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

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ABSTRACT

Cyclin D1 is a critical gene involved in the regulation of progression through the G1 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 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 new 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, 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 G1 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). Oncogenic properties of cyclin D1 have been suggested by its cooperation with ras or adenovirus E1A to transform cultured cells (6, 7). Oncogenic properties of cyclin D1 have been suggested by its cooperation with ras or adenovirus E1A to transform cultured cells (6, 7).

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2 To whom requests for reprints should be addressed, at National Cancer Center, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Phone: 81-3-3542-2511, extension 2211; Fax: 81-3-3541-2685; E-mail: mterada@ncc.go.jp.

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.

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pCAGGS, resulting in a transgene named CALNL-Cyclin D1. The transgene was linearized and injected into C57BL/6 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/6 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 littersmates (10 weeks of age) were shaved with surgical clippers 1 day before *Cyclin D1* was switched on locally in the skin by intradermal administration of AxCANCre at 5 × 10^8 pfu/4–5 cm². 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-specific/clone-specific conditional gene activation by Cre recombinase was assessed by Northern blot analysis. 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.

**RT-PCR Analysis.** Total RNAs were prepared using ISOGEN solution (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 ^32P-labeled human Cyclin D1 cDNA fragment. The same filter was rehybridized with ^32P-labeled β-actin probe.

**Statistical Analysis.** Enzyme activities 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 cm²) intradermally. As a control, littersmates from the same litter carrying a nontransgene that were treated with AxCANCre were 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.

**RESULTS**

**Generation of Mice with Conditional Regulation of Cyclin D1 Expression.** To establish an animal model in which human *Cyclin D1* 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 loxP site, thereby initiating target gene expression in the target tissue (Fig. 1A). 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 cm²) intradermally. As a control, littersmates from the same litter carrying a nontransgene that were treated with AxCANCre were

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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. 2A, indicated by arrows), whereas expression of the transgene could not be demonstrated in control wild-type mice treated with AxCANCre (Fig. 2B). Western blot analyses showed that Cyclin D1 protein was detected in the skin injected with AxCANCare, whereas control AxCAwt did not induce overexpression of cyclin D1 (Fig. 2C). 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 AxCANCare 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 AxCANCare and 5.4 ± 1.8% in wild-type mice with AxCANCare. 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 AxCANCare. The first skin tumor appeared in AxCANCare-treated mice at 9 weeks after DMBA treatment, whereas the control wild-type mice with AxCANCare

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**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)/ethidium. The same RNA samples were subjected to RT-PCR analysis of products amplified with specific primers were electrophoresed and visualized with

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**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 AxCANCare in conditional transgenic mouse (A) and in control wild-type mouse (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.

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"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, but not with mRNA from the control mice (Fig. 1B). 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."
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. 4A). 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. 4B). 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.

**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 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. 6B, 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. 6B, 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. 6B, 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.
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 pre-malignant 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. 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.
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.

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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).

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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