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Enhanced Skin Carcinogenesis in Cyclin D1-conditional Transgenic Mice: Cyclin D1 Alters Keratinocyte Response to Calcium-induced Terminal Differentiation[1]

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ABSTRACT

***Cyclin D1* is a critical gene involved in the regulation of progression through the G_1 phase of the cell cycle, thereby contributing to cell proliferation. Gene amplification and abnormal expression of *Cyclin D1* have been described in several human cancers. To understand their biological significance in skin carcinogenesis, we established *Cyclin D1*-conditional transgenic mice with C57BL/6J background, in which skin-specific overexpression of *Cyclin D1* transgene was observed. The mice were subjected to dimethylbenz[*a*]anthracene complete skin carcinogenesis studies. After 40 weeks of repeated administration of dimethylbenz[*a*]anthracene on the skin (once a week), all of the mice with high *Cyclin D1* expression had papillomas, whereas only 9.5% of the control mice without the transgene developed papillomas. Primary cultured keratinocytes with induced *Cyclin D1* transgene expression showed resistance to calcium-induced terminal differentiation and continued to replicate *in vitro*. These results clearly provide us with direct experimental evidence that overexpression of *Cyclin* D1 induces excessive dermal cell proliferation via the altered differentiation state of keratinocytes. The conditional transgenic mice described here provide excellent *in vivo* and *in vitro* model systems to understand the role of cyclin D1 and deregulation of the cell cycle in carcinogenesis.**

INTRODUCTION

The cyclins and their catalytic partners, the cdks,[3] have been identified as key regulators of the mammalian cell cycle (1–3). Cyclin D1 in cooperation with its major catalytic partners, cdk4 and cdk6, facilitates progression through the G_1 phase of the eukaryotic cell cycle, in part through phosphorylation of retinoblastoma protein (4, 5). Oncogenic properties of cyclin D1 have been suggested by its cooperation with ras or adenovirus E1A to transform cultured cells (6, 7), as well as its overexpression in transgenic mice, resulting in the generation of breast cancer (8, 9). Our laboratory is the first reporting amplification of chromosome 11q13 containing *Cyclin D1* in esophageal cancers (10–12). Cyclin D1 overexpression is found in up to 50% of primary breast cancers (13, 14), and its incidence is increased in cases of malignant lesions (15).

It has been shown that suppression of cyclin D1 activity in malignant cells can result in the disappearance of the malignant phenotype (16). On the other hand, *Cyclin D1* overexpression results in a normal cell's acquisition of malignant phenotype (17, 18), and such deregulated expression of cyclin D1 overrides antimitotic signals of tumor growth factor β-1 (19). Previous reports concerning *Cyclin D1* transgenic animals (SENCAR background) have suggested that overexpression of *Cyclin D1* with the bovine keratin 5 promoter showed no enhancing ability to produce skin tumors by DMBA/12-*O*-tetradecanoylphorbol-13-acetate two-stage carcinogenesis, although *Cyclin D1* overexpression corresponded with increased proliferation of cells in the epidermis of these transgenic mice (20). It has been suggested that oral-esophageal tissue-specific expression of cyclin D1 with the EBV ED-L2 promoter in transgenic mice results in the generation of dysplasia (21), which is associated with abnormalities of the cell cycle, epidermal growth factor receptor, and p53 (22). Moreover, *Cyclin D1* overexpression with ED-L2 promoter and a chemical carcinogen, *N*-nitrosomethylbenzylamine, may cooperate to increase the severity of esophageal squamous dysplasia, a prominent precursor to carcinoma (23). Despite these experimental results, the functional consequences of aberrant *Cyclin D1* overexpression are not entirely understood apart from increased cell proliferation.

To study the functional consequences of *Cyclin D1* overexpression in tumorigenesis, we have established Cre recombinase-regulated conditional transgenic mice with a C57BL/6J background. C57BL/6J mice are well known for their relative resistance to phorbol-induced skin carcinogenesis (24–26). When a sufficient amount of Cre is supplied by use of adenoviruses, it initiates target gene expression in the target tissue at a specified time. In this newly developed animal model, the *Cyclin D1* transgene is overexpressed specifically in the skin. The mice were subjected to DMBA complete skin carcinogenesis experiments by repeated administration of DMBA on the skin once every week for 40 weeks. The epidermis of these mice has a higher proliferative activity than *Cre*-untransduced animals. After 40 weeks, all of the mice with *Cyclin D1* transgene overexpression had papillomas, whereas the frequency of papillomas in wild-type mice without high *Cyclin D1* expression was as low as 9.5% after DMBA administration. Mice not treated with DMBA had no papillomas.

In vitro-cultured primary keratinocytes from transgenic mice were resistant to calcium-induced terminal differentiation when cells switched on their *Cyclin D1* transgene expression. It was suggested that deregulated *Cyclin D1* overexpression and subsequent alterations in cell cycle machinery provide keratinocytes with the ability to at least partially override terminal differentiation processes, which provides a basis for skin tumors induced by DMBA. These results with transgenic mice indicated that *Cyclin D1* overexpression provides mice with increased sensitivity to a carcinogen. The results also possibly support the notion that *Cyclin D1* in the 11q13 amplicon is a responsible gene for the malignant phenotype of human cancer with 11q13 amplification.

