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# Proprotein Convertase Inhibition Results in Decreased Skin Cell Proliferation, Tumorigenesis, and Metastasis[1,2]

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## Abstract

PACE4 is a proprotein convertase (PC) responsible for cleaving and activating proteins that contribute to enhance tumor progression. PACE4 overexpression significantly increased the susceptibility to carcinogenesis, leading to enhanced tumor cell proliferation and premature degradation of the basement membrane. In the present study, we sought to evaluate a novel approach to retard skin tumor progression based on the inhibition of PACE4. We used decanoyl-RVKR-chloromethylketone (CMK), a small-molecule PC inhibitor, for *in vitro* and *in vivo* experiments. We found that CMK-dependent blockage of PACE4 activity in skin squamous cell carcinoma cell lines resulted in impaired insulin-like growth factor 1 receptor maturation, diminished its intrinsic tyrosine kinase activity, and decreased tumor cell proliferation. Two-stage skin chemical carcinogenesis experiments, together with topical applications of CMK, demonstrated that this PC inhibitor markedly reduced tumor incidence, tumor multiplicity, and metastasis, pointing to a significant delay in tumor progression in wild-type and PACE4 transgenic mice. These results identify PACE4, together with other PCs, as suitable targets to slow down or block tumor progression, suggesting that PC inhibition is a potential approach for therapy for solid tumors.

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## Introduction

Proprotein convertases (PCs) are serine proteases that participate in the posttranslational activation of inactive proteins leading to mature, active proteins [1,2]. The family comprises several members: PC1/3, PC2, PC4, PC5, PC7, furin, PACE4, subtilisin kexin isoenzyme 1, and neuronal apoptosis-regulated convertase 1. Many of the PCs protein substrates such as matrix-degrading enzymes [3], growth factors, growth factor receptors, and adhesion proteins [4,5] have a direct role in tumor progression. Overexpression of PCs has been documented in a variety of neoplasms, including those from skin, lung, ovary, nervous system, and colon [6–9], some leading to increased processing of their cognate substrates [10]. Although all of these proteases recognize and cleave at the C-terminus of the motif RXKR, differences in substrates preferences [11], inhibition kinetics [12,13], and the phenotypes of the different PC's knockout animals [14] suggest some degree of heterogeneity in PC functions. In this context, we demonstrated a preferential overexpression of PACE4 in chemically induced skin tumors and its role promoting cell proliferation and basement membrane degradation, pointing to a unique role of this PC in murine skin cancer [15]. The tumor-enhancing characteristic of PACE4 suggested that inhibition of this PC's activity would result in decrease proliferation and tumor development. PACE4, as the other PC's, is activated in the trans-Golgi network and transported into vesicles to the extracellular compartment [16,17]. PACE4 is an extracellular PC that binds several components of the extracellular matrix, such as heparan sulfate proteoglycans [18]. PACE4 anchors to extracellular matrix components through its cysteine-rich domain, facilitating its localization to the cell membrane and the processing of its membrane associated substrates [19]. Also, it has been proposed that PACE4 may be activated in the cell surface pointing to a novel mechanism for PC action [20].

Abbreviations: AMC, aminomethylcoumarine; CMK, decanoyl-RVKR-chloromethylketone; PC, proprotein convertase; SCC, squamous cell carcinoma

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Insulin-like growth factor 1 receptor (IGF-1R) is a crucial PC substrate involved in cell proliferation and is the first component of the IGF-1–mediated signal transduction pathway [8,21]. Because blockage of PACE4 activity *in vivo* may lead to the impairment of the IGF-1 signal transduction pathway [22], we sought to demonstrate the feasibility of this novel therapeutic approach to delay skin tumor progression in a paradigmatic animal model of human cancer, that is, a two-stage skin carcinogenesis.

Experiments based on tumor cell transfections demonstrated that the Portland variant of $\alpha_1$-antitrypsin protein [12] mediated the inhibition of PC activity, resulting in decreased proliferation [8,9,23,24], invasion [23], or adhesion to extracellular matrix proteins [21,25] or endothelial cells [21]. To determine the usefulness of PACE4 as a target for cancer therapy, we evaluated the effects of its inhibition on epidermal cell proliferation and *in vivo* tumor development. To this effect, we selected the small-molecule decanoyl-RVKR-chloromethylketone (CMK) [26] that successfully blocked cell proliferation [10,27] and invasion in cell-based assays [24,28,29], contains a hydrophobic moiety that may favor its absorption into the skin, and could potentially be used as a topical agent. Furthermore, CMK is commercially available.

In this article, we provide evidence that CMK-dependent *in vivo* inhibition of PACE4 activity results in decreased epidermal basal keratinocyte proliferation, tumor development, and metastasis, demonstrating that inhibition of PCs has the potential to be a therapeutic tool.

