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Expression of cyclin D1 in epithelial tissues of transgenic mice results in epidermal hyperproliferation and severe thymic hyperplasia

(keratin 5/skin/thymus/cell cycle/oncogene)

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ABSTRACT **To study the involvement of cyclin D1 in epithelial growth and differentiation and its putative role as an oncogene in skin, transgenic mice were developed carrying the human cyclin D1 gene driven by a bovine keratin 5 promoter. As expected, all squamous epithelia including skin, oral mucosa, trachea, vaginal epithelium, and the epithelial compartment of the thymus expressed aberrant levels of cyclin D1. The rate of epidermal proliferation increased dramatically in transgenic mice, which also showed basal cell hyperplasia. However, epidermal differentiation was unaffected, as shown by normal growth arrest of newborn primary keratinocytes in response to high extracellular calcium. Moreover, an unexpected phenotype was observed in the thymus. Transgenic mice developed a severe thymic hyperplasia that caused premature death due to cardio-respiratory failure within 4 months of age. By 14 weeks, the thymi of transgenic mice increased in weight up to 40-fold, representing 10% of total body weight. The hyperplastic thymi had normal histology revealing a well-differentiated cortex and medulla, which supported an apparently normal T-cell developmental program based on the distribution of thymocyte subsets. These results suggest that proliferation and differentiation of epithelial cells are under independent genetic controls in these organs and that cyclin D1 can modulate epithelial proliferation without altering the initiation of differentiation programs. No spontaneous development of epithelial tumors or thymic lymphomas was perceived in transgenic mice during their first 8 months of life, although they continue under observation. This model provides *in vivo* evidence of the action of cyclin D1 as a pure mediator of proliferation in epithelial cells.**

Cyclin D1 has been described as a growth-factor sensor whose expression is induced during the early G_1 phase of the cell cycle after stimulation of quiescent cells (for review, see ref. 1). Cells engineered to overexpress cyclin D1 showed accelerated G_1 phase (2–4), suggesting that expression of this cyclin might be a rate-limiting step for G_1 phase progression. Several lines of evidence indicate that cyclin D1 functions to phosphorylate the retinoblastoma protein (Rb) near the G_1/S-phase transition (ref. 4; for review, see ref. 5). Perturbation of the cyclin D1/Rb pathway either by loss of Rb or overexpression of cyclin D1 has been proposed as one of the processes leading to tumor development (5). In fact, such aberrations seem to constitute alternative mechanisms of carcinogenesis in esophageal (6) and lung (7) cancers. Moreover, expression of wild-type Rb (8), as well as introduction of p16 (9), a recently cloned inhibitor of cyclin D-associated kinase activity (10), can prevent transformation by *ras*, while cyclin D1 has been implicated as a target of *ras* activation in intestinal epithelial cells (11) and in NIH 3T3 cells (12).

Cyclin D1 alterations have been reported in a variety of human and experimental cancers. Human cyclin D1 was originally cloned as *Prad1*, the gene at the site of the rearrangement characteristic of a subset of parathyroid adenomas (13–15). Cyclin D1 rearrangement, amplification, and overexpression have also been reported in B-cell lymphomas (16–18), squamous cell carcinomas of the head and neck (19) and esophagus (20), mammary carcinomas (19, 21), colorectal carcinomas (22), and chemically induced mouse skin tumors (23, 24). The role of cyclin D1 in tumorigenesis has been further investigated in transgenic mice carrying a cyclin D1 gene under the control of tissue-specific promoters. When expressed in lymphoid tissues, however, cyclin D1 presented a mild phenotype and failed to induce tumors by itself, although it cooperated with *myc* in the development of lymphomas (25, 26). Overexpression of cyclin D1 in mammary epithelial tissues resulted in abnormal proliferation and an increased incidence of mammary adenocarcinomas that were evident in a subset of mice after a latency time of more than 1 year (27). Interestingly, recently developed cyclin D1-deficient mice showed defective mammary-tissue proliferation (28). Thus, these data suggest that the up-regulation of a gene critical for normal proliferation can indeed induce growth and promote tumor development.