MATERIALS AND METHODS

Recombinant DNA Constructs and Conditional Transgenic Mice. The CALNL*Cyclin D1* switching unit was constructed according to the original method (27). In brief, 1.2 kb of a full-length human *Cyclin D1* cDNA fragment (28) was cloned into the *Swa*I site of plasmid pCALNLw, consisting of the CAG promoter, a *loxP* sequence, a neo-resistant gene (1.0 kb of the *Bgl*II-*Sma*I fragment from pSV2neo), a second *loxP* site, and the poly(A) signal from

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[3] The abbreviations used are: cdk, cyclin-dependent kinase; DMBA, dimethylbenz[*a*]anthracene; pfu, plaque-forming unit(s); MOI, multiplicity of infection; RT-PCR, reverse transcription-PCR; BrdUrd, bromodeoxyuridine; GFP, green fluorescent protein.

pCAGGS, resulting in a transgene named CALNL*Cyclin D1*. The transgene was linearized and injected into C57BL/6J mouse (CLEA Japan Inc., Tokyo, Japan) zygotes at a concentration of 2 ng/μl to generate transgenic mice according to an established procedure (29). Transgenic founder mice were mated to C57BL/6J mice, and offspring were screened for the presence of the transgene by Southern blot analysis of genomic DNA isolated from tail biopsies at the age of 3 weeks.

Switching On of the Transgene by AxCANCre. A recombinant adenovirus expressing Cre recombinase (AxCANCre) was prepared as described (27). The virus stock was concentrated and purified at 1.0×10^9 pfu/ml as described previously (30). C57BL/6J conditional transgenic mice and their littermates (10 weeks of age) were shaved with surgical clippers 1 day before *Cyclin D1* was switched on locally in the skin on the back by intradermal administration of AxCANCre at 5×10^8 pfu/4–5 cm^2. As a control, an equal volume of physiological saline solution was administered to the skin on the backs of mice. Mice were sacrificed at various times, and transgene expression in the skin was investigated.

Tumor Experiments. Beginning 3 days after the *Cyclin D1* was switched on or after administration of saline, the backs of the mice were painted once weekly with 0.2 ml of 100 nM DMBA dissolved in acetone. A total of 64 mice were used, divided into four groups: one group included conditional transgenic mice that received AxCANCre (*Cyclin D1* switched on) and repeated administration of DMBA; the second group included conditional transgenic mice that received physiological saline solution as a control (*Cyclin D1* switched off) and repeated administration of DMBA; the third group included wild-type mice that received AxCANCre and repeated administration of DMBA; and the last group included wild-type mice that received physiological saline solution and repeated administration of DMBA. Observations on tumor formation were made weekly. When tumors had formed, biopsies were made if necessary, and these biopsies were sectioned and fixed with 4% formaldehyde-PBS(-) for histological analysis.

Keratinocyte Isolation and Culture. Adult mouse epidermal keratinocytes were isolated from 10-week-old transgenic mice according to the trypsin flotation procedure of Yuspa and Harris (31) and cultured in low Ca^{2+}-enriched MEM supplemented with human epithelial growth factor (100 pg/ml), insulin (5 μg/ml), hydrocortisone (500 ng/ml), transferrin (10 ng/ml), bovine pituitary extract (0.04 mg/ml), and antibiotics (0.1 mg/ml penicillin; 0.06 mg/ml kanamycin). All reagents were purchased from BioWittaker, Inc. (Walkersville, MD).

The experimental design of the terminal differentiation assay and the preparation of medium containing various Ca^{2+} concentrations have been detailed previously (32, 33). In brief, cultured keratinocytes at a Ca^{2+} concentration as low as 0.05 mM were infected with AxCANCre at a MOI of 50. Two days after the Cre switching on, cells were treated with 1.2 mM Ca^{2+} and then cultured for studies on cell number and morphology.

Detection of *Cyclin D1* mRNA. Evaluation of tissue- or cell-specific conditional gene activation by Cre recombinase was assessed by Northern blot analysis. Two days after the administration of AxCANCre or control AxCAwt, total RNA was extracted from the skin and primary cultured keratinocytes by ISOGEN (NipponGene, Tokyo, Japan). Each RNA sample (10 μg) was separated on a 1% agarose denaturing gel and transferred to a NitroPlus nitrocellulose membrane (Osmonics, Westborough, MA); the blot was hybridized with the ^{32}P-labeled human *Cyclin D1* cDNA fragment. The same filter was rehybridized with ^{32}P-labeled *β-actin* probe.

RT-PCR Analysis. Total RNAs were prepared using ISOGEN solution (NipponGene) according to the manufacturer's protocol. Aliquots of total RNA isolated from skin dermis or cultured cells were treated with DNase (DNase I, amplification grade; Life Technologies, Inc., Gaithersburg, MD). RT-PCR was performed with the SuperScript One-Step RT-PCR system (Life Technologies) with the following primers: 5′-CCGATGCCAACCTCCTCAA-3′ and 5′-GAAATCGTGCGGGGTCATTG-3′ for human *Cyclin D1*. After RT-PCR, aliquots were electrophoresed on 3.0% agarose gels, stained with ethidium bromide, and then photographed under UV illumination. *β-actin* cDNA was amplified as an internal control, using primers 5′-GACATCAAA-GAGAAGCTGTGC-3′ and 5′-TAGGAGCCAGAGCAGTAATC-3′.