## Materials and Methods

### *Cell Lines and Transfection Procedures*

We used mouse skin cell lines, including line 308 obtained form normal adult keratinocytes [30], a line derived from a skin papilloma MT1/2, and lines derived from carcinomas JWF-2, CH72, and CH72T3, generously provided by Dr Susan Fischer (MDACC Science Park, Smithville, TX). These cells were selected because they originated from papillomas and squamous cell carcinomas (SCCs) from mice treated with chemical carcinogens. In addition, we included two isogenic cell lines, isolated in our laboratory, derived form a low-grade SCC (CC4B) and spindle cell carcinoma (CC4A) [31]. All cells were grown in spinner minimal essential medium eagle (s-MEM) medium containing 10% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin (Pen-Strep, Cellgro, Manassas, VA) (100 U/ml and 100 mg/ml, respectively). Primary epidermal keratinocytes were prepared as described in a previous publication [15].

### *Small Interference RNA*

PACE4 small interference RNA (siRNA) was synthesized using the pSilencer siRNa Construction Kit (Ambion, Austin, TX). The template sequences were obtained from RNAi Central, a Web page from Hannon Laboratory (http://katahdin.cshl.org:9331/homepage/portal/scripts/main2.pl).

The sequences used as templates are shown in Table 1.

### *PACE4 Enzymatic Assay*

PACE4 catalyses the hydrolysis of the synthetic substrate Boc-RXKR-AMC into Boc-RXKR and aminomethylcoumarine (AMC). The latter is a fluorescent compound that can be used to monitor the extent of the reaction. Reactions were performed as previously described [32]. Briefly, conditioned medium from cell cultures was incubated for 17 hours at 30°C in the presence of Boc-RXKR-AMC (50 μM), 100 mM HEPES (pH 7.5), 0.5% Triton X-100, and 1 mM $CaCl_2$. To assess the extent of CMK inhibition, we evaluated PACE4 activity in the presence of CMK (0.1-200 μM). After completion of the reaction, we measured of AMC liberated in a plate reader spectrofluorometer, using the program Cary Eclipse, Varian Inc ($\lambda_{absorption}$ = 380 nm, $\lambda_{emission}$ = 460 nm, 800 V of sensitivity). Experiments were performed in triplicates.

**Table 1.** SiRNA Templates.

| Template | | Sequence |
|---|---|---|
| mPACE4 1 | Forward | 5′AACGCGAAGTGACTCTCTTTACCTGTCTC 3′ |
| | Reverse | 5′AATAAAGAGAGTCACTTCGCGCCTGTCTC 3′ |
| mPACE4-2 | Forward | 5′AAGCGAGCGACTGGAAAGTCACCTGTCTC 3′ |
| | Reverse | 5′AATGACTTTCCAGTCGCTCGCCCTGTCTC 3′ |
| mPACE4 scr | Forward | 5′AACGGTGCAGACTGAACGAAGCCTGTCTC 3′ |
| | Reverse | 5′AACTTCGTTCAGTCTGCACCGCCTGTCTC 3′ |

### *Western Blot Analyses*

Cell lysis and Western blot were performed using 50 μg of cell lysates as described previously [33]. Antibodies used for immunoblot analysis included anti–IGF-1R β-subunit (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and a monoclonal antibody anti–phospho-IGF-1R, anti–extracellular signal–regulated kinase 1/2 (ERK1/2), and anti–phospho-ERK1/2 (Cell Signaling Inc, Beverly, MA). Filters were incubated with the appropriate secondary horseradish peroxidase–conjugated antirabbit or antimouse (Amersham, Piscataway, NJ) antibodies.

### *Proliferation Assay*

*In vitro* cell proliferation was measured as incorporation of [$^3$H] methyl thymidine into DNA as described elsewhere [8,23].

### *CMK Labeling, Absorption Experiments, and Fluorescence Microscopy*

CMK was diluted to 2 mM in DMSO and 0.01 M carbonate buffer (pH 9). Equimolecular quantities of fluorescein isothiocyanate (FITC; 10 mM in DMSO) were added with constant stirring. The reaction mix was incubated for 2 hours at room temperature protected from light. A thin precipitate corresponding to the FITC derivative separated from the reaction mixture. The reaction was stopped by the addition of 2 μl of 0.1 M phosphate buffer pH 7.4, and the crude precipitate was filtrated through Microcon 10 filters (Millipore, Billerica, MA) and washed with methanol to remove the excess of FITC and DMSO. The filtrate was analyzed for the presence of FITC by thin-layer chromatography (silica gel, developed with acetone, $R_f$ FITC = 0.91, $R_f$ neutralized product = 0.3). The precipitate was washed until FITC was no longer detectable using this system. The washed precipitate containing FITC-labeled CMK (FITC-CMK) was finally dissolved in acetonitrile/2% formic acid; the purity of the product was confirmed by C-18 HPLC column and analyzed by mass spectrometry (calculated mass = 1133, found = 1133, main peak = 1079).

