Epidermal Expression of the Translation Inhibitor Programmed Cell Death 4 Suppresses Tumorigenesis

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Abstract

Programmed cell death 4 (Pdcd4) is a novel repressor of *in vitro* transformation. Pdcd4 directly inhibits the helicase activity of eukaryotic translation initiation factor 4A, a component of the translation initiation complex. To ascertain whether Pdcd4 suppresses tumor development *in vivo*, we have generated transgenic mice that overexpress Pdcd4 in the epidermis (K14-Pdcd4). K14-regulated Pdcd4 expression caused a neonatal short-hair phenotype due to early catagen entry compared with matched wild-type siblings. In response to the 7,12-dimethylbenz(*a*)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) mouse skin carcinogenesis protocol, K14-Pdcd4 mice showed significant reductions in papilloma formation, carcinoma incidence, and papilloma-to-carcinoma conversion frequency compared with wild-type mice. The translational efficiency of an mRNA engineered to form a structured 5′ untranslated region (UTR) was attenuated in primary keratinocytes when Pdcd4 was overexpressed. Pdcd4 inhibited by 46% TPA-induced activator protein-1 (AP-1)–dependent transcription, an event required for tumorigenesis. CDK4 and ornithine decarboxylase (ODC) are candidates for Pdcd4-regulated translation as their mRNAs contain 5′ structured UTRs. In K14-Pdcd4 primary keratinocytes expressing activated Ha-Ras to mimic DMBA-initiated epidermis, ODC and CDK4 protein levels were decreased by 40% and 46%, respectively. Expression of a protein encoded by 5′ unstructured mRNA showed no change. These results extend to an *in vivo* model the observations that Pdcd4 inhibits both translation initiation and AP-1 activation while decreasing benign tumor development and malignant progression. The K14-Pdcd4 mice seem to validate translation initiation as a novel target for cancer prevention. (Cancer Res 2005; 65(14): 6034-41)

Introduction

Eukaryotic cells regulate gene expression at multiple levels, among them transcription, splicing, stability, and translation of mRNA. Whereas transcriptional dysregulation is a well-studied contributor to cancer pathogenesis, increasing evidence implicates altered translational regulation as well (1, 2). Translation initiation is the predominant rate-limiting step for most mRNAs during protein synthesis. Expression of a translation initiation factor, eukaryotic translation initiation factor 4E (eIF4E), malignantly transforms rodent fibroblasts via downstream activation of Ras and acts directly as an oncogene in mice (3–5). Exposure to skin tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), enhances the formation of the translation initiation complex (6). Several tumors and tumor cell lines show elevated levels of components of the translation initiation complex, including eIF4A (7, 8), eIF4E (9), and eIF4G (10). Translation-dependent transcription has been suggested as a possible immediate early outcome of combined oncogenic assault by Akt and Ras. Coexpression of Akt with Ras produces increased recruitment of specific transcripts to the ribosome and consequently enhanced translation rate before induction of gene transcription (11). Therefore, inhibitory regulation of translation initiation factors may prevent or inhibit cancer induction.

Several eukaryotic translation initiation factors, including the eIF4F complex, participate in the translation initiation process. eIF4F is a multiple-subunit complex comprised of the scaffold protein eIF4G, the cap-binding protein eIF4E, and the RNA helicase enzyme eIF4A. eIF4E recruits capped mRNA to the initiation complex, completing the formation of eIF4F and facilitating the unwinding of structured mRNA by eIF4A, thus making possible ribosome binding. Current evidence suggests that the cap-binding activity of eIF4E is a limiting factor in recruitment of mRNA to the ribosome. However, efficient translation requires the unwinding of the mRNA secondary structure by the ATP-dependent helicase activity of eIF4A (12). Whether eIF4A may also be rate-limiting for translation of specific transcripts is currently unknown.

A novel eIF4A binding partner, programmed cell death 4 (Pdcd4), has been identified. Pdcd4 binds to and inhibits the helicase activity of eIF4A, subsequently inhibiting cap-dependent translation (13). Pdcd4 also binds eIF4G independently of its interaction with eIF4A although the consequence of Pdcd4 binding to eIF4G is not well understood. Pdcd4 seems to be the first example of a protein in mammalian cells that inhibits translation through attenuation of eIF4A activity. Pdcd4 is a novel inhibitor of transformation in cell culture models (14–16). *Pdcd4* was initially cloned from differential display analysis of JB6 transformation response variants derived from mouse epidermal cells (14). The JB6 model includes variants that are sensitive (P+) or insensitive (P−) to cellular transformation by tumor promoters (17, 18). *Pdcd4* is highly expressed in transformation-resistant (P−) JB6 cells and functions to inhibit transformation in the JB6 model (14–16).

Pdcd4 inhibits tumor promoter–induced transformation, at least in part, by inhibiting transactivation of the activator protein-1 (AP-1) transcription factor (15). AP-1–dependent transcription is required for transformation as expression of dominant-negative AP-1 is sufficient to inhibit tumor promotion/progression in the JB6 cell model, in a human keratinocyte progression series, and in a transgenic mouse line expressing an inhibitor of AP-1 (19–21). The inhibition of the AP-1 transactivation by Pdcd4 seems to be by an upstream indirect mechanism (15). Synthesis of AP-1 proteins is not inhibited by Pdcd4 (16). In addition, Jun/Fos

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protein concentration is not limiting as ectopic expression of individual AP-1 proteins fails to relieve inhibition of AP-1–dependent transcription by Pdcd4 (15). eIF4A and AP-1 seem to be linked via Pdcd4. A Pdcd4 mutant that is inactivated for binding to eIF4A is inactivated not only for inhibiting translation, but also for inhibiting AP-1 transactivation (13).