Labeling Index and Immunohistochemistry. Animals received an i.p. injection of 5-BrdUrd (100 mg/kg of body weight; Sigma-Aldrich Japan, Tokyo, Japan) 24 h before being sacrificed. To determine the BrdUrd labeling index, the dermis of each animal was fixed in 4% formaldehyde fixative and embedded in paraffin. Sections were prepared and processed immunohistochemically using the BrdUrd staining kit (Roche Diagnostics, Mannheim, Germany) according to the recommended method. Using a microscope, we determined the percentage of BrdUrd-labeled cells by counting at least 1000 dermal epithelial cells.

Tissues (and cells) were fixed with 4% formaldehyde-PBS(-), and sections (or cells) were boiled for 25 min in 10 mM citrate buffer (pH 6.0) in a microwave oven and cooled down for at least 2 h on a magnetic stirrer. Section were then subjected to immunohistochemical analysis using primary antibody for cyclin D1 (M-20; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution and incubated overnight at 4°C. A routine immunoperoxidase staining method was performed with peroxidase-conjugated streptavidin (Cosmo Bio Co., Ltd., Tokyo, Japan) and a DAB staining kit (DAKO Japan Co., Ltd., Kyoto, Japan). For examination of skin and tumor histology, standard H&E staining was performed.

Protein Analysis. Protein was extracted from skin tissues. Tissues were weighed and ground into powder while in liquid N_2 and then were lysed in ice-cold RIPA buffer (50 mM Tris, 1.0% NP40, 150 mM NaCl, 0.1% SDS, 5 mM EDTA, 0.1 mM sodium orthovanadate, 0.1 mM NaF) containing protease inhibitors phenylmethylsulfonyl fluoride (1 mM), DTT (1 mM), and protease inhibitor cocktail (Sigma-Aldrich). Lysates were subsequently clarified by centrifugation. The protein concentration was determined by a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA). Approximately 10 μg of protein were subjected to SDS-PAGE in 10% gels, after which the protein was electroblotted to polyvinylidene difluoride membranes. The blot was blocked with 5% skim milk in Tris-buffered saline [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20]. The antibody used for Western blot analysis was antihuman cyclin D1 antibody (M-20). For Western blot analysis, detection was carried out with the ECL detection kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Sequencing Analysis of *ras* Gene Mutation. Genomic DNAs isolated from papillomas and a carcinoma were used as templates for a PCR reaction for detection of *ras* gene mutations. Exon sequences containing codons 12 and 13 (exon I) and codon 61 (exon II) of the H-*ras* gene were amplified, and nucleotide sequences were determined by dye terminator cycle sequencing (Applied Biosystems Japan Ltd., Tokyo, Japan). The primers for amplifying the exon sequences are as follows: Ha-*ras* exon I, 5′-GGCCTTGGCTAA-GTGTGCTTCTCAT-3′ and 5′-TGGTCATTTACCCATGACCACTGCC-3′; Ha-*ras* exon II, 5′-CCCCACTAAGCCGTGTTGTTTTGC-3′ and 5′-TCAGT-GTGCACACGGAACCTTCCT-3′. Amplification conditions included premelting at 94°C for 2 min, 30 cycles of melting at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, with extension at 72°C for 7 min in the last cycle. The primers for sequencing are as follows: Ha-*ras* exon I, 5′-AAGGGCCTTGGCTAAGTGTG-3′ and 5′-ACCTCTGGCAGGTAG-GCAG-3′; Ha-*ras* exon II, 5′-GGACTCCTACAGGAAACAAG-3′ and 5′-GGCAAATACACAAAGAAAGC-3′.

Statistical Analysis. The results are represented as means ± SD. Student's *t* test was performed for statistical evaluation, with $P < 0.05$ considered significant.

RESULTS

Generation of Mice with Conditional Regulation of Cyclin D1 Expression. To establish an animal model in which human *Cyclin D1* gene expression could be regulated, Cre/*lox* conditional transgenic mice were produced. In this system, the recombinase is provided by the adenovirus-carrying *Cre* gene (AxCANCre; Ref. 27). When a sufficient amount of Cre is supplied, the stuffer sequence is excised as circular DNA and then the CAG promoter and the *Cyclin D1* gene are joined via a single *lox*P site, thereby initiating target gene expression in the target tissue (Fig. 1*A*). Five independent transgenic mouse founder lines harboring the switching unit were obtained. No abnormalities among founders and/or their progeny were observed. To determine whether conditional gene expression was attained in the skin of transgenic mice, we administered AxCANCre (5.0×10^8 pfu/4–5 cm^2) intradermally. As a control, littermates from the same litter carrying a nontransgene that were treated with AxCANCre were