To determine the skin absorption of FITC-CMK, female K5-PACE4 transgenic mice that overexpress the PC under the control of the keratin-5 promoter in squamous epithelia [15] were used after shaving the dorsal skin. The following day, the animals were treated by topical application of a solution of either 100 μM or 1 mM of FITC-CMK. As controls, animals were treated with acetone or 1 mM FITC alone. One hour thereafter, the animals were killed; the skin was dissected and immediately frozen in OCT embedding medium (Tissue-Tek, Torrance, CA). Samples were mounted with fluorescent mounting medium (Prolong Gold antifade reagent; Molecular Probes, Eugene, OR).

Frozen sections (5 μm) from the FITC-CMK–labeled skins were analyzed by fluorescence microscopy. Imaging was done in a microscope (Eclipse TE 2000-U; Nikon, Melville, NY) using a magnification of 60× (NA = 1.4) by excitation at 364 nm. Pictures were obtained with a camera (CoolSNAP HQ; Roper Scientific, Tucson, AZ), with an exposure of 1 second, and processed using Metamorph software (Molecular Devices Inc, Downingtown, PA). For quantitative measurements of fluorescence, the images were visualized in pseudocolor to enhance the levels of fluorescence, and the difference between the fluorescence of the image and the background was recorded and plotted in a bar diagram.

### *Skin Carcinogenesis*

We used wild-type FVB × FVB mice purchased from Taconic as controls. Transgenic K5-PACE4 animals were generated in house as described elsewhere [15]. These transgenic mice express PACE4 under the control of the keratin 5 promoter, which restricts its expression to the basal layer of squamous epithelia.

A single 100-nmol initiating dose of 7,12-dimethylbenz(*a*)anthracene (DMBA) in 0.2 ml of acetone was applied topically to the shaved dorsal skin of 6- to 8-week-old female mice. One week after DMBA treatment, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (4 μmol) in 0.2 ml of acetone or acetone alone was applied twice weekly to the skin for the duration of the experiment (30 weeks). Wild-type and PACE4 transgenic animals were subdivided in two groups: control (treated with TPA only, twice a week) or CMK (Alexis Corp, San Diego, CA; treated five times a week, 100 μM final concentrations).

Tumor incidence and multiplicity were observed weekly starting at 8 weeks of TPA promotion. Three sets of experiments were performed. The combined number of mice per group was as follows: DMBA + TPA, 20 wild-type mice and 35 PACE4 transgenic mice; DMBA + TPA + CMK, 25 wild-type mice and 57 transgenic mice. Papillomas and SCCs were recorded by gross observation as exophytic cauliflower-like small tumors or as infiltrating and/or ulcerating lesions, respectively, and were confirmed by histopathologic analysis. Autopsies of carcinoma-bearing mice were done, and metastasis in axial lymph nodes, lung, liver, and spleen was recorded. All experiments have been reviewed and approved by the Fox Chase Cancer Center IACUC. Tumor volume was calculated as described before [34].

### *Histology and Immunohistochemistry*

Paraffin sections obtained from control (untreated) and CMK-treated skin from K5-PACE4 mice were fixed in phosphate-buffered 10% formaldehyde and embedded in paraffin. Histologic and immunohistochemical analysis was performed using 5-μm-thick paraffin sections. Immunohistochemistry was performed using the IGF-1R, pIGF-1R, antibodies described under Western blot analysis. The proliferative marker Ki-67 was detected with a monoclonal rat anti-67 antibody from DAKO (cl.T-3; Glostrup, Denmark). An avidin-biotin-peroxidase kit