We reported (23, 24) that cyclin D1 is dramatically overexpressed in chemically induced tumors of mouse skin and postulated that it is a downstream mediator of Ha-*ras* activation (11). Thus, we hypothesized that cyclin D1 is a key regulator of proliferation in epithelial tissues and is responsible for the increase in proliferation generated after *ras* transformation. To study the role of cyclin D1 in normal epithelial proliferation and differentiation, we have generated transgenic mice that constitutively overexpress cyclin D1 in stratified epithelia. These mice presented a remarkable phenotype of a pure hyperproliferative response that did not affect normal differentiation and function of the target organs.

MATERIALS AND METHODS

Generation of Transgenic Mice. A 1.3-kb *Eco*RI fragment containing the human cyclin D1 (*Prad1*) cDNA was excised from the plasmid pPL-8 (14) and introduced in the polylinker of the vector p163/7 (29). The *Sal*I/*Kpn*I fragment containing the 5′ β-globin intron 2, the cDNA, and the 3′ polyadenylylation sequences were inserted 3′ downstream of the 5-kb bovine keratin 5 (K5) regulatory sequences cloned in pBlue-

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Abbreviations: Rb, retinoblastoma; K5, keratin 5.

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script (30). This construct was designated pK5prad1. The transgene was excised from the plasmid vector by digestion with *Kpn*I, separated by low-melting-point agarose electrophoresis, and purified. Three laboratories performed the microinjections, as detailed in Table 1.

DNA Extraction. Genomic DNA was extracted from mouse tail clips by using the QIAamp tissue kit (Qiagen, Chatsworth, CA) according to instructions from the manufacturer.

Cyclin D1 cDNA-Specific PCR. For screening of positive transgene integration by PCR, we used an upstream primer (5′-ATGGAACACCAGCTCCTGTGCT-3′) specific for the exon 1 and a downstream primer (5′-GGCGGCAAGCTTC-CACTTGAGC-3′) specific for the exon 3 of the cyclin D1 gene (31). These primers bind to both human and murine sequences, but only the human cDNA present in the construct is amplified rendering a 462-bp PCR product. PCR was performed by denaturation at 94°C for 1 min, followed by 30 cycles of amplification as follows: denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The founders generated by The University of Texas, M. D. Anderson Cancer Center (Houston) and by DNX (Princeton), and their subsequent litters were identified by this technique using DNA extracted from tail clips.

Southern Blot. The 5-kb fragment corresponding to the bovine K5 promoter was used as a probe in Southern blot analysis of *Bam*HI-digested tail DNA, to identify transgenic mice generated in Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas and to confirm the integrity of the transgene and the presence of only one integration site in all founders.

Cyclin D1 Immunohistochemistry. Immunohistochemical staining of formalin-fixed paraffin-embedded tissues with polyclonal human cyclin D1 antibody (Upstate Biotechnology, Lake Placid, NY) has been described (24).

Protein Extraction from Thymus. Proteins were extracted from frozen thymi essentially as described (32) in a low salt buffer. The extracts were sonicated for 1 min, ultracentrifuged at 100,000 × *g* for 45 min at 4°C, quantitated, distributed in aliquots, and stored at −70°C.

Flow Cytometry. Thymocytes were obtained by pressing thymic tissue through a nylon mesh. Less than 2% of the thymocyte population consisted of B cells and macrophages. Thymocytes were stained with anti-CD4-coupled phycoerythrin and anti-CD8-coupled fluorescein isothiocyanate and analyzed by two-color immunofluorescence with a Coulter Elite Flow cytometer.

Culture of Newborn Primary Keratinocytes. Keratinocytes were isolated from newborn mice and cultured in Eagle's medium containing 8% Chelex-treated serum and 0.05 mM Ca^{2+} as described (33). For immunoblot analysis, 5-day cultures were washed with ice-cold PBS and lysed in 50 mM Tris·HCl (pH 7.4) containing 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 2 mM Na_2VO_4, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (25 μg/ml). Protein extracts were quantitated, distributed in aliquots, and stored at −70°C. For differentiation studies, cells were grown in 0.05 mM Ca^{2+} for 5 days and then in 0.05 mM Ca^{2+} or 1.3 mM Ca^{2+} for 24 h, adding 100 μM 5-bromo-2′-deoxyuridine (BrdU) (Sigma) to the medium during the last hour.