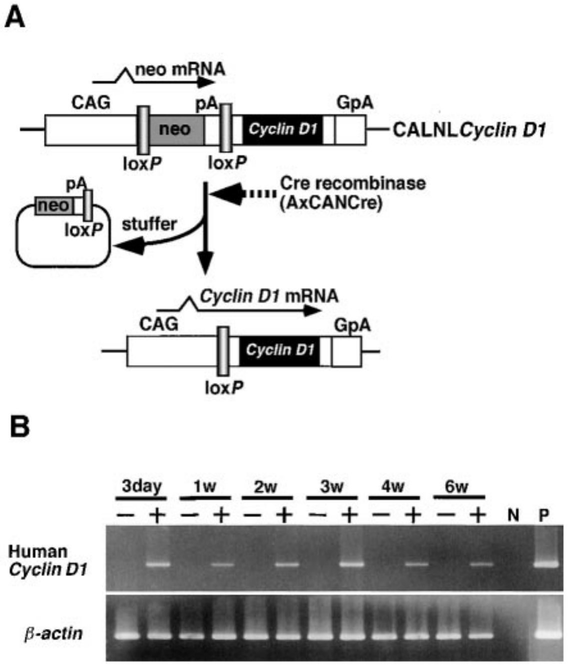

Fig. 1. Cre-mediated activation of *Cyclin D1* gene in mice. *A*, activation of the human *Cyclin D1* gene in the CALNL*Cyclin D1* switching unit by Cre recombinase. The Cre-mediated excisional deletion removes both a neo-coding region and a poly(A) sequence, consequently generating a functional *Cyclin D1* gene expression unit. *SpA*, SV40 early poly(A) site; *GpA*, rabbit-β-globin poly(A) site. *B*, RT-PCR analysis of activation of the dormant human *Cyclin D1* gene in the skin of transgenic mice. RT-PCR products amplified with specific primers were electrophoresed and visualized with ethidium. The same RNA samples were subjected to RT-PCR analysis of *β-actin* transcripts to verify their integrity. *N*, no-RNA samples; *P*, human *Cyclin D1* cDNA used as a positive control template; +, AxCANCre-injected transgenic mice; −, AxCANCre-injected control mice carrying nontransgene.

used. No apparent inflammatory response was observed in the injected sites. One day after administration, the skin of the treated area was excised, and total mRNA was obtained. Activation of the dormant *Cyclin D1* transgene by Cre was assessed by RT-PCR analysis of transgene sequences in transgenic mice. The expected 428-bp human *Cyclin D1* fragment was indeed detected in Cre-injected transgenic mice skin samples, but not with mRNA from the control mice (Fig. 1*B*). RT-PCR analysis revealed that switching on of the transgene activation in mouse skin continued up to at least 6 weeks after AxCANCre administration. These Cre-mediated gene activations were confirmed in three independent transgenic mouse founders. Southern blot analysis revealed that two lines had 5 copies and the other had 10 copies of the transfected genes. The one five-copy mouse line that transmitted stably to the next generation was used for the following experiments.

To determine the infection efficiency of the adenovirus, an adenovirus carrying the *GFP* gene (AxCAGFP) was injected intradermally into C57BL/6J mice. One day after administration, the skin of the injected area was excised, and skin sections were prepared for analysis of *GFP* gene expression. The results indicated that a variety of cell types in the skin, including stratum basale cells, hair follicle cells, and blood vessels, were positive for GFP. These results suggest that adenovirus injection allows efficient gene transfer in the skin, including keratinocytes. Our previous report suggested that the Cre-mediated switching on of gene activation was nearly 100% in cultured cells.

In the next experiment, we performed immunohistochemical analysis to determine the frequency of *Cyclin D1* gene activation and cell type in mouse skin. The results showed that human cyclin D1 products were detected in skin, especially in some stratum basale in Cre-injected transgenic mice (Fig. 2*A*, indicated by *arrows*), whereas expression of the transgene could not be demonstrated in control wild-type mice treated with AxCANCre (Fig. 2*B*). Western blot analyses showed that *Cyclin D1* protein was detected in the skin injected with AxCANCre, whereas control AxCAwt did not induce overexpression of cyclin D1 (Fig. 2*C*). Although the data are not shown, other tissues, such as brain, lung, and liver, showed no transgene expression. These results confirm that *Cyclin D1* transgene activation had occurred in the Cre-injected skin regions only, as predicted, via precise site-directed deletion of the stuffer sequences in the conditional transgenic mice carrying the dormant transgene.

Cyclin D1 Increases the Proliferative Index of Skin Epidermal Cells. To estimate cell proliferation activity in skin from *Cyclin D1*-overexpressing mice, BrdUrd incorporation in skin was assessed 7 days after the administration of AxCANCre in conditional transgenic mice and control wild-type mice. The results showed that the stratum basale cells in transgenic mice contained an increased number of BrdUrd-positive cells relative to the control group (Fig. 3). The labeling indices were 12.5 ± 4.3% in transgenic mice with AxCANCre and 5.4 ± 1.8% in wild-type mice with AxCANCre. These results suggest that forced *Cyclin D1* gene expression in skin may affect basal cell proliferation.