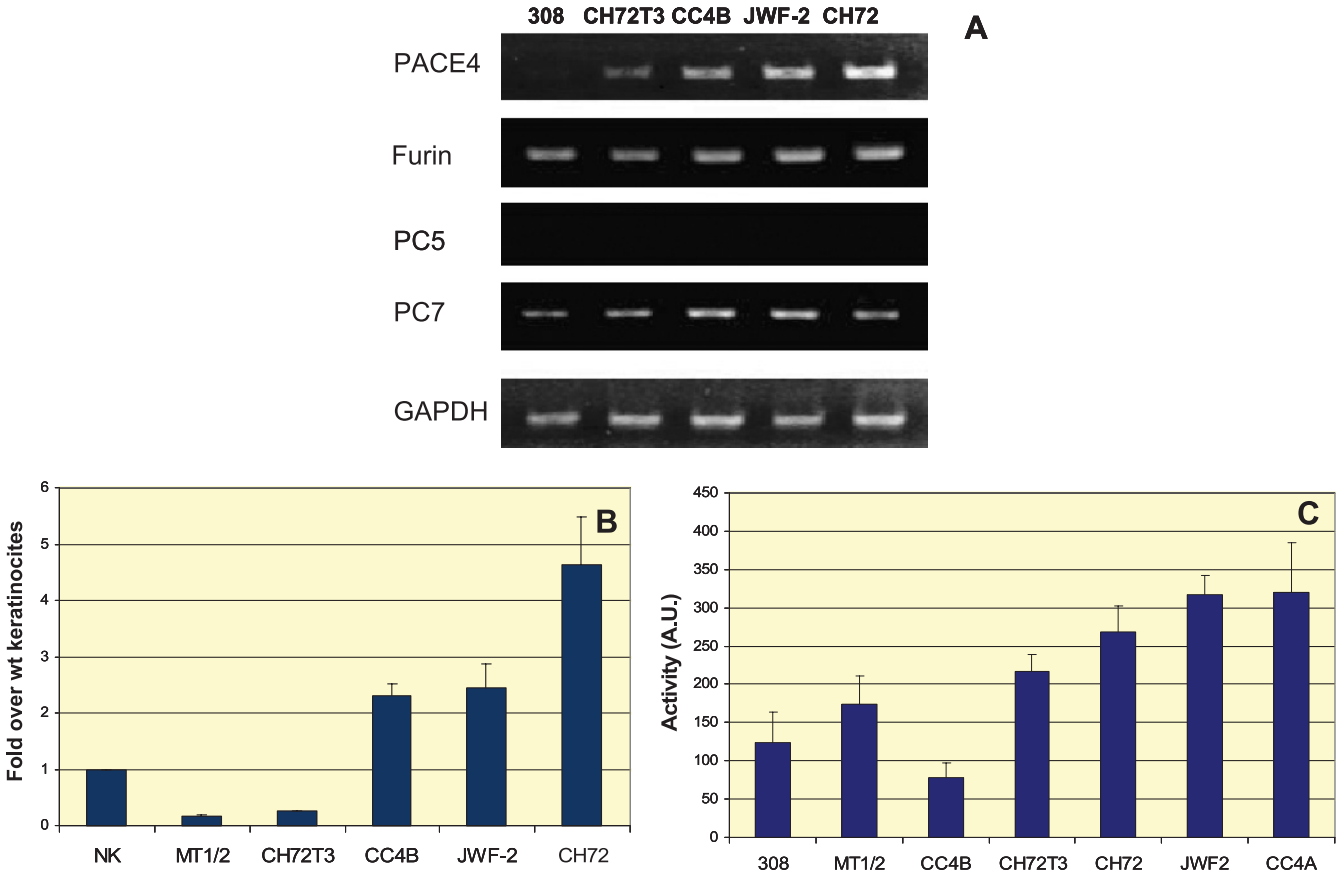


**Figure 1.** PACE4 expression and activity in murine skin cell lines. NK and 308, normal murine keratinocytes; MT1/2, papilloma cell line; all others, SCC cell lines. DNAse-treated messenger RNA was analyzed for PACE4 expression by reverse transcription–polymerase chain reaction (A) and quantified by real-time quantitative reverse transcription–polymerase chain reaction (B). The conditioned media from these cells were tested for PC proteolytic activity and evaluated as production of the fluorescent product AMC (C). Note that PACE4 expression directly correlates with enzymatic activity. *GAPDH* indicates glyceraldehyde-3-phosphate dehydrogenase.

(Vectastain Elite, Burlingame, CA), followed by the chromagen 3′,3′-diaminobenzidine, was used following the manufacturer's instructions. Negative controls, not incubated with the primary antibodies, were first incubated with either normal rabbit or mouse serum. All sections were counterstained with hematoxylin and were mounted.

### *Statistical Analysis*

To test the statistical significance of the differences of tumor development a Wilcoxon two-sample tests and two-tailed *t*-test were used. The differences in metastasis were determined by a Fisher exact test. The *P* values for the differences in the slope of the tumor development curves, the simple linear regression techniques, and the *t*-test were used to calculate the slope, the SD of the slope, and the confidence interval ($\alpha = 0.05$).

## Results

### *PACE4 Expression and Activity Correlated with Cell Line Aggressiveness*

Previous studies demonstrated that ectopic expression of PACE4 conferred benign tumors cells the ability to invade surrounding normal tissues [35]. To demonstrate the association of PACE4 and neoplastic transformation, we determined its expression levels and activity in several murine tumor cell lines and compared them with primary cultures of normal epidermal keratinocytes. Most of the cancer cell lines tested expressed higher levels of PACE4 messenger RNA, proteins, and enhanced proteolytic activity than a cell line derived from normal adult murine keratinocytes (308; Figure 1, *A* and *B*). The other PCs did not show this clear tendency in murine cell lines. Furin was expressed in nearly all cell lines, whereas PC5 was not detected. PC7 expression increased moderately from normal to cancer cell lines (Figure 1*A*). For further studies, we selected JWF-2 and CH72, cell lines of rapid growth and invasiveness both *in vivo* and *in vitro* [36], characterized by high PACE4 activity (Figure 1*C*). This activity was clearly attributable to this PC because silencing PACE4 expression using a combination of two specific siRNA resulted in a 50% decrease of the extracellular PACE4 proteolytic activity (Figure 2*A*).

To inhibit PACE4 activity, we used the small PC inhibitor molecule, CMK. First, we established the CMK doses that were effective in reducing the cleavage of Boc-RVRR-AMC, a synthetic substrate for PCs, in JWF-2 and CH72 cell lines. Treatment of both cell lines with CMK showed that PACE4 activity was significantly reduced in a dose-dependent manner (Figure 2*B*).

