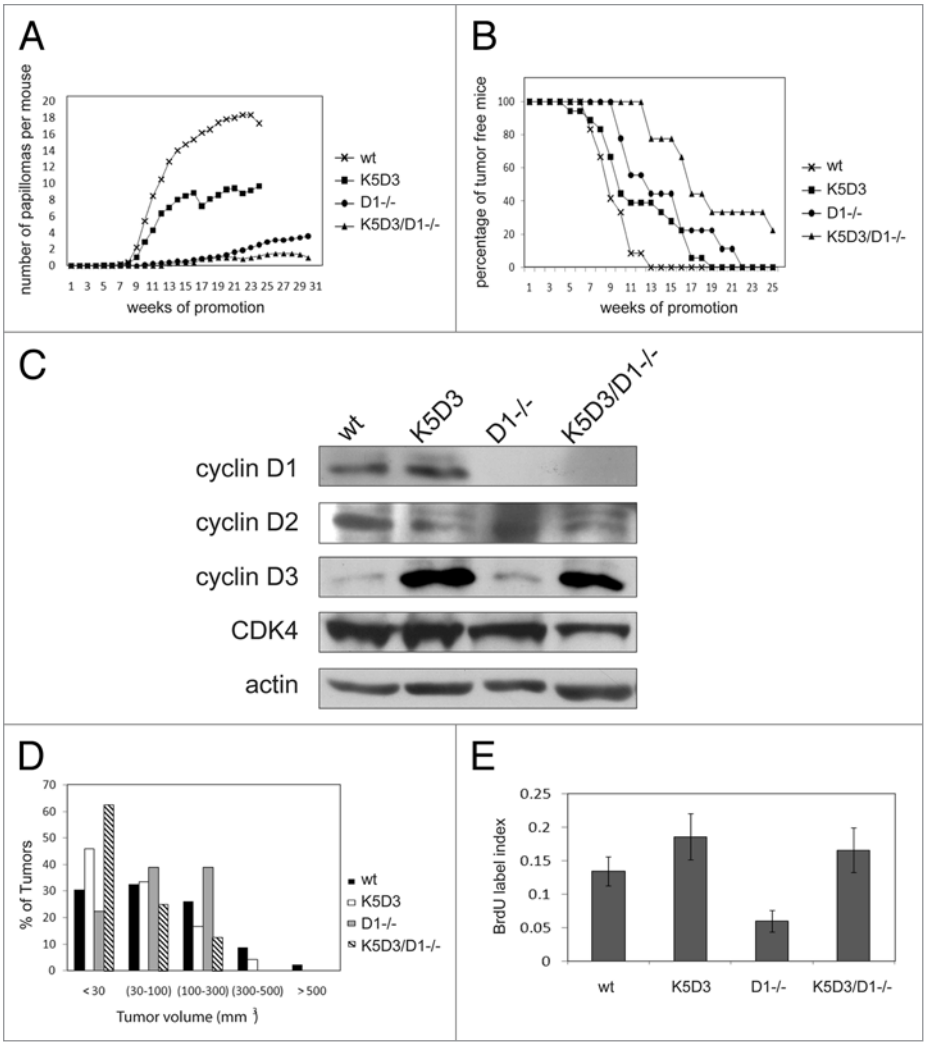

Figure 3. Kinetics of papilloma formation and biochemical analysis of skin tumors. Wild-type (wt), K5D3, Cyclin D1$^{-/-}$ (D1$^{-/-}$) and K5D3/D1$^{-/-}$ siblings were initiated with DMBA and promoted with multiple applications of TPA on dorsal mouse skin. (A) Average number of papillomas per mouse (multiplicity) as a function of weeks of study. (B) Percentage of tumor-free mice as a function of weeks of study (incidence). (C) 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 control. (D) Skin papillomas were classified according their volume and depicted as percentage of the total number of tumors for each genotype. (E) BrdU label index of immunostained paraffin-sections of papillomas from wild-type (wt), K5D3, cyclin D1$^{-/-}$ and K5D3/D1$^{-/-}$.

increased keratinocyte proliferation, we analyzed the BrdU incorporation in the interfollicular epidermis. Consistent with previous observations,[30] 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 with wild-type siblings ($p < 0.0001$, t-test) (**Fig. 2C**). 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 interfollicular epidermis and hair follicles.