Skin Tumorigenesis. To observe the effects of up-regulation of cyclin D1 in formation of the skin tumors in mice, repeated administration of DMBA was performed. Because overexpressed *Cyclin D1* is expected to act as an initiator gene through the unregulated cell cycle of target cells in the skin, our conditional gene activation in the skin might lead to an increase in the incidence of chemically induced papillomas and carcinomas. Fig. 4 shows a comparison of DMBA complete skin carcinogenesis results obtained for transgenic mice and control wild-type mice pretreated with AxCANCre. The first skin tumor appeared in AxCANCre-treated mice at 9 weeks after DMBA treatment, whereas the control wild-type mice with AxCANCre

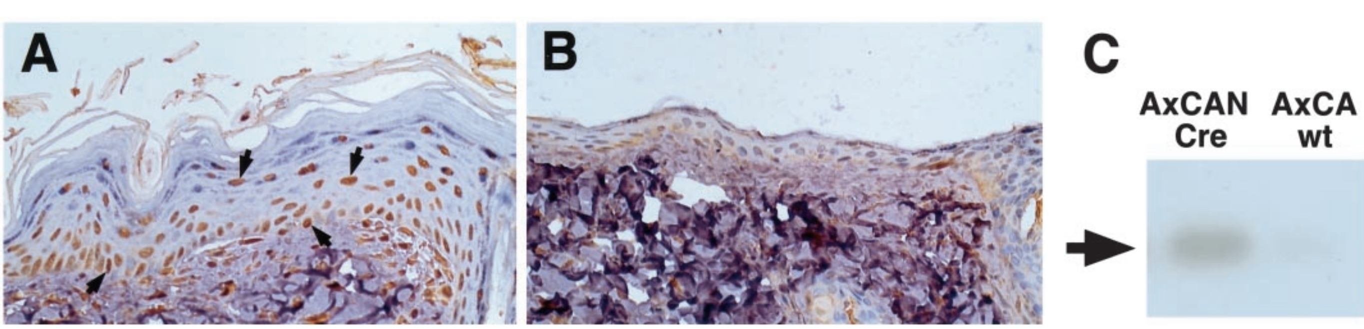

Fig. 2. Immunological analyses of cyclin D1 in the skin. The cross-sections of skin from transgenic mice 7 days after the intradermal administration of AxCANCre in conditional transgenic mouse (*A*) and in control wild-type mice (*B*) were immunostained with antihuman cyclin D1 antibody. Cyclin D1-immunopositive cells are indicated by *arrows*. Western blot analysis showed *Cyclin D1* expression in the skin (*C*). Each *lane* was loaded with 10 μg of total protein.

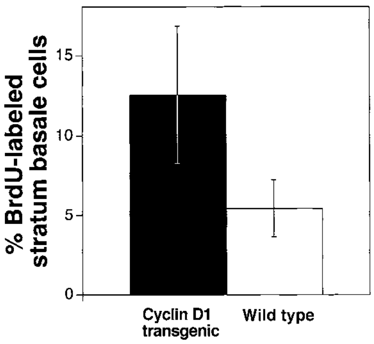

Fig. 3. Proliferative index of skin epidermal cells. Both transgenic mice and their littermates carrying the nontransgene treated with AxCANCre for 7 days received i.p. injection of 5-BrdUrd 2 h before being sacrificed. The labeling index in epidermal regions was determined and compared between transgenic mice (■) and wild-type mice (□). *Bars*, SE.

showed papillomas at 10 weeks after DMBA treatment. There was no obvious difference in the time when a papilloma was first observed between transgenic mice and wild-type mice. After 40 weeks, however, all of the transgenic mice with AxCANCre had papillomas (100% mice with papillomas), whereas papillomas had formed in only 9.5% of the control wild-type mice ($P < 0.005$; Fig. 4*A*). The average number of tumors per surviving transgenic mouse with AxCANCre was significantly higher than in the control mice ($P < 0.005$): 4.6 for transgenic mice and 0.6 for control mice (Fig. 4*B*). Littermates carrying transgenes and nontransgenes that were treated with a physiological saline solution instead of AxCANCre did not show any tumors even after 40 weeks of treatment with DMBA (data not shown). These results suggest that *Cyclin D1* gene activation contributed to enhanced DMBA-induced skin tumorigenesis in mice with a C57BL/6J background.

Tumor Phenotype and *ras* Mutations. Tumor tissues that appeared in transgenic mice with high expression of *Cyclin D1* gene were removed for routine histological analysis. At the end of the carcinogenesis experiments (40 weeks), histological examination of 15 randomly selected tumors (all tumors were >5 mm in diameter) revealed that 14 were papillomas (Fig. 5, *A* and *B*) and 1 was carcinoma (Fig. 5, *C* and *D*). We then evaluated these 15 tumors for the presence of an activating mutation at codons 12, 13, and 61 of the Ha-*ras* gene by PCR amplification and direct sequencing of PCR products. We could not find any mutations at codons 12, 13, and 61 of the Ha-*ras* gene.

Properties of Cyclin D1-altered Keratinocytes Resistant to Calcium-induced Terminal Differentiation. To assess possible alterations of dermal keratinocytes from *Cyclin D1* transgenic mice, we harvested keratinocytes from the epidermis, after which the primary culture was prepared. The purified cells were epithelium-like cells, which grew well in specified medium with a lower Ca^{2+} concentration (Fig. 6*B*, *top panel*). Cells were infected with AxCANCre at a MOI of 50 for 1 h. Two days after the infection, conditional expression of human *Cyclin D1* gene in primary cultured keratinocytes was determined by Northern blot analysis (Fig. 6*A*). A 1.2-kb transcript corresponding to the transgene was detected 2 days after the cells were infected with AxCANCre at a MOI of 50. In contrast, cells treated with control saline revealed no *Cyclin D1* gene activation. These results again confirm that human *Cyclin D1* transgene activation had occurred in the skin, as predicted, and allowed transgene switching on/off as desired.