Pdcd4 is a novel candidate for cancer prevention yet to be assessed *in vivo* in animal models. The mouse skin two-stage carcinogenesis model has been productive in defining the irreversible genetic events of initiation and progression and the required threshold signaling events of tumor promotion during neoplastic development (22–26). To investigate whether overexpression of Pdcd4 may prevent or inhibit tumorigenesis *in vivo*, we generated a transgenic mouse model in which expression of Pdcd4 was directed to the epidermis by the human keratin 14 promoter. Pdcd4 expression inhibited translation of a luciferase transcript containing a structured 5′ untranslated region (UTR) and inhibited tumor promoter–induced AP-1 activity *in vivo*. Moreover, Pdcd4 expression provided resistance to tumor promotion and tumor progression in response to a multistage carcinogenesis regimen. These results support the hypothesis that Pdcd4 is an inhibitor of multistage carcinogenesis and suggest that targeting translation initiation may be a promising approach to cancer prevention.

Materials and Methods

Materials. TPA was purchased from Alexis Biochemical (San Diego, CA). 7,12-Dimethylbenz(*a*)anthracene (DMBA), anti-FLAG antibody, and $_{met}$FLAG cDNA sequence were purchased from Sigma-Aldrich (St. Louis, MO). Peptide purified anti-Pdcd4 antibody was described previously (27). Anti-CDK4 and anti–ornithine decarboxylase (ODC) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-eIF4A antibody was a generous gift from Bill Merrick (Case Western Reserve University, Cleveland, OH).

Generation and identification of K14-Pdcd4 transgenic mice. Murine $_{met}$FLAG-Pdcd4 cDNA was inserted into the hK14-hGH vector. DNA sequence for murine *pdcd4* was identical to previously published sequence (accession no. NM 011050). The hK14-hGH vector was characterized previously (28). The 6.5 kb K14-$_{met}$FLAG-Pdcd4 expression cassette was removed from the vector by digestion with *Kpn*I and *Hin*dIII, purified, and microinjected into the pronuclei of fertilized FVB/N mouse oocytes. Eight founders were positive for the transgene determined by Southern analysis of *Bam*HI-digested tail DNA. Subsequent identifications of transgenic mice were determined by PCR utilizing human specific K14 promoter based primers previously described (21). Mice were maintained in accordance with NIH guidelines.

Histology and immunohistochemistry. For the histology done to examine the hair cycle, animals were sacrificed at postnatal days 9, 12, 16, and 19. The dorsal skin was excised from approximately identical regions among the mice and fixed overnight in 10% buffered formalin, transferred to 70% ethanol, paraffin-embedded, and H&E stained. Age- and sex-match littermates were grouped, and the hair status was determined by morphologic examination.

For immunohistochemistry to show Pdcd4 expression, tissues were fixed in 4% paraformaldehyde (24 hours) and embedded in paraffin. Four-micrometer sections were cut for H&E staining or immunostaining. Deparaffinized slides were hydrated, trypsinized, blocked for endogenous peroxidase activity, preincubated with goat serum, incubated with anti-Pdcd4 antibody (1:200), and visualized with Rabbit IgG Vectastain ABC kit and DAB Peroxidase Substrate kit (Vector Laboratories, Burlingame, CA).

TPA-induced epithelial proliferation was measured by topically treating the dorsal skin with 5 nmol TPA or solvent control (acetone). Twenty-two hours after TPA treatment, mice were given by i.p. injection with 5-bromodeoxyuridine (BrdUrd) at 250 mg/kg body weight in 100 μL PBS and sacrificed 2 hours postinjection. Dorsal skin from approximately the same area on each mouse was fixed for 20 hours in 10% neutral buffered formalin, transferred to 70% ethanol, and processed immediately. BrdUrd incorporation was detected by immunohistochemical staining of paraffin-embedded sections. The percentage of positive staining was determined by observing 1,000 interfollicular basal keratinocytes for each mouse skin. Each treatment was done in three mice. To determine the percentage of apoptotic cells, the same skin sections were analyzed for positive staining by terminal deoxynucleotidyl transferase (TdT)–mediated nick-end nucleotide labeling (TUNEL).

Isolation of primary keratinocytes. Primary keratinocytes were harvested from 2- to 4-day-old postnatal pups as described previously (29).

Western blot analysis. Mice were euthanized and the shaved; the dorsal skin was removed. Epidermis was separated from the dermis by scraping or papillomas were ground in liquid nitrogen. Both types of samples were homogenized with 5 volumes of Tris/SDS extraction buffer [10 mmol/L Tris-HCl (pH 7.4), 0.1% SDS plus protease inhibitors]. The homogenate was centrifuged at 14,000 × *g* for 30 minutes at 4°C, and the supernatant was fractionated on 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and incubated with specific antibodies, detected using the appropriate secondary antibodies and visualized with ECL (Amersham Bioscience, Piscataway, NJ).

Virus production and candidate Pdcd4 target analysis. To investigate candidate Pdcd4 target proteins encoded by 5′ structured mRNAs, primary keratinocytes from either Pdcd4 transgenic or wild-type mice were infected with a replication-defective retrovirus containing the activated ras^{Ha} gene, called pBabepuroRasV12 or control pBabepuro (30). High-titer retroviral supernatants were obtained through transient transfection of retroviral plasmid DNA into the Ecopak packaging cell line (BD Biosciences, Franklin Lakes, NJ). After retroviral infection at a multiplicity of infection of 0.5 to 1, cells underwent puromycin selection. Cells were grown in puromycin-free media for 48 hours before harvest. The extracts were analyzed for steady-state protein levels of candidates for Pdcd4-specific attenuation via Western blot analysis.