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 induced by chemical carcinogenesis.[29,31] Similarly, transgenic expression of cyclin D3 also decreased skin tumorigenesis as a result of cyclin D2 down-regulation.[29] 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.[29,31] 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 in reference 29, after 24 weeks of promotion, K5D3 mice displayed a significantly lower number of tumors per mouse compared with wild-type mice (45% reduction, $p = 0.03$, t-test) (**Fig. 3A**). 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 with wild-type mice and 36% compared with cyclin D1$^{-/-}$ mice) (**Fig. 3A**). 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) (**Fig. 3A**). 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, tumor development in K5D3/cyclin D1$^{-/-}$ mice was severely delayed, beginning at week 13 and reaching an incidence of 50% by week 16.5 (**Fig. 3B**). The size of the

tumors also varied among the genotypes. Wild-type papillomas were the largest, reaching of up to 300 mm^3. Conversely, the K5D3/cyclin $D1^{-/-}$ papillomas were much smaller than the other groups, and 63% of the tumors never reached a volume of 30 mm^3 (**Fig. 3D**). The tumor size of the cyclin $D1^{-/-}$ and K5D3 animals was between the size of the wild-type and the K5D3/cyclin $D1^{-/-}$ papillomas (**Fig. 3D**). 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 5-fold reduction compared with wild-type mice, respectively) (**Fig. 3C**).

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) (**Fig. 3E**). Notably, overexpression of cyclin D3 appeared to compensate for the lack of cyclin D1, as K5D3/cyclin $D1^{-/-}$ exhibited a comparable number of proliferative keratinocytes as shown by wild-type and K5D3 tumors (**Fig. 3E**). 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.[36] 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 as 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 with the malignant progression observed in wild-type tumors.

Table 1. Histopathological analysis of skin tumors

Mice	No of tumors/ group	No (%) of tumors classified as:	
		Papilloma[a]	SCC[b]
Wild type	16	8 (50)	8 (50)
K5D3	12	6 (50)	6 (50)
Cyclin $D1^{-/-}$	9	9 (100)	0 (0)
K5D3/Cyclin $D1^{-/-}$	14	14 (100)	0 (0)

[a]No atypia in basal layers, basal cell hyperplasia, mild acanthosis and hyperkeratosis and no invasion of epidermal cells into the dermis.
[b]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.

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 feedback loop as a tumor suppressor mechanism that prevents ras-induced carcinogenesis.

Discussion

We have previously reported that overexpression and/or ablation of D-type cyclins results in varied sensitivities to chemically induced mouse skin tumors.[27,29,37,38] 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.[29,31] In fact, the ablation of cyclin D1 and cyclin D2 results in a 75% and 45% reduction in the number of skin papillomas, respectively.[29,31] 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.[27,29] In contrast, overexpression of cyclin D3 in mouse keratinocytes decreased skin tumorigenesis, which is associated with downregulation of cyclin D2.[29] 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,[29] 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 with 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 in skin tumorigenesis. 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.[29] 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 remain constant compared with normal and hyperproliferative skin.[21]