When the keratinocytes were shifted to culture medium containing 1.2 mM Ca^{2+}, growth was inhibited and cells were terminally differentiated. As could be seen by phase-contrast microscopy, the cells derived from a control littermate showed significant morphological changes within 3 days, and the most evident terminal differentiation was seen 7 days after Ca^{2+} induction (Fig. 6*B*, *middle panel*). In contrast, cells derived from a conditional transgenic mouse treated with AxCANCre were resistant to Ca^{2+}-induced terminal differentiation and were actively proliferating even 7 days after the addition of Ca^{2+} to the culture (Fig. 6*B*, *bottom panel*).

Growth profiles of keratinocytes under different conditions are shown in Fig. 7. In three separate experiments, keratinocytes growth was inhibited when cells were treated with 1.2 mM Ca^{2+}, and inhibition was calculated to be 50%. On the other hand, in accordance with cell morphology, as can be seen in Fig. 6*B*, cells in which *Cyclin D1* was overexpressed were resistant to Ca^{2+} treatment, and growth inhibition was only 20%. These results suggest that *Cyclin D1* overexpression induces alteration of Ca^{2+}-induced keratinocyte terminal differentiation, which may cause unregulated cell proliferation in the skin when cells with a high cyclin D1 concentration are further affected by chemical carcinogens such as DMBA.

DISCUSSION

Amplification and expression of 11q13, including the *Cyclin D1* gene, are required for generation of esophageal and head and neck cancers (10–12). Although mice that overexpress the *Cyclin D1* oncogene have been developed and used for elucidating the functional significance of this gene in the development of esophageal squamous dysplasia (21–23), the notion that skin carcinogenesis is promoted by overexpression of *Cyclin D1 in vivo* has yielded conflicting results. Rodriguez-Puebla *et al.* (20) have suggested that *Cyclin D1* overexpression in SENCAR mouse epidermis does not affect skin tumor development. On the other hand, the same group has reported that

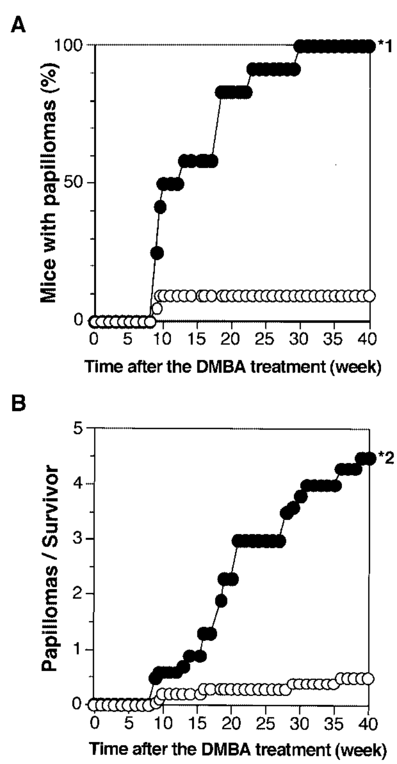

Fig 4. DMBA-dependent complete skin carcinogenesis. Conditional transgenic mice were treated topically once a week with 100 ng/ml DMBA beginning 7 days after the postactivation of *Cyclin D1* gene. Data are represented as percentage of mice with papillomas (*A*) and papillomas per survivor mouse (*B*). ●, transgenic mice; ○, wild-type mouse. Significance of differences relative to control noted by symbols: **1*, $P < 0.005$; **2*, $P < 0.005$.