Two-stage chemical carcinogenesis. Seven- to eight-week-old mice were initiated with a single topical application of DMBA (100 nmol/0.2 mL acetone) to the shaved backs. Two weeks later, TPA (5 nmol/0.2 mL acetone) or acetone alone was applied twice weekly to the initiated skin for 26 weeks (K14-Pdcd4-C1, $n = 16$; K14-Pdcd4-G6, $n = 14$; wild-type FVB/N, $n = 37$). Carcinomas were recorded grossly as downward-invading lesions, and malignancy was confirmed histologically as invading the panniculus carnosus.

Activator protein-1 luciferase activity. AP-1 luciferase activity was measured in primary keratinocytes derived from a cross between AP-1–Luciferase (AP-1–Luc) reporter mice (31) and K14-Pdcd4 mice, which produced AP-1–Luc/K14-Pdcd4 bitransgenic pups and AP-1–Luc transgenic siblings. Primary keratinocytes were plated overnight and treated for 13 hours with the indicated dose of TPA dissolved in DMSO or DMSO alone (0.1% final DMSO concentration). Cellular extracts were collected after lysis with Passive Lysis Buffer (Promega, Madison, WI). Luciferase activity was determined from lysates (25 μL) in a MLX Luminometer (Dynex Technologies, Chantilly, VA) and normalized to total DNA content.

Translational activity. A translation assay of the luciferase and structured luciferase reporter system was described previously (13). Primary keratinocyte cells from K14-Pdcd4-G6 and wild-type siblings were seeded in a 24-well plate and were transfected with 80 ng of pcDNA-LUC (luciferase) or pcDNA-SL-LUC (structured luciferase) and 20 ng RL-TK using Fugene6 (Roche Diagnostics, Indianapolis, IN). Cells were serum starved for 24 hours and incubated with medium + 10% chelexed fetal bovine serum for 20 hours. Cells were lysed in 1× passive lysis buffer. Luciferase activity was measured and normalized to renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) and an MLX luminometer.

Statistical analysis. Two-sided *P* values were calculated for papilloma multiplicity by Wilcoxon rank sum test, for tumor incidence and progression frequency by Fisher's exact test, and for AP-1 luciferase and stem-loop luciferase activity by Student's *t* test.

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Results

Transgenic gain of Pdcd4 expression in epidermis is cytoplasmic and causes transient short-hair phenotype. *In vitro* models suggest that Pdcd4 may negatively regulate cellular transformation. To determine whether overexpression of the Pdcd4 conferred resistance to multistage carcinogenesis, we generated transgenic mice expressing FLAG epitope-tagged Pdcd4 in the epidermis directed by the human keratin 14 promoter (hK14-hGH, called K14; refs. 21, 28).

All founders were bred to wild-type FVB/N mice to produce F_1 offspring in which the characterization of each line was done. Relative transgenic expression levels were determined by Western blot analysis of epidermal protein extracts with anti-Pdcd4 antibody. Two lines, hK14-FLAG-Pdcd4-G6 (Pdcd4-G6) and hK14-FLAG-Pdcd4-C1 (Pdcd4-C1), showed the highest levels of transgenic and total Pdcd4 expression. Of note, both lines independently displayed short-hair phenotype, which was evident during the first hair cycle up until 7 weeks of age (Fig. 1*B*).

Wild-type and transgenic primary keratinoctyes were cultured and protein extracts were collected for the purpose of determining relative Pdcd4 protein levels in basal keratinocytes. Overall Pdcd4 protein levels in Pdcd4-C1 and Pdcd4-G6 primary keratinocytes were increased 4-fold and 10-fold, respectively, as determined after Western blot analysis with anti-Pdcd4 antibody followed by densitometric analysis of Pdcd4-specific bands (Fig. 1*A*, *middle*). Both Pdcd4-C1 and Pdcd4-G6 keratinocytes displayed an increase in exogenous protein level determined after Western blot analysis with anti-FLAG antibody (Fig. 1*A*, *top*). Note that a low-expressing FLAG cross-reactive protein migrated as a doublet at approximately the same location as Pdcd4 (54 kDa). Although Pdcd4 binds to eIF4A and inhibits its activity, no compensatory elevation in eIF4A protein was detected in either Pdcd4-C1 or Pdcd4-G6 transgenic mice compared with wild-type. Thus, eIF4A served as a suitable loading control (Fig. 1*A*, *bottom*).

To determine the pattern of expression of Pdcd4 in the transgenic mouse skin, we did immunohistochemical staining using the anti-Pdcd4 antibody on sections of mouse skin. Pdcd4 was expressed in the outer root sheath of the hair follicle and interfollicular epidermis. Pdcd4 displayed diffuse cytoplasmic staining and an apparent lack of nuclear staining in wild-type mice, as indicated by the lack of Pdcd4 staining in hematoxylin-positive nuclei (Fig. 1*C*). Pdcd4 staining of transgenic Pdcd4-G6 skin showed localization similar to wild-type siblings but seemed markedly increased (Fig. 1*D*).

To further characterize the neonatal short-hair phenotype of Pdcd4-G6 and Pdcd4-C1 mice, skin of age- and sex-matched pups was harvested from the mid-dorsal region beginning at postnatal day 9 and continuing on days 12, 16, and 19. At day 16 postpartum during the primary hair cycle, the wild-type hair follicles remained in anagen with hair follicles embedded in the subcutis (Fig. 1*E*). However, Pdcd4 transgenic mice exhibited premature termination of anagen as determined by the complete regression of the hair follicles into the upper dermis (Fig. 1*F*). The temporal reduction in the primary hair cycle of Pdcd4 transgenic animals was consistent with presentation of the neonatal short-hair phenotype.