Table 1. Generation of transgenic lines

Laboratory	Line	Genetic background
U.T.M.D.A.C.C.	917	(C57BL/6 × DBA/2) F_1
DNX	7108	(C57BL/6 × SJL) F_2
DNX	7111	(C57BL/6 × SJL) F_2
CIEMAT	K5-D1	(C57BL/6 × DBA/2) F_2

U.T.M.D.A.C.C., University of Texas, M. D. Anderson Cancer Center; CIEMAT, Centro de Investigaciones Énergéticas, Medioambientales y Tecnológicas.

Immunoblot Analysis. Total proteins (150 μg) were electrophoresed through a 10% SDS/PAGE gel and transferred to Hybond-ECL (Amersham). To differentially detect endogenous cyclin D1 and human cyclin D1 expressed by the transgene, the following antibodies were used sequentially: monoclonal mouse anti-cyclin D1 (Zymed, San Francisco), which reacts only with the mouse protein and does not detect human cyclin D1, and polyclonal human anti-cyclin D (Upstate Biotechnology), which reacts with cyclin D1 of both human and mouse origins. Specific horseradish peroxidase-conjugated secondary antibody was applied at a 1:3000 dilution followed by chemiluminescent detection using the ECL kit (Amersham).

K5 Immunohistochemistry. K5 immunostaining was performed on ethanol-fixed paraffin-embedded tissues as described for other keratin antibodies (34).

BrdU Immunostaining. The animals were injected i.p. with BrdU (50 mg/kg) in PBS 30 min before they were killed. Sections of formalin-fixed paraffin-embedded samples and ethanol-fixed tissue culture samples were incubated with a 1:25 dilution of anti-BrdU antibody (Becton Dickinson), followed by peroxidase staining.

RESULTS AND DISCUSSION

Generation of Cyclin D1 Transgenic Mice. The construct used to generate transgenic mice is depicted in Fig. 1*A*. The expression of cyclin D1 was targeted to stratified epithelia by the 5′ regulatory fragment of the bovine K5 gene. As reported (30, 35), this fragment drives expression of a reporter gene in basal cells of squamous stratified epithelia, where K5 is normally expressed. Eight mice with positive integration of the transgene were identified by PCR and Southern blot analysis

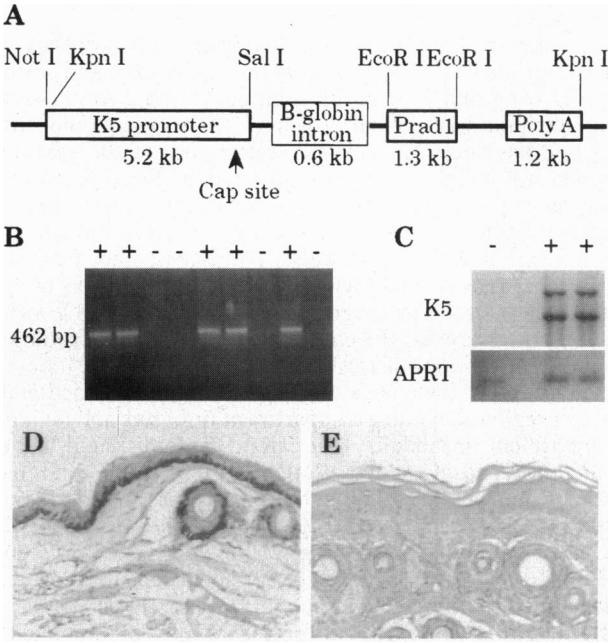

FIG. 1. pK5prad1 transgenic construct and screening techniques. (*A*) Diagram of the pK5prad1 construct. (*B*) PCR amplification of DNA extracted from mouse tails. Human cyclin D1 cDNA was amplified as a 462-bp product. Genotypes are shown above the lanes. (*C*) Southern blot analysis of DNA extracted from mouse tails. K5, 5-kb fragment of the bovine K5 promoter; APRT, loading control. Genotypes are shown above the lanes. (*D*) Cyclin D1 immunohistochemistry of a tail section from a transgenic founder. (*E*) Cyclin D1 immunohistochemistry of a tail section from a normal sibling. (×95.)