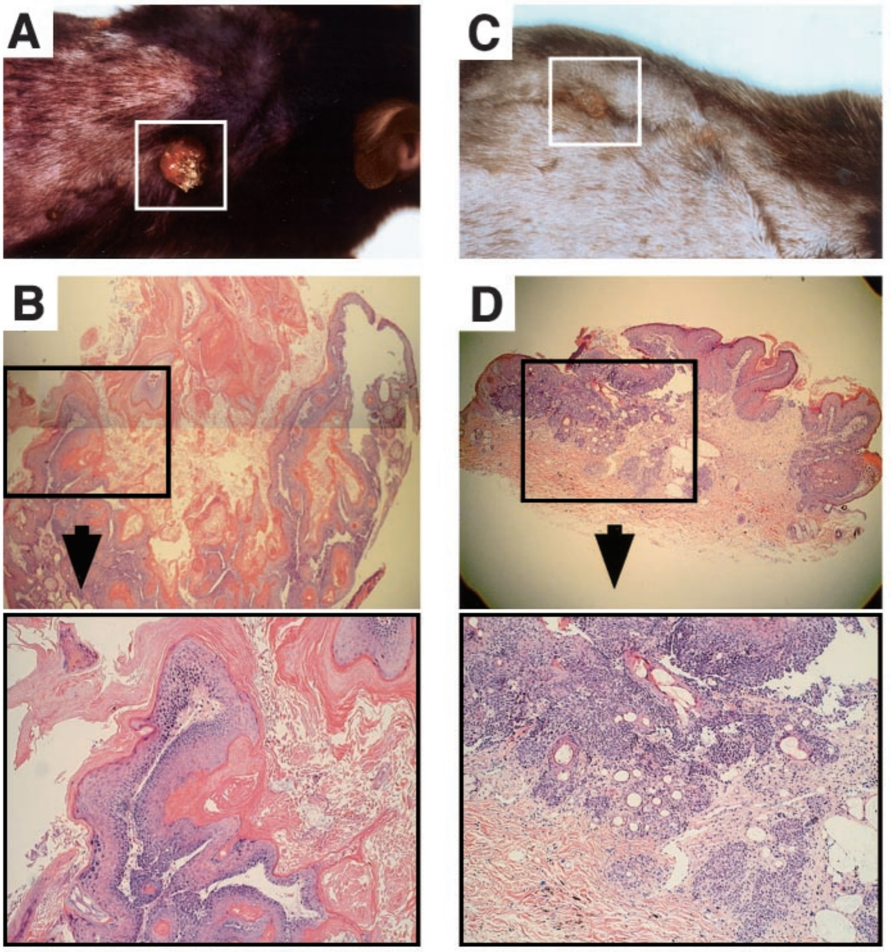

Fig. 5. Histological findings of mouse skin tumors. H&E-stained paraffin sections were used for morphological evaluation of skin tumors. A papilloma produced in the back skin of conditional transgenic mice 30 weeks after the treatment with AxCANCre and its histology are shown in *A* and *B*, respectively. A carcinoma produced in the back skin of conditional transgenic mice 40 weeks after the treatment with AxCANCre and its histology are shown in *C* and *D*, respectively. Enlargements of the sections indicated by *squares* in the *middle panels* of *B* and *D* are shown in the *bottom panels*.

expression of cyclin D1 in epithelial tissues of transgenic mice leads to epidermal hyperproliferation (34) and that cyclin D1 deficiency leads to reduced skin carcinogenesis (35). To understand the biological significance of *Cyclin D1* overexpression in squamous cell carcinomas, we successfully established conditional C57BL/6J transgenic mice with human *Cyclin D1*, using the conditional Cre/*lox* system. Precise genetic switches can be efficiently generated in a straightforward manner with an adenovirus that carries Cre recombinase (27). Our recombination-based conditional gene activation strategies *in vivo* can be used to design induced expression of the *Cyclin D1* gene to targeted skin epidermis at any desired time by a simple intradermal injection of adenoviruses.

The first goal of this study was to determine whether cyclin D1 and chemical carcinogens cooperate in the development and progression of skin tumors. In our skin-specific cyclin D1-overexpressing transgenic mice, we performed DMBA complete carcinogenesis because our mouse strain C57Bl/6J is insensitive to typical two-stage carcinogenesis induction (24–26). The results showed that *Cyclin D1* overexpression significantly enhanced skin carcinogenesis. The increased sensitivity of a skin tumor was accompanied by a statistically significant increase in BrdUrd incorporation of the stratum basale cells in mice with *Cyclin D1* overexpression. These results suggest that cyclin D1 may participate in cell cycle regulation of stratum basale cells and allowed enhancement of papilloma formation. This notion was partially supported by our previous results indicating that NIH3T3 cells that overexpressed *Cyclin D1* exhibited an unregulated growth profile and anchorage-independent phenotypic changes (28). Additional studies are required to determine whether our transgenic mice with C57BL/6J background showed increased sensitivity against DMBA/12-*O*-tetradecanoylphorbol-13-acetate two-stage carcinogenesis. Furthermore, as shown in the second part of our experiment, primary cultured keratinocytes from our transgenic mice in which *Cyclin D1* expression was induced were resistant to calcium-induced terminal differentiation and continued cell growth *in vitro*. These results from a novel animal model of human skin carcinogenesis provide us with direct experimental evidence that overexpression of *Cyclin D1* induces excessive dermal cell proliferation via an altered differentiation state of keratinocytes.

Mutations of the *ras* genes are frequently found both in human and in animal tumors (36, 37). The mouse model system for skin carcinogenesis has been used for the study of tumor initiation, promotion, and progression processes (38). Activation of the Ha-*ras* gene is sufficient to produce the papilloma phenotype at the time of initiation, whereas additional genetic changes are required for malignant conversion. DMBA induces predominant mutation of an A-to-T transversion in the mouse Ha-*ras* gene at codon 61 (39). However, we found that our DMBA-induced skin papillomas from *Cyclin D1*-overexpressing mice did not contain a mutation of codon 61 of Ha-*ras* or of codons 12 and 13. These results may suggest that overexpression of *Cyclin D1* gene could replace the function of an activated Ha-*ras* gene because mutant Ha-*ras* genes are found in a high frequency in premalignant tumors, meaning that the Ha-*ras* mutation is required for the initial step of carcinogenesis (40). They also suggest that cyclin D1 has a role as a downstream mediator of ras activity in tumor development (41). Animal model experiments in two-stage skin carcinogenesis showed that *Cyclin D1* overexpression occurred only in