Pdcd4 inhibits helicase-dependent translation. Pdcd4 directly binds to and inhibits the helicase activity of eIF4A (13). The translational requirement for eIF4A is directly proportional to the degree of mRNA 5′ secondary structure (32). In JB6 cells, Pdcd4 reduces the translation efficiency of a luciferase reporter

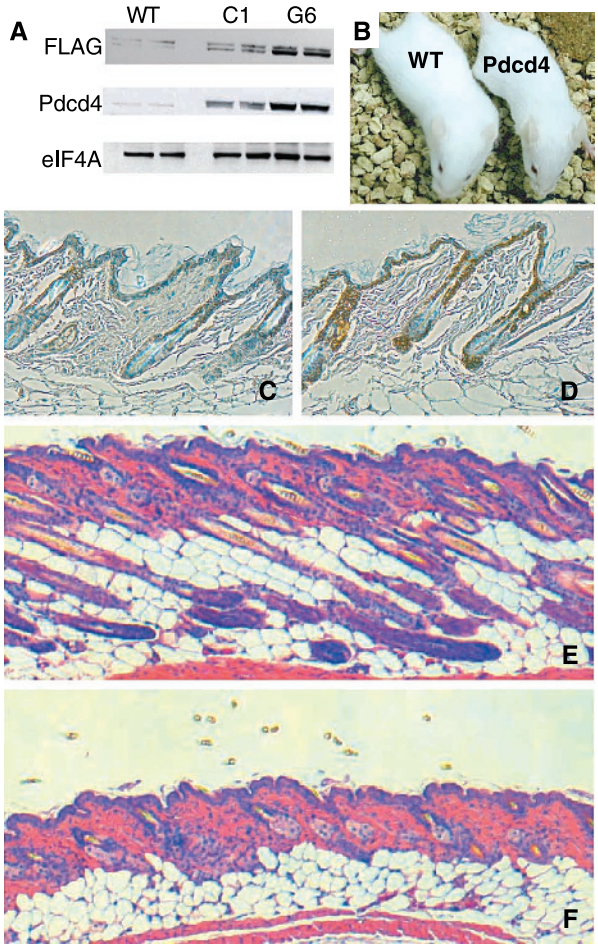

Figure 1. Transgenic expression of Pdcd4 decreases length of hair through the first hair cycle. *A,* Western blot of protein extract from primary keratinocyte cultured lines (wild type, Pdcd4-C1, and Pdcd4-G6), displaying endogenous Pdcd4 and exogenous FLAG epitope–tagged Pdcd4 expression by immunoblotting with anti-FLAG, anti-Pdcd4, or anti-eIF4A antibodies. Note the lack of change in eIF4A protein levels. *B,* photograph of wild-type and Pdcd4-G6 siblings (28 days old) revealing a shorter hair phenotype due to Pdcd4 overexpression. *C* and *D,* immunohistochemical staining of Pdcd4 in wild-type (*C*) and Pdcd4-G6 (*D*) mouse skin. Note the increase of brown staining within the epidermis and hair follicle in Pdcd4-G6 skin compared with wild-type skin. *E* and *F,* skin sections were made at 16 days postpartum. The follicles pictured are from similar mid-dorsal regions on both wild-type (*E*) and Pdcd4-G6 (*F*) mice. Pdcd4-G6 mice have almost completed catagen and follicles have regressed into the upper dermis. In contrast, the wild-type follicles remain in anagen.

transcript engineered with a 24 bp stem loop (ΔG = −44.8 kcal/mol), inserted 97 nucleotides downstream of the cap of the mRNA (33). The free energy of the 24 bp stem loop seems to be physiologically relevant due to its similarity to the TAR (+111) mRNA of HIV (ΔG = −49.9 kcal/mol; ref. 32). To determine whether the transgenic increase in Pdcd4 expression altered the *in vivo* translation efficiency of the stem-loop luciferase reporter transcript, wild-type and Pdcd4-G6 primary keratinocytes were cultured and transfected with plasmids carrying either stem-loop or non–stem-loop luciferase transcripts under the regulation of the CMV promoter. Elevated transgenic Pdcd4 expression inhibited translation of the stem-loop transcript by 43%, whereas translation of the unstructured mRNA was essentially unaffected.

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(Compare Pdcd4-G6 and wild-type primary keratinocytes; Fig. 2*A*.). The translation of the stem-loop luciferase was 2% as efficient as the translation of the non–stem-loop luciferase transcript in wild-type mouse keratinocytes. The Pdcd4-specific inhibition of translation of the stem-loop reporter suggests that translation of mRNA transcripts with structured 5′ UTRs may be particularly sensitive to Pdcd4 expression in transgenic mice.

Both CDK4 and ODC have been reported to be controlled partially at the level of translation due to 5′ structured UTRs (34). Comparing K14-Pdcd4 and wild-type primary keratinocytes subsequently infected with a retroviral construct expressing activated Ha-Ras to mimic DMBA-initiated epidermis, the immunoreactive protein of candidate genes *ODC* and *CDK4* was decreased by 40% and 46%, respectively (Fig. 2*B*). There was no difference in 5′ unstructured mRNA-encoded eIF4A protein (Fig. 2*B*) or actin (data not shown) protein levels in transgenic and wild-type primary cultures, suggesting specificity toward mRNA with greater secondary structure. In contrast to protein levels, candidate gene mRNA levels remained equal as determined by gene array analyses.[1] Thus, translation of both structured luciferase and structured endogenous mRNAs, but not of unstructured mRNAs, is inhibited by Pdcd4 expression.