(Fig. 1 *B* and *C*, respectively). A second screening to verify transgene expression was performed by cyclin D1 immunohistochemical staining of tail clips (Fig. 1*D*). Since cyclin D1 is undetectable in normal epidermis by immunohistochemistry (Fig. 1*E*), positive cyclin D1 staining was used as an indication of transgene expression. Based on those results, four of the integration-positive mice were selected as founders and crossed with Sencar line B inbred mice. The identity and genetic background of the four transgenic founders are indicated in Table 1. Aberrant expression of cyclin D1 was detected in transgenic mice by immunohistochemical staining in a variety of tissues that bear stratified squamous epithelia, including skin, tongue, esophagus, thymus, cervix, and vagina (data not shown).

Development of Thymic Hyperplasia. All the founders and their litters were born with normal body size and developed normal hair. However, the founder of line 917 died unexpectedly at 15 weeks of age. The autopsy revealed a severe hyperplasia of the thymus that represented a 40-fold increase over the normal size of the organ. The only transgenic mouse from this founder had to be sacrificed at 8 weeks due to severe respiratory distress resulting from the same type of hyperplasia. No other transgenic mice were born from this line, which was, therefore, lost. The founder of line 7108 died at 20 weeks of age. It also showed a dramatic hyperplasia of the thymus that weighed approximately 2 g, whereas the weight of a normal age-matched thymus is approximately 0.05 g. All of the 54 transgenic mice generated from this founder and none of the normal siblings have shown the same thymic phenotype, with the first signs of respiratory distress appearing at 8–10 weeks of age. A photograph of a hyperplastic thymus from an 18-week-old transgenic animal is shown in Fig. 2*A* along with the thymus of a normal sibling. Thymic hyperplasia was also developed by the transgenic line K5-D1. Therefore, this phenotype was observed in three integration events on different genetic backgrounds (Table 1). The transgenic line 7111 was the only one that did not develop respiratory distress, thymic hyperplasia, or premature death.

To study the time frame of development of hyperplasia, we harvested and weighed thymi from transgenic mice and normal siblings from line 7108 at different ages from 2 to 21 weeks. The maximum time of survival for a transgenic animal from this line has been 22 weeks. A scattergram of the values of thymus wet weight vs. age is depicted in Fig. 2*B*. Normal littermates presented a maximum thymus weight of approximately 0.1 g between 6 and 10 weeks of age and a reduction to approximately 0.05 g by 14 weeks. Transgenic mice from line 7108 developed normal thymus weights up to 2 weeks of age, but afterwards, the thymi grew dramatically to 2–2.3 g within 4–6 weeks, occupying the entire thoracic cavity and leading to death by respiratory distress.

The normal thymus is organized into two functionally distinct regions, an outer compartment or cortex and an inner compartment or medulla, that can be easily distinguished on hematoxylin/eosin-stained sections (Fig. 2*D*). Thymi harvested from each of the three transgenic lines that developed hyperplasia presented histological features consistent with their normal counterparts (Fig. 2*E*). The thymic cortex and medulla are mainly composed of epithelial cells that together with other stromal elements support thymopoiesis and T-cell differentiation. A subset of the epithelial cells (predominantly, but not exclusively in the medulla) expresses K5 (35) (Fig. 2*F*), and therefore, it is primarily these cells that express the cyclin D1 transgene (Fig. 2*G*).