### *In Vitro Inhibition of PACE4 Leads to Decreased IGF-1R Maturation and Phosphorylation*

One of the main processes altered during tumor development is the IGF-1 mediated proproliferative signals transduced through its receptor IGF-1R, a known PC substrate [8]. Complete activation of this receptor and phosphorylation was observed in both CH72 and JWF-2 cell lines. Decreased IGF-1R processing (Figure 3*A*) resulted in impairment of its intrinsic tyrosine kinase activity (Tyr 1131 autophosphorylation) as evidenced by a remarkable dose-dependent reduction of receptor phosphorylation levels, indicating that cells became refractory to IGF-1 signaling. Decreased receptor phosphorylation resulted in diminished phosphorylation of ERK1 and ERK2 (Figure 3*B*), well-known downstream substrates of the IGF-1R signal transduction pathway. CMK-dependent inhibition of IGF-1R processing altered this signal transduction pathway and its biologic end point, cell proliferation, measured as incorporation of [$^3$H]-thymidine into newly synthesized DNA (Figure 4, *A* and *B*).

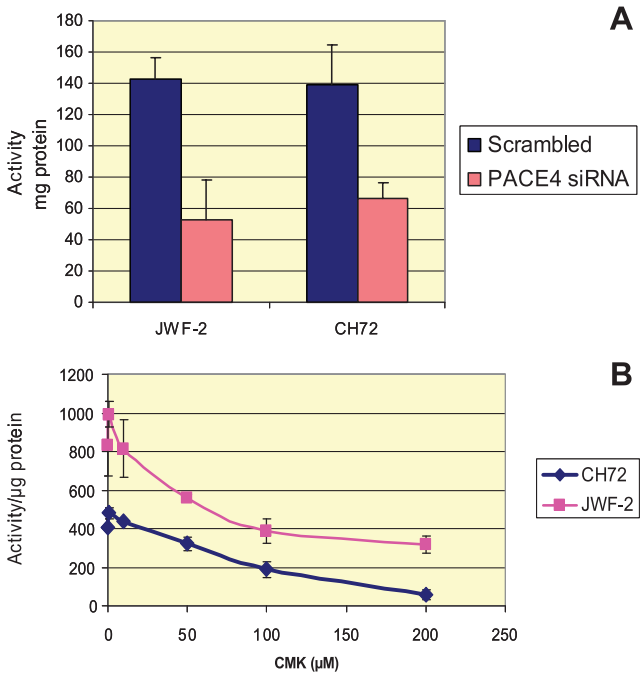


**Figure 2.** (A) Small interference RNA–mediated silencing of PACE4 expression resulted in decreased proteolytic activity in the conditioned medium extracted form the highly PACE4-expressing cells JWF-2 and CH72: $P < .0001$, $P < .05$, respectively. (B) CMK-dependent inhibition of PC activity. Conditioned medium from the JWF-2 and CH72 cell lines were collected and assayed for PC proteolytic activity in the presence of increasing amounts of CMK. The enzymatic activity was evaluated as the production of the fluorescent product AMC: $P < .002$.

### *FITC-CMK Penetrates through the Epidermis in a Dose-Dependent Manner*

The epidermis represents an effective barrier, protecting the underlying tissues from exogenous chemical injuries, and limiting drug administration through topical application. Before the use of the two-stage skin chemical carcinogenesis protocol, we determined whether CMK was able to overcome this natural barrier. To determine whether CMK reaches the basal cells of the epidermis where the transgene is expressed, we derivatized CMK by the sole addition of FITC in the basic lysine residue. The more basic arginine residues (three basic residues per arginine; total six basic residues) are not modified because being ionized at the conditions of the addition reaction (pH = 9) indicates being poor nucleophiles. The addition of FITC adds a fluorescent label to facilitate its visualization in frozen sections. However, addition of FITC to the lysine groups renders the compound inactive because blockage of this basic residue abrogates its ability to bind to the active site of PCs.

FITC-labeled CMK was topically applied to the skin surface at 0.1 and 1 mM, respectively. The levels of penetration were assessed by fluorescence microscopy. As shown in Figure 5*B*, CMK penetrated through the skin at a concentration of 0.1 mM and could be detected homogeneously distributed throughout the epidermis. Application of a 1-mM solution resulted in a notorious increased penetration through the epidermis and some areas of fluorescent accumulation (Figure 5*C*). Untreated skin showed no detectable levels of fluorescence, demonstrating that skin autofluorescence was not present (Figure 5*A*), even after photographic exposures of up to 10 seconds (Figure W1). FITC alone (without CMK) remained at the surface of the epidermis, unable to pass through the stratum corneum, a behavior previously reported with other

nondiffusible molecules [37], thus excluding that the addition of moderately hydrophobic moiety, FITC, to the drug was responsible for its penetration through the epidermis (Figure W1*D*). Furthermore, the addition of FITC, which increases the molecular weight in approximately 300, does not seem to impair the ability of the CMK moiety to drive the absorption of CMK through the skin.