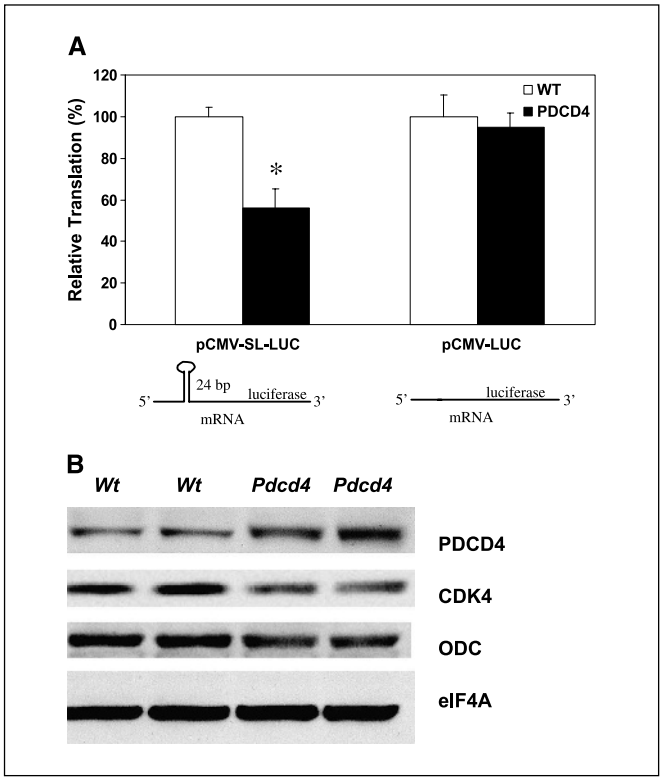

Figure 2. Pdcd4 expression inhibits translation of 5′ UTR structured mRNA. *A,* basal keratinocytes were cultured from wild-type and Pdcd4-G6 transgenic mice. pCMV-LUC or pCMV-SL-LUC were cotransfected with pRL-TK (reporter used to control for variations in transfection efficiency). Cellular extract was collected and luciferase activity was measured. All luciferase activity was normalized to individually determined renilla luciferase activity. Translational activity of wild-type mice was arbitrarily expressed as 100%. Pdcd4 overexpression decreased the translation of the stem loop luciferase transcript by 43%. *Statistical significance compared with wild-type keratinocytes ($P < 0.001$). Data are representative of three independent experiments. *B,* activated-ras^{Ha} was introduced via retroviral infection of both Pdcd4-G6 and wild-type primary keratinocytes. Western blot analyses were done utilizing extracts with anti-Pdcd4, anti-eIF4A, anti-CDK4, and anti-ODC antibodies. Samples from replicate retroviral transfections were analyzed upon the same SDS-PAGE and shown. The immunoreactive protein of ODC and CDK4 was decreased by 40% and 46%, respectively, in the K14-Pdcd4 cells. Note that under conditions of activated Ras, the relative overexpression of the Pdcd4 transgene seems only 2-fold higher.

Pdcd4 inhibits both basal and tumor promoter–induced transactivation of activator protein-1–dependent transcription. AP-1–dependent transcription is required for tumor promotion in the mouse skin (21). Pdcd4 has been shown to inhibit TPA-induced AP-1 transactivation in tissue culture models in a manner dependent on Pdcd4 interaction with eIF4A (13, 15). To address whether Pdcd4 inhibition of AP-1 transactivation extends to an *in vivo* model, the K14-Pdcd4 transgenic mice were bred to AP-1–Luc reporter transgenic mice, which ubiquitously express a luciferase transcript under the regulation of an AP-1–dependent promoter (31). Primary keratinocytes were harvested from pups and pooled according to genotype. AP-1–Luc/Pdcd4 bitransgenic cultures were compared with those from AP-1–Luc transgenic siblings negative for Pdcd4 transgenic expression. Both basal and TPA-induced AP-1 activity were attenuated in Pdcd4-C1/AP-1–Luc and Pdcd4-G6/AP-1–Luc bitransgenic keratinocytes (Fig. 3). The inhibition of luciferase activity in transgenic keratinocytes is unlikely to arise from nonspecific inhibition of luciferase mRNA translation, as Pdcd4 does not alter translation of the luciferase transcript in JB6 RT101 cells transfected with pCMV-luciferase (33). To determine the specificity of the inhibition of AP-1, the K14-Pdcd4 transgenic mice were crossed to nuclear factor-κB (NF-κB)–luciferase promoter transgenic mice (35). Keratinocytes were harvested and the relative level of NF-κB–dependent activity was measured. Although this model could not be analyzed for tumor promoter–induced NF-κB (as none occurs), analysis of the high basal levels of NF-κB revealed no inhibition by Pdcd4 (data not shown). Thus, as seen in cell culture (15), Pdcd4 specifically inhibits AP-1–dependent transcription *in vivo*.

Expression of Pdcd4 attenuates both tumor development and tumor progression. Because Pdcd4 inhibits transformation and maintenance of tumor phenotype in the JB6 cell culture model, Pdcd4 might be expected to provide genetic resistance to multistage carcinogenesis in an *in vivo* animal model. The tumorigenesis responses of Pdcd4-C1 and Pdcd4-G6 transgenic mice were compared with wild-type siblings during a DMBA/TPA chemical carcinogenesis regimen. Both Pdcd4-C1 (n = 16) and Pdcd4-G6 (n = 14) transgenic mice displayed reduced papilloma burden compared with wild-type siblings (n = 37) by 45% and 65%, respectively (Fig. 4*A*). No tumors developed in any group with DMBA/acetone (data not shown). Benign tumor latency was delayed by 4 weeks in both transgenic Pdcd4-C1 and Pdcd4-G6 lines (Fig. 4*B*). Despite the delay in latency, the papilloma number for all mice reached a maximal level by week 24. This suggests that the papillomas are inhibited for formation rather than simply growing more slowly.