To determine whether the development of thymic hyperplasia in line 7108 and the lack of this phenotype in line 7111 was due to differential expression of the transgene in thymus, total protein extracts were analyzed in Western blots using two cyclin D1 antibodies with different species crossreactivity. Fig. 2*C* shows that normal and transgenic mice from both lines

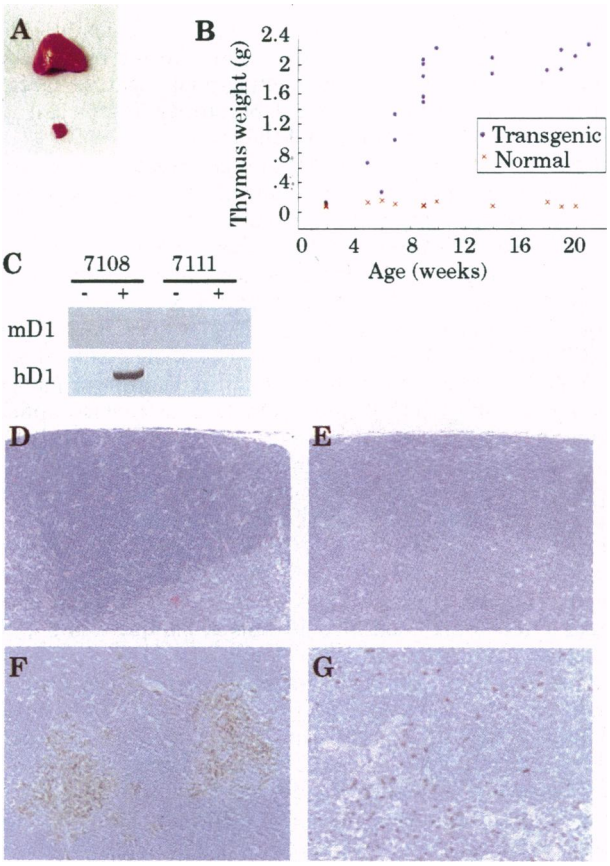

FIG. 2. Thymic hyperplasia in line 7108. (*A*) Photograph of a thymus from a representative 18-week-old transgenic mouse and a normal littermate. (*B*) Scattergram of thymus wet weight versus age in transgenic and normal mice from line 7108. (*C*) Expression of endogenous murine cyclin D1 (mD1) and transgenic cyclin D1 (hD1) in thymus protein extracts from 10-week-old mice. Transgenic line identity and genotypes are shown above the lanes. Hematoxylin/eosin staining of sections of thymi from a normal mouse (*D*) and from a transgenic sibling with thymic hyperplasia (*E*). Expression of K5 (*F*) and cyclin D1 (*G*) in a transgenic thymus. (*D–F*, ×47.5; *G*, ×95.)

expressed low levels of endogenous cyclin D1 in the thymus. However, a similar analysis with a polyclonal anti-human cyclin D1 antibody revealed that only line 7108 expressed the transgene in the thymus. Therefore, the absence of the hyperplastic thymus phenotype in line 7111 appears to be due to lack of transgene expression in this tissue, although the transgene is, in fact, expressed in other target organs of these mice (data not shown).

The thymocytes present in the transgenic thymi appeared normal and did not show histological evidence of transformation. To determine whether expression of the cyclin D1 transgene in thymic epithelium altered T-cell development, we analyzed the thymocyte subset distribution in normal mice and in transgenic mice at 2 weeks of age, when no thymic hyperplasia was evident, and at 14 weeks, when the thymi were hyperplastic. The results in Table 2 show that each of the four

Table 2. Thymocyte subset distribution from line 7108

	Percentage of thymocytes			
pK5prad1	$CD4^-CD8^-$	$CD4^+CD8^+$	$CD4^+CD8^-$	$CD4^-CD8^+$
−*	2.5	84	11	2.5
+†	2	87	9	2
+‡	5	67	19	9

*Average of three normal mice (thymus weight, ≈0.05 g).
†Average of two 2-week-old transgenic mice (thymus weight, ≈0.05 g).
‡Average of two 14-week-old transgenic mice (thymus weight, ≈2 g).

major thymocyte subsets, defined by CD4 and CD8 expression, is present in transgenic mice regardless of age. However, hyperplastic thymi harvested from 14-week transgenic mice showed an increase in the proportion of mature or single positive thymocytes. Since thymic lymphoma cells are large and consist of a single predominant phenotype (36), while thymocytes from cyclin D1 transgenic mice are small and consist of each of the major T-cell phenotype subsets, we conclude that thymocytes in the hyperplastic thymi are not transformed cells.