### *In Vivo PACE4 Inhibition Impairs Epidermal Proliferation*

After assessing the epidermal absorption of CMK, we sought to determine the short-term effects of CMK on skin physiology. As we expected to apply CMK to the skin, we first analyzed this organ for putative toxic effects. Altered cell morphology, pyknotic nuclei, or inflammatory cells were not apparent even after topical skin treatment with a high dose (0.5 mM) of CMK for five consecutive days. Furthermore, no systemic effects were detected in any animals treated with CMK in the complete autopsies that were performed in all treated animals.

To study the possible blockage in proliferation *in vivo*, before the CMK application, the skin was topically treated with the hyperplasiogenic phorbol ester TPA to increase the usually low-basal keratinocyte proliferation level. IGF-1R phosphorylation was assessed by immunohistochemistry after a 2-day treatment either with vehicle alone (acetone) or with CMK. Although the expression of total IGF-1R was not affected by CMK (Figure 6, *A* and *B*), a complete absence of phosphorylation was observed after CMK treatment (Figure 6, *C* and *D*), mirroring the inhibition of IGF-1–mediated signal transduction observed *in vitro*. These results support the fact that CMK-mediated inhibition of PACE4

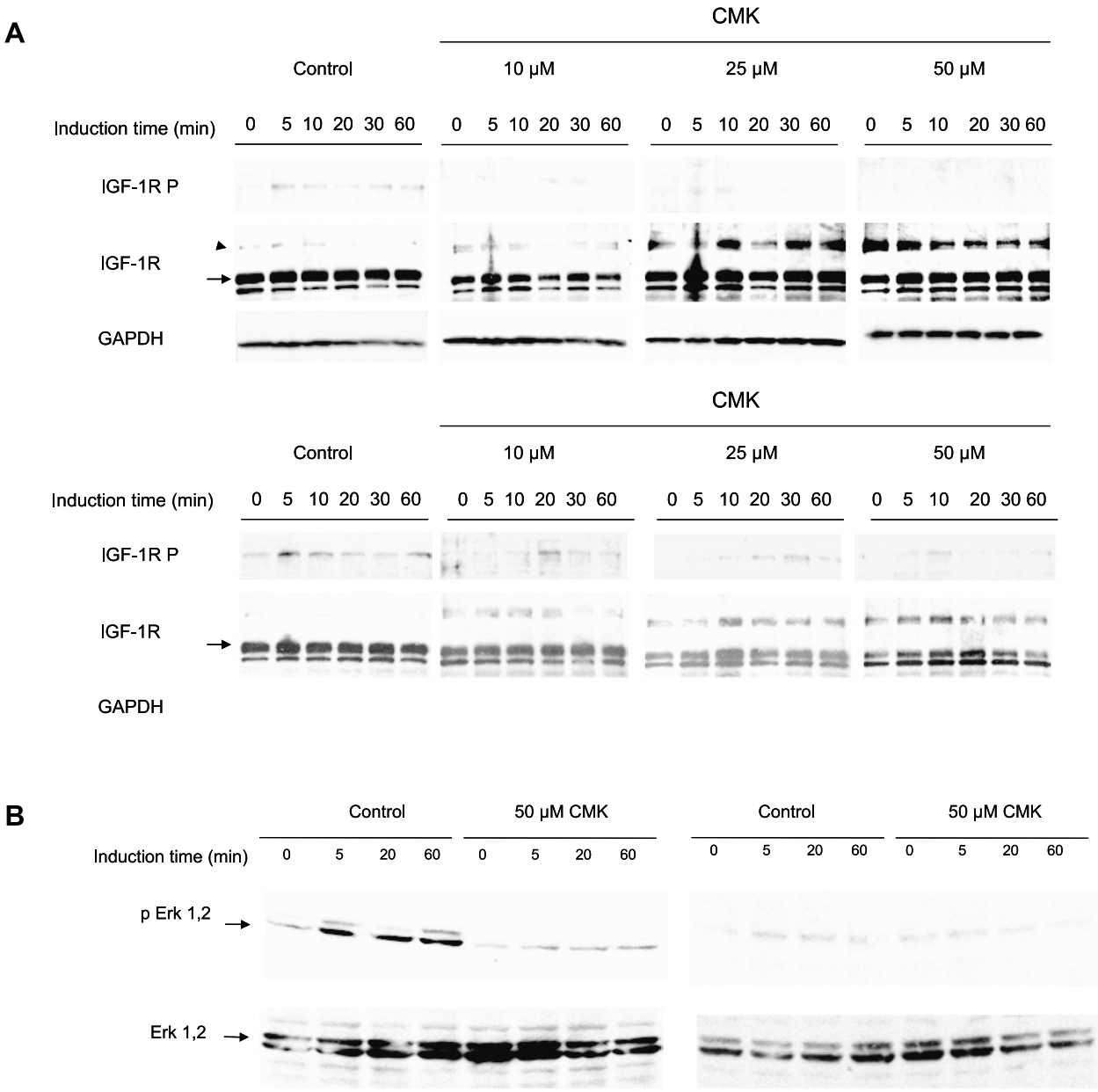


**Figure 3.** CMK inhibits IGF-1R maturation and phosphorylation. Cells were incubated in the absence or presence of different concentrations of CMK. Cells were plated and incubated with CMK and serum-containing media. After 24 hours, the medium was replaced with serum-free medium containing the same CMK concentrations. On the third day, cells were induced with 50 nM IGF-1 for different incubation periods as indicated. pIGF-1R and the extent of processing of IGF-1R (A) or ERK1/2 phosphorylation (B) were evaluated by Western blot. Note that CMK abolished almost completely IGF-1R phosphorylation even at the lowest CMK concentration.