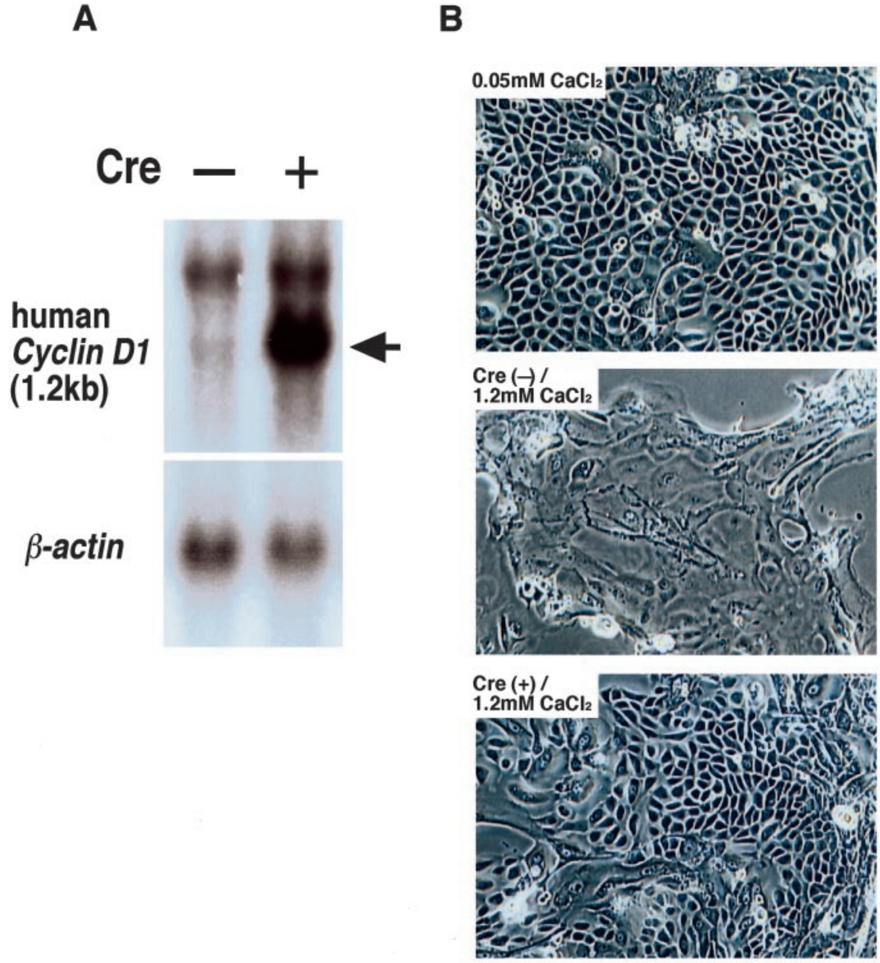

Fig. 6. AxCANCre-induced *Cyclin D1* gene overexpression in primary cultured keratinocytes. The keratinocytes were purified from conditional transgenic mice and then cultured in the presence of 0.05 mM Ca^{2+}. *A*, Northern blot analysis of mRNA from keratinocytes treated with AxCANCre (MOI of 50) and a control saline. A 1.2-kb specific transcript for human *Cyclin D1* can be detected. The same filter was rehybridized with an *β-actin* probe. *B*, phase-contrast morphology of Ca^{2+}-induced terminal differentiation of keratinocytes. Primary keratinocytes from *Cyclin D1* transgenic mice were treated with AxCANCre (*bottom panel*) or control saline (*middle panel*). Data presented are after 7 days of culture in the presence of 1.2 mM Ca^{2+}. As an undifferentiated morphological control, untreated cells were cultured in the presence of 0.05 mM Ca^{2+} (*top panel*).

premalignant lesions (42–44), supporting our results that early overexpression of *Cyclin D1* is important for enhanced skin carcinogenesis. In fact, although the data are not shown, post switching on of *Cyclin D1* gene activation followed by 40 weeks of treatment with DMBA exhibited no enhancement of tumorigenesis events thereafter.

Finally, we report here for the first time that a simple inoculation of Cre-expressing adenovirus into a target organ allows genetic switching on of human *Cyclin D1* in animals. Our adenovirus-mediated, recombinase-based conditional gene activation strategies *in vivo* can be used to design induced overexpression of *Cyclin D1* gene to any tissue at any desired time and will have a profound impact on fundamental biology and the design of better therapeutic models of human diseases.

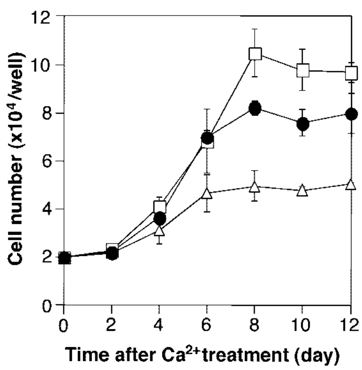

Fig. 7. Growth profiles of keratinocytes at different Ca^{2+} conditions. Cell growth of keratinocytes from conditional transgenic mice was monitored periodically after cells were treated with 1.2 mM Ca^{2+}. ●, AxCANCre plus 1.2 mM Ca^{2+}; △, control saline plus 1.2 mM Ca^{2+}; □, control culture in 0.05 mM Ca^{2+}. *Bars*, SD.

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