Thus, these data suggest that the thymic phenotype developed by three of four independent transgenic lines can be attributed to a subset of thymic epithelial cells expressing exogenous cyclin D1 by virtue of the K5 promoter. Cyclin D1 overexpression might enable thymic epithelial cells to escape negative growth regulatory signals and continue to proliferate, providing an ever growing environment for T-cell development. In this regard, a recent study showed that thymocytes could inhibit thymic epithelial cell proliferation in culture, in part through production of transforming growth factor β (TGF-β) (37). Also, cyclin D1 overexpression in an esophageal epithelial cell line resulted in reduced TGF-β growth inhibition (38). Thus, it is possible that loss of growth inhibition by TGF-β in thymic epithelial cells overexpressing cyclin D1 could be a mechanism underlying the development of thymic hyperplasia. Further studies are necessary to test this hypothesis. Thymic hyperplasia was also observed in transgenic mice expressing the simian virus 40 large tumor antigen in thymic epithelial cells (39) and attributed to immortalization of those cells, probably through Rb binding and inactivation by the viral antigen (40). The data provided herein further support the action of cyclin D1 and Rb in a common growth regulatory pathway *in vivo*.

The Skin Phenotype: Epidermal Hyperproliferation and Hyperplasia. K5 is normally expressed in the basal cell compartment of the skin (Fig. 3*A*), consistent with the expression of cyclin D1 in transgenic mice. Positive mice from all four lines generated (including line 7111) showed detectable cyclin D1 protein in basal cells (Fig. 3*B*), while normal littermates were negative (data not shown). The skin phenotype was observed in all transgenic lines developed, with high variability among siblings. Consistent with its role as a cyclin rate-limiting for G_1-phase progression (4), overexpression of cyclin D1 increased the rate of epidermal proliferation, which was assessed by the percentage of BrdU incorporation and by number of nucleated cells in adult mice epidermis. There was a significant increase in the percentage of BrdU-positive or S-phase basal cells in transgenic mice compared with normal siblings (Fig. 3*C*), in lines 7108 and 7111 ($P \leq 0.003$, Mann–Whitney), that can be observed in representative BrdU-stained sections of normal and transgenic skin (Fig. 3 *E* and *F*, respectively). A third line, K5-D1, showed 3.8-fold higher BrdU labeling in transgenic mice epidermis (data not shown). Significant difference was also found in the number of nucleated cells in interfollicular epithelia (Fig. 3*D*), in lines 7108 and 7111 ($P \leq 0.00001$ and $P \leq 0.0003$, respectively), illustrated by histological sections of normal and transgenic skin from two littermates (Fig. 3 *G* and *H*). Typically, transgenic mice showed a more densely packed basal cell compartment or basal cell hyperplasia (Fig. 3*H*) and most of them also presented suprabasal proliferative cells (Fig. 3*F*). This resulted in a moderate acanthosis (increase in the thickness of the nucleated layers of the epithelia) with mild hyperkeratosis (accumulation of keratinized cells in the epidermal surface).

Normal skin is a very appropriate system in which to study regulation of proliferation vs. differentiation, since the proliferative compartment is clearly separated from the differentiated and keratinized layers (Fig. 3*G*). Deregulation of cyclin D1 in basal cells of the skin resulted in increased rate of proliferation and expansion of the proliferative compartment