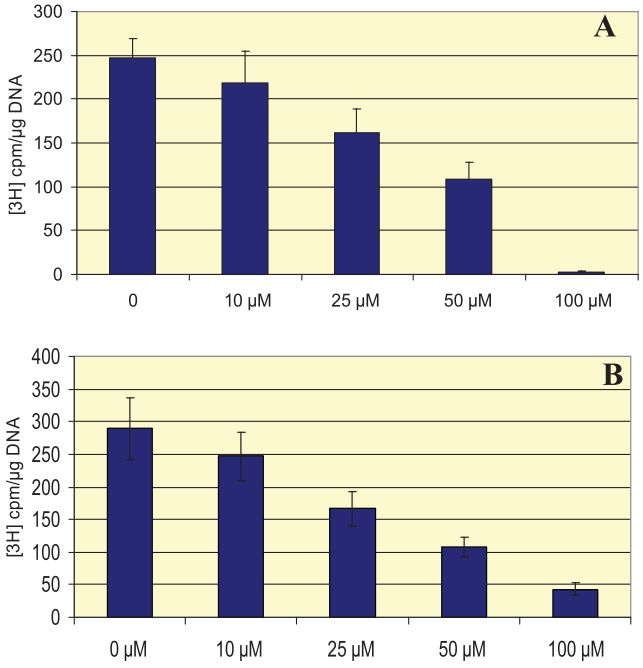


**Figure 4.** Inhibition of IGF-1R processing and phosphorylation results in decreased *in vitro* proliferation rates. Cells were subsequently treated with the indicated CMK concentrations for 24 hours, starved an additional 16 hours, and incubated in the presence of serum for 4 hours. Cells (A) JWF-2 and (B) CH72 were labeled with [$^3$H]-methyl thymidine for 4 hours. DNA was extracted, and the levels of radioactivity associated with the newly synthesized DNA were measured in a scintillation counter. Note the dramatic decreased in proliferation in CMK-treated cells ($P < .0001$).

results in disruption of the IGF-1 signaling pathway by decreasing the receptor's tyrosine kinase activity. No significant differences in the rate of proliferation between treated and untreated epidermis was observed after 2 days. To better evaluate the effects of CMK on cell proliferation *in vivo*, CMK treatment was thus extended for 3 weeks. WT and K5-PACE4 transgenic mice were treated with TPA to induce epidermal cell proliferation. Immunohistochemistry with the proliferation marker Ki-67 showed that CMK treatment was very effective in decreasing the number of Ki-67–labeled cells in both K5-PACE4 mice epidermal basal epidermal keratinocytes (approximately 57% less proliferative than non–CMK-treated epidermis) and wild-type mouse epidermis treated with CMK (45%; Figure 7, *A*–*E*).

### *In Vivo PACE4 Inhibition Results in Decreased Skin Tumor Development*

The inhibition of basal keratinocyte proliferation observed in the short-term experiments described may result in delayed tumor development and progression. To test this hypothesis, wild-type mice and K5-PACE4 transgenic mice were treated with a two-stage carcinogenesis protocol to induce tumor formation and concomitantly treated five times weekly either with CMK (1 mg/kg, or 100 µM CMK, 200 µL) dissolved in acetone, or with the vehicle alone, starting at week 2.

As previously described, wild-type mice developed more papillomas than transgenic mice [15], suggesting that PACE4 expression favors the formation of SCCs without undergoing the usual papilloma-SCC sequence. Although CMK only marginally affected papilloma growth (Figure 8, *A* and *B*), it exerted a clear and consistent inhibition of tumor progression to SCC (Figure 8, *C* and *D*). As expected, CMK treatment of PACE4 transgenic mice showed a reduction of 37% in SCC multiplicity, exhibiting 0.9 SCC/mouse after 30 weeks of treatment (Figure 8*D*). This contrasted with 1.4 SCC/mouse observed in the control non–CMK-treated group of transgenic mice ($P < .01$; Figure 8*C*). Wild-type mice were less responsive to CMK, showing a reduction of 29% in the number of SCC per mouse (Figure 8*C*; $P = .059$). Furthermore, in CMK-treated K5-PACE4 mice, the rate of carcinoma growth decreased 25%, when compared with the untreated mice as shown by the reduced slopes in the linearized curves of multiplicity *versus* time (Figure 9). In addition, CMK applications resulted in decreased tumor volumes of approximately 50% in both wild-type and transgenic mice (Figure 10*A*).