To address the possibility that the tumors that did form might have escaped Pdcd4-mediated inhibition by losing Pdcd4 expression, we collected a random sampling of papillomas and uninvolved epidermis from Pdcd4-G6 mice. For each sample, whole-cell protein extract was harvested and the relative amounts of Pdcd4 protein were determined by Western blot analysis. Interestingly, four of four Pdcd4-G6 papillomas measured displayed markedly reduced Pdcd4 protein levels when compared with

[1] A. Jansen et al., in preparation.

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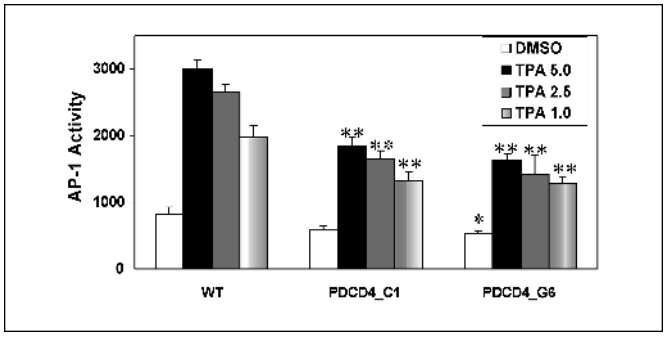

Figure 3. Pdcd4 expression inhibits AP-1 transactivation. Wild-type, Pdcd4-C1, and Pdcd4-G6 mice were bred to AP-1 luciferase reporter mice. Primary keratinocyte cultures were treated with the indicated TPA dose (or 0.1% DMSO) for 13 hours. AP-1 activity refers to luciferase activity per microgram of DNA. Pdcd4 expression was inhibited (46%) both the basal and TPA-induced AP-1 activity in either Pdcd4-C1 or Pdcd4-G6 transgenic cells compared with wild-type cells. *Statistical significance compared with wild-type DMSO alone ($P < 0.05$). **Statistical significance compared with wild-type respective TPA treatment ($P < 0.01$). Data are representative of three independent experiments.

uninvolved epidermis (Fig. 4*C*). This suggests that either long-term TPA treatment down-regulates Pdcd4 or that spontaneous down-regulation of elevated Pdcd4 protein is selected for during papilloma development.

Following discontinuation of TPA at week 26, papillomas were followed for progression to squamous cell carcinoma (SCC). Carcinoma incidence was decreased at weeks 25 and 35 in Pdcd4-C1 and Pdcd4-G6 lines. Whereas 57% of wild-type siblings were positive for SCC formation, only 25% of Pdcd4-C1 and 7% of higher-expressing Pdcd4-G6 mice were SCC positive (Table 1). To further address the reduction of progression-to-carcinoma incidence, each papilloma was defined as an individual event and the number of carcinomas per papilloma was calculated and converted to a percentage (conversion frequency). By week 35, wild-type siblings exhibited a conversion frequency rate of 13%, whereas Pdcd4-C1 and Pdcd4-G6 displayed rates of 9% and 3%, respectively (Table 1). Thus, elevated Pdcd4 protein levels not only decrease benign tumor development but also decrease the rate of malignant conversion.

In a number of mitotically active mouse tissues, including the glandular stomach, colon, and cervix, Pdcd4-positive staining inversely correlated with positive proliferating cell nuclear antigen staining within the tissue.[2] This suggests that Pdcd4 protein levels are higher in amitotic or more differentiated cells. To test whether Pdcd4 decreased the TPA-induced mitogenic response in the epidermis, groups of three mice were treated either once or multiple times (eight times) with TPA (5 nmol/0.2 mL acetone) or acetone alone, then injected i.p. with BrdUrd 22 hours after the final TPA treatment and subsequently harvested 2 hours later. Pdcd4 transgenic expression was insufficient to decrease either basal or TPA-induced mitogenic DNA replication in the epidermis.

Because increases in Pdcd4 steady-state mRNA levels have been associated with cell death in certain models, we asked whether Pdcd4 increased the number of apoptotic cells in the epidermis as determined by TUNEL assay. Like the BrdUrd results, with or without TPA treatment, Pdcd4 transgenic expression had no effect on the percentage of TUNEL-positive cells.

[2] Unpublished data.

Elevated Pdcd4 protein expression was also not sufficient to induce basal primary keratinocytes from Pdcd4-C1 or Pdcd4-G6 transgenic lines to differentiate prematurely in low-calcium medium. Thus, Pdcd4 transgenic expression inhibits skin tumorigenesis and tumor progression without altering TPA-induced mitogenic response, apoptosis, or terminal differentiation.

Discussion

Two lines of mice harboring a human K14 promoter–driven Pdcd4 show increased Pdcd4 expression targeted to the keratinocytes of the basal epidermis and the outer root sheath of the hair follicle. The K14-Pdcd4 transgenic mouse shows that targeting translation initiation can be effective for cancer prevention *in vivo*. As in cell culture models, mRNA-specific inhibition of translation initiation and specific inhibition of AP-1 induction seem to contribute to the suppression of tumorigenesis by Pdcd4.