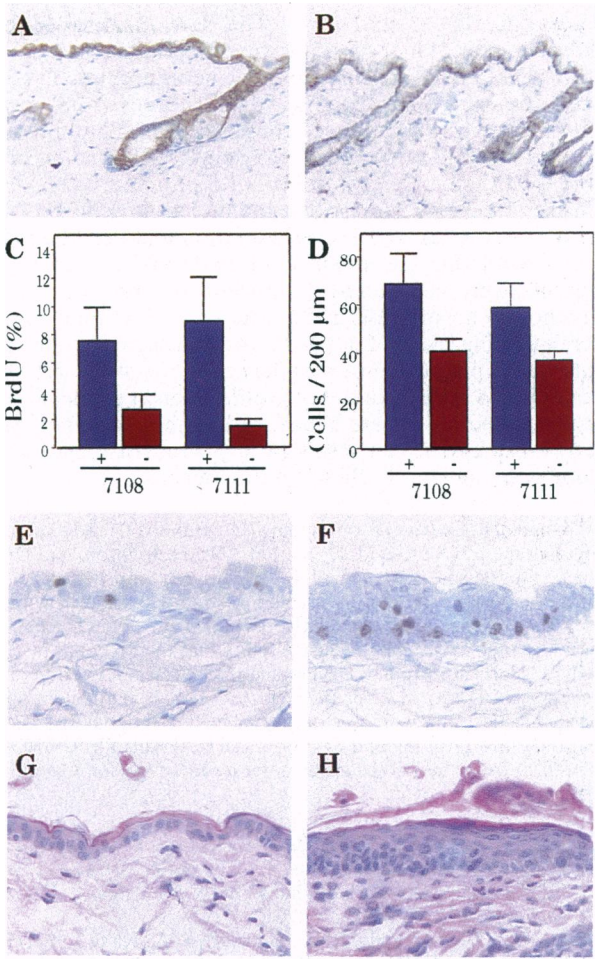

FIG. 3. Skin phenotype of transgenic mice. (*A*) Expression of K5 in skin. (*B*) Expression of cyclin D1 in transgenic skin. (*C*) Increase in epidermal proliferation. The bars indicate the percentage of BrdU incorporation in basal cells from interfollicular epithelia. Values are the mean ± SD of 7 adult mice. Transgenic line identity and genotypes are shown below. (*D*) Basal cell hyperplasia. The bars indicate the number of nuclei in 200-μm interfollicular epithelia. Values are the mean ± SD of 18 and 9 adult mice (7108 and 7111, respectively). Transgenic line identity and genotypes are shown below. Representative sections showing BrdU incorporation in normal (*E*) and transgenic (*F*) skin. Hematoxylin/eosin staining of representative sections of skin from a normal mouse (*G*) and from a transgenic littermate (*H*). (*A* and *B*, ×47.5; *E–H*, ×190.)

with only a mild effect in the differentiated layers. Therefore, cyclin D1 seems to increase the fraction of cells that are retained in the proliferative compartment. This observation was confirmed by immunohistochemical staining for K1, a keratin whose expression is restricted to terminally differentiated cells. In spite of the increase in the overall thickness and cellularity of the epidermis, the number of differentiated layers remained essentially identical between transgenic mice and normal siblings (data not shown). This phenotype is markedly different from that observed in hyperproliferative skin generated by chemical treatment. In the later case, for a comparable increase in the rate of proliferation, there is a mild expansion of the proliferative compartment that is compensated by a concomitant expansion of the differentiated compartments (41).

To further analyze the response of transgenic keratinocytes to differentiation-induced growth arrest, we subjected cultures of primary keratinocytes isolated from newborn mice to high extracellular calcium. Control and transgenic keratinocytes responded normally to growth arrest/differentiation induced

by exposure to 1.3 mM Ca^{2+}. The percentage of S-phase inhibition after 24 h of exposure was 83% (line 7108) and 84% (line 7111), for cultures of transgenic keratinocytes, and 83% (line 7108) and 80% (line 7111) for cultures from normal siblings. This observation is consistent with that reported previously in transgenic mice carrying the Rb-binding oncoprotein E1a under the control of a K5 promoter (42).

Thus, these data suggest that the effect of cyclin D1 overexpression was restricted to proliferation and that the mechanisms regulating the initiation of epidermal differentiation programs were not altered. Consistent with previous studies that showed an increase in the rate of proliferation in cells overexpressing cyclin D1 (2–4), the transgenic model presented here provides direct evidence *in vivo* of the action of cyclin D1 as a pure mediator of proliferation in epithelial cells. Ongoing experiments are aimed to determine whether cyclin D1 overexpression can result in an increased sensitivity to tumor development by chemical carcinogens.

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