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 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 with cyclin D2 downregulation) compensates for the absences of cyclin D1 and cyclin D2 in keratinocyte proliferation but not in skin tumor development, it is tempting to speculate that cyclin D1/D2 are essential for proliferation/expansion of the chemically initiated hair follicle stem cells. Therefore, understanding the differences between the cell cycle regulation in keratinocyte stem cells vs. normal keratinocytes should permit appreciation of 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 mm^3 and tumors > 300 mm^3 were not observed in the K5D3/cyclin D1$^{-/-}$ compound mice. Conversely, wild-type mice develop a considerable number of tumors > 300 mm^3. 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 abrogates the oncogenic function of cyclin D3. These results suggest that cyclin D1 plays a unique role during the malignant conversion to SCC, since overexpression of each D-type cyclin member can induce malignant progression to SCC,[27,29] 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 "addicted" to cyclin D1 expression, which is regulated by Ha-ras.[39,40] Supporting this idea, early studies on skin tumorigenesis showed that cyclin D1 is expressed in small incipient papillomas, but overexpression was more notable in advanced stages of skin tumorigenesis.[41,42] Moreover, the kinetics of cyclin D1 overexpression coincide with the expression of the mutated Ha-ras allele.[22] 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-translational mechanisms are likely to be involved.[29] 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.[29,30] 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.[18-20,43]

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.[44] In addition, a genetic-proteomic screen has revealed that cyclin D1 plays a transcriptional role during development.[45]

In summary, we developed a new animal 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.[5] 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 epidermal 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 a 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.

Materials and Methods

Experimental animals. The generation of the cyclin D1 knockout (cyclin D1$^{-/-}$) and K5-cyclin D3 (K5D3) transgenic mice has been previously described in references 30 and 32. To generate K5D3/cyclin D1$^{-/-}$ compound mice, we crossed cyclin D1$^{+/-}$ and K5D3 mice. Subsequently the K5D3/cyclin D1$^{+/-}$ mice were mated with cyclin D1$^{+/-}$ siblings. The genotype of each mouse was screened by PCR.[30,32]

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 2 x 2 Chi-square 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 $D1^{-/-}$, K5D3/cyclin $D1^{-/-}$ and wild-type mice) at 24 weeks using the t-test.

Protein gel blotting and kinase assays. After the mice were sacrificed, the dorsal skins were treated with a depilatory agent for 1 min and washed with tap water. The epidermal tissue was scraped off with 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 14,000x g at 4°C to collect the supernatant, which was used directly for protein gel 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 10,000x g for 10 min. The supernatants were collected and used directly for protein gel blot analysis. The protein concentration was measured with the Bio-Rad protein assay system (Bio-Rad Laboratories). 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% non-fat 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) and monoclonal antibodies against cyclin D1 (DCS-6), cyclin D3 (Ab-1) (NeoMarkers) and CDK6 (DCS-83) (Santa Cruz Biotech). Incubation with secondary antibodies followed by exposure to enhanced chemiluminescence (ECL detection kit; GE Health Care) 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, 1 mmol EDTA, 25 mmol EGTA, 10% Glycerol, 0.1% tween 20, 1 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L DTT and 1 mmol/L PMSF] with precoated antibodies against CDK2, CDK4 and CDK6 for 2 h 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 $MgCl_2$, 5 mmol/L $MnCl_2$]. Subsequently, 30 μL of kinase buffer, 1 μg of pRb or histone H1 (Upstate Biotechnology Inc.) substrate, 5 μCi of [γ-^{32}P] ATP (6,000 Ci/mmol), 1 mmol/L DTT and 5 μmol/L ATP were added to the bead pellet and incubated for 30 min at 30°C. SDS sample buffer was added and each sample was boiled for 3 min to terminate the reaction and they were electrophoresed through polyacrylamide gels. The bands obtained by protein gel blot and the kinase assay were quantified using UNSCANT IT gel software for Windows.

Immunostaining. Epithelial cell proliferation was measured by intraperitoneal injection of 60 μg/g (body weight) of 5-bromodeoxyuridine (BrdU) 30 min 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), biotin-conjugated anti-mouse antibody (Vector Laboratories) 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 μm^2 with a reticule grid.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 4 Software (GraphPad Software Inc.).

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