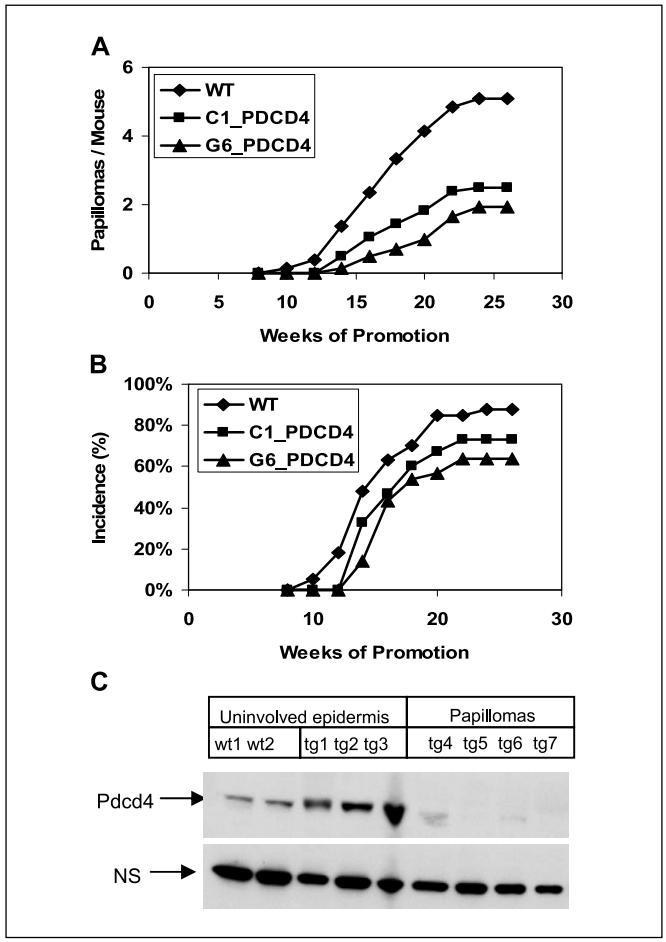

Figure 4. Pdcd4 delays and attenuates papilloma development in a dose-dependent manner. Pdcd4-C1, Pdcd4-G6, and wild-type siblings (7-8 weeks old) were initiated topically with DMBA (100 nmol). Mice were treated topically twice weekly with TPA (5 nmol per treatment) for 26 weeks. *A,* papilloma multiplicity (measured through 25 weeks) was reduced with Pdcd4-C1 and Pdcd4-G6 transgenic mice by 45% and 65%, respectively. *B,* papilloma incidence (percentage mice bearing papilloma) was delayed by 4 weeks in both Pdcd4-C1 and Pdcd4-G6 transgenic mice. All acetone mice were negative for papilloma development and not plotted. *C,* as mice were terminated from the experiment due to carcinoma invasion, uninvolved epidermis and papillomas were randomly chosen from wild-type and Pdcd4-G6 mice. Western blot analysis of extracts was done with anti-Pdcd4 antibody. An invariant nonspecific band was used for loading control comparisons.

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Table 1. Pdcd4 suppresses benign tumor outgrowth and malignant conversion rate

Tumors	Wild type	C1	G6
Papillomas/mouse	5.1	2.8*	1.8*
Papilloma incidence	88%	73%	64%
Carcinoma incidence			
(25 wk)	16%	6%	0%
(35 wk)	57%	25%†	7%†
Conversion frequency			
(25 wk)	6%	2%	0%
(35 wk)	13%	9%	3%‡
Mice, *n*	37	16	14

NOTE: Mouse skin was initiated with a single application of DMBA (100 nmol), followed by twice weekly application of TPA (5 nmol) in 0.2 mL acetone for 26 weeks. Changes in papilloma and carcinoma development between Pdcd4-G6 and Pdcd4-C1 mice were compared to respective wild-type littermates.

*Two-sided $P < 0.05$ and $P < 0.005$ for comparison of papilloma burden between wild-type and C1 and wild-type and G6, respectively.

†Two-sided $P < 0.05$ and $P < 0.001$ for comparison of carcinoma incidence between wild-type and C1 and wild-type and G6, respectively.

‡Two-sided $P < 0.001$ for comparison of conversion frequency between wild type and G6.

The observation that Pdcd4 inhibited both tumor promotion and tumor progression seems to be consistent with the evidence that translation initiation factors are linked to cancer pathogenesis (1, 2). Because Pdcd4 interacts with and inhibits the RNA helicase eIF4A, it is plausible that Pdcd4 decreases the translation efficiency of a small set of target mRNAs having highly structured 5′ UTRs that require the unwinding activity of eIF4A helicase. It seems that without Pdcd4, eIF4A would be left unchecked to increase the rate of inefficiently translated mRNAs, thereby causing the cells to proliferate aberrantly leading to cancer. Genes known to drive oncogenesis whose mRNAs are translationally regulated by structured 5′ UTRs include insulin-like growth factor II, transforming growth factor-β, androgen receptor, CDK4, cyclin D1, p53, and ODC (reviewed in ref. 34). We show in this model that Pdcd4 causes a decrease in steady-state protein levels of two of the above factors, ODC and CDK4, in primary keratinocytes infected with activated Ha Ras. The reduction in protein levels does not seem to be mediated by enhanced degradation because protein levels of ODC and CDK4 remain relatively unchanged during proteasome treatment (data not shown). However, use of proteasome inhibitor, even at low doses, causes significant death to the primary keratinocyte cultures within 8 hours. Decreases in CDK4 protein levels have been reported in Bon 1 carcinoid cells upon overexpression of Pdcd4 (36). Our observations with the K14-Pdcd4 mice are consistent with those for cdk4 null mice in which skin tumor development, but not induction of keratinocyte proliferation, is inhibited (37). Whereas increased proliferation can be an important contributor to tumorigenesis, it is not targeted by Pdcd4 when Pdcd4 inhibits tumorigenesis. This is consistent with multiple models in which inhibition of tumorigenesis can occur *without* affecting tumor promoter–induced hyperplasia caused by multiple promoters, including TPA, okadaic acid, UV, and human papillomavirus 16 E7 (21, 38–40).