Topical applications of CMK also decreased the metastatic potential of skin SCCs. When evaluated after 30 weeks of promotion, 30% of the untreated animals developed lung metastases. This contrasted with 13% of mice with pulmonary metastases in the CMK-treated group ($P = .045$). The difference in lymph node metastasis incidence was less impressive, that is, 60% of untreated animals *versus* 40% of CMK-treated mice presented with this type of metastasis. These differences were marginally significant ($P = .057$, Fisher exact test; Figure 10*B*).

## Discussion

Increased activity and expression of metalloproteases and growth factors often precedes the ability of tumor cells to proliferate, invade, and metastasize. The serine protease PACE4 activates several tumor-related biomolecules necessary to accomplish these newly acquired abilities. Overexpression of PACE4 in murine cells leads to increased invasive abilities both *in vitro* [31] and *in vivo* [15] has been described by our laboratory, suggesting that inhibition of this PC's activity may slow down tumor development. The results presented herein, using a PC inhibitor, indicate that inhibition of PACE4 activity lowered cell proliferation *in vitro* and decreased skin proliferation and tumor development *in vivo*. Decreased cell proliferation rates can be ascribed to a reduction in IGF-1R processing and activation, leading to a less efficient transduction of the IGF-1–mediated proproliferative signals.

PACE4 expression correlates with increased murine cell line invasiveness [31]. Its expression was two- to four-fold higher in tumor cell lines than in normal primary keratinocytes and its higher expression correlated with increased PC proteolytic activity *in vitro* [31]. One exception to the rule was the papilloma-derived cell line MT1/2, which expressed very low levels of PACE4 and exhibited moderate PC activity, indicating that enzymes other than PACE4 might be operating in this cell line. Nevertheless, this atypical activity does not translate in an aggressive phenotype because this particular cell line exhibits low cell proliferation and invasiveness [38].

Because PACE4 is an extracellular protease, it can be detected and assayed in the conditioned medium of cultured cells. Incubation of conditioned medium with CMK effectively decreased the cleavage of the PC's synthetic substrate, Boc-RVKR-AMC. This activity may be mainly attributed to PACE4 because specific silencing of this PC's expression by a combination of two murine PACE4 siRNA resulted in a significant reduction of Boc-RVKR-AMC cleavage.

CMK-dependent inhibition of PACE4 resulted in decreased cleavage of a prominent PC substrate, IGF-1R, that is directly associated with tumor cell proliferation, followed by decreased IGF-1R phosphorylation, pointing to a reduced ability to transmit IGF-1–mediated signals. IGF-1R is responsible for many proproliferative and prosurvival

responses, involving the MAPK or the PIK3/Akt pathways, respectively [39,40]. In this context, CMK-treated cells showed reduced ERK1/2 phosphorylation, key proteins in the signal transduction pathway associated with IGF-1. IGF-1R processing and activation were not completely abolished by CMK even at the highest CMK concentrations tested (100 μM), suggesting that a fraction of mature receptor could still be capable of some levels of autophosphorylation. However, the receptor phosphorylation and the consequent tumor cell proliferation were greatly reduced, indicating that the presence of noncleaved receptor might interact with the mature receptor, thus inhibiting the intrinsic kinase activity. Many mechanisms for this dominant-negative–like activity were proposed, such as the sequestering of normal IGF-1R substrates including IRS-1 [41] or the assembling of nonfunctional hybrids between the noncleaved and cleaved receptors [42].

The reduction in proliferation rates observed *in vitro* suggested that inhibition of PACE4-mediated activation of IGF-1R could derive in a diminished susceptibility to carcinogens, resulting in decreased tumor development. IGF-1–mediated signaling plays an important role in skin carcinogenesis. For instance, transgenic mice overexpressing IGF-1 in basal keratinocytes exhibited increased susceptibility to chemical carcinogenesis [43,44]. Moreover, IGF-1R expression is necessary for inducing transformation in fibroblasts [45] and is responsible for decreased keratinocyte differentiation and enhanced proliferation [46].

After confirming that PACE4 activity could be efficiently blocked *in vitro* by CMK, we sought to determine whether CMK counteracts the increased susceptibility of PACE4 transgenic mice to chemical carcinogenesis [15]. CMK was applied five times a week for 30 weeks, together with a two-stage chemical carcinogenesis protocol. Experiments were terminated at 30 weeks because of the high tumor burden displayed by control animals. The development of benign tumors (papillomas) was not significantly altered by CMK treatment, suggesting that PACE4 activity is more prominent during the later stages of tumor conversion and progression. Previous results [15] showed that PACE4 transgenic mice developed twice as many malignant tumors as wild-type animals without increased growth of benign tumors. In this report, we showed that the application of this PC inhibitor resulted in a significant decrease in the number and in the rate of SCC development, suggesting that CMK counteracts the proproliferative effects of PACE4.

PACE4 also activates substrates involved in cell invasiveness, such as membrane type-matrix metalloproteases [35]. After PC-mediated activation, metalloproteases degrade the epithelial basement membrane,