Tumor promoter–induced AP-1 activity is necessary for tumor promotion in mice (21). Whereas inhibition of TPA-induced AP-1 activity provides a possible mechanism for the carcinogenesis-resistant phenotype of the K14-Pdcd4 mice, it may not suffice as the two transgenic mouse lines inhibit AP-1 activity to similar levels. Reduction in tumor burden is greater in the higher-expressing Pdcd4-G6 than in the lower-expressing Pdcd4-C1 transgenic line. This suggests either that the carcinogenesis-resistant phenotype is independent of AP-1 inhibition or that AP-1 inhibition by Pdcd4 is necessary, but not sufficient, for the reduction of tumor multiplicity to the level seen in the higher-expressing Pdcd4-G6 line. Clarifying the role of AP-1 inhibition in the mechanism by which Pdcd4 confers resistance to tumorigenesis and tumor progression would call for transgenic expression of Pdcd4 mutants that fail to inhibit AP-1 activity and/or translation (13, 33). Whether translational inhibition via eIF4A and reduction in AP-1 activity are linked to each other in this model is not established. However, in an *in vitro* tissue culture model, Pdcd4 mutants that fail to bind to eIF4A fail to inhibit both translation and TPA-induced AP-1 activity (13), providing evidence that Pdcd4-specific inhibition of AP-1 activity is dependent upon and downstream of translation inhibition.

Although Pdcd4 suppresses tumor induction and tumor progression as do some of the known tumor suppressors, Pdcd4 differs from these tumor suppressors in apparently not being inactivated by genetic mutation in human cancer.[3] Pdcd4 overlaps with established tumor suppressors in the following respects: (*a*) Overexpression of Pdcd4 in mouse skin attenuates both benign tumor development and malignant progression. (*b*) In a transformed cell line (mouse RT 101), expressing Pdcd4 causes a reversion of tumor phenotype (16). (*c*) Inactivation of *PDCD4* by down-regulated protein expression accompanies metastatic progression in certain of the NCI60 human tumor cell lines (27). (*d*) Loss of *PDCD4* expression in human lung cancer correlates with tumor progression and poor patient prognosis (41). The necessity of a threshold of Pdcd4 seems to be supported by the intermediate response to chemical carcinogenesis of the lower-expressing Pdcd4-C1 (4-fold) transgenic line compared with the Pdcd4-G6 (10-fold) line and wild-type FVB/N siblings. The relative potency of Pdcd4 as a tumorigenesis inhibitor is expected to be influenced not only by protein level but also by activity status. Determining the activity of Pdcd4 in papillomas or SCCs will call for the generation of stem-loop luciferase transgenic reporter mice to determine *in vivo* translation inhibitory activity. Ascertaining the levels of transgenic Pdcd4 expression and activity in SCCs will provide insight into the potency and context dependency of Pdcd4 suppressor activity.

Pdcd4 overexpression prematurely induced withdrawal from anagen in the initial postnatal hair cycle of neonates. The regression of the hair follicle during catagen requires programmed cell death (42). Evidence suggests that premature induction of catagen is caused by early induction of apoptosis rather than decrease in proliferative capability of the hair follicle (43). The premature induction of catagen occurs in both lines of Pdcd4 transgenic mice. Therefore, 4-fold overexpression of Pdcd4 as determined in the Pdcd4-C1 line is sufficient to cause the hair growth phenotype. Whether Pdcd4 is causing premature catagen by induction of apoptosis or decrease in proliferation is under

[3] A. Jansen et al., unpublished data.

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investigation.[4] Despite its name, the role of Pdcd4 in programmed cell death and apoptosis remains unclear. Conflicting evidence exists as to whether the Pdcd4 transcript is up- or down-regulated during induced apoptosis. Expression of the Pdcd4 transcript is up-regulated in a number of apoptosis models and in senescent human diploid fibroblasts (44, 45). However, treatment of mouse lymphoma cells with topoisomerase inhibitors, which induce apoptosis, down-regulates Pdcd4 transcript (46). Recently, Pdcd4 expression was shown to be up-regulated by retinoic acid receptor agonists in human breast cancer cells, and Pdcd4 expression was sufficient to induce apoptosis in the T47D breast cancer cell line (47). In addition, Pdcd4 alters renal cancer cell response to geldanamycin from cytostatic to cytotoxic via an apoptotic mechanism mediated by G_2-M arrest (27).

Mice genetically engineered for resistance to carcinogenesis, such as Pdcd4 transgenic mice, provide tools to validate molecular targets for cancer prevention (48). The generation of the K14-Pdcd4 transgenic mouse and the ability of Pdcd4 to target a rate-limiting process in multistage carcinogenesis provide a valuable tool for understanding the role of translational regulation in cancer development. In contrast to housekeeping genes, many transcripts involved in growth control or oncogenesis have highly structured 5′ UTRs. As Pdcd4 inhibits eIF4A helicase, it is plausible to hypothesize that drugs designed to mimic Pdcd4 in inhibiting eIF4A may target the altered regulation in cancer cells without changing the translation of housekeeping genes. Such a drug might have a therapeutic dose window toxic to cancer cells, yet be relatively harmless to normal tissue. Building on the premise that the protein synthesis machinery is an attractive target for therapeutic intervention, discovering specific mRNA molecules and their protein products that are regulated by Pdcd4 may reveal important future targets for therapeutic consideration.

[4] A. Jansen et al., in preparation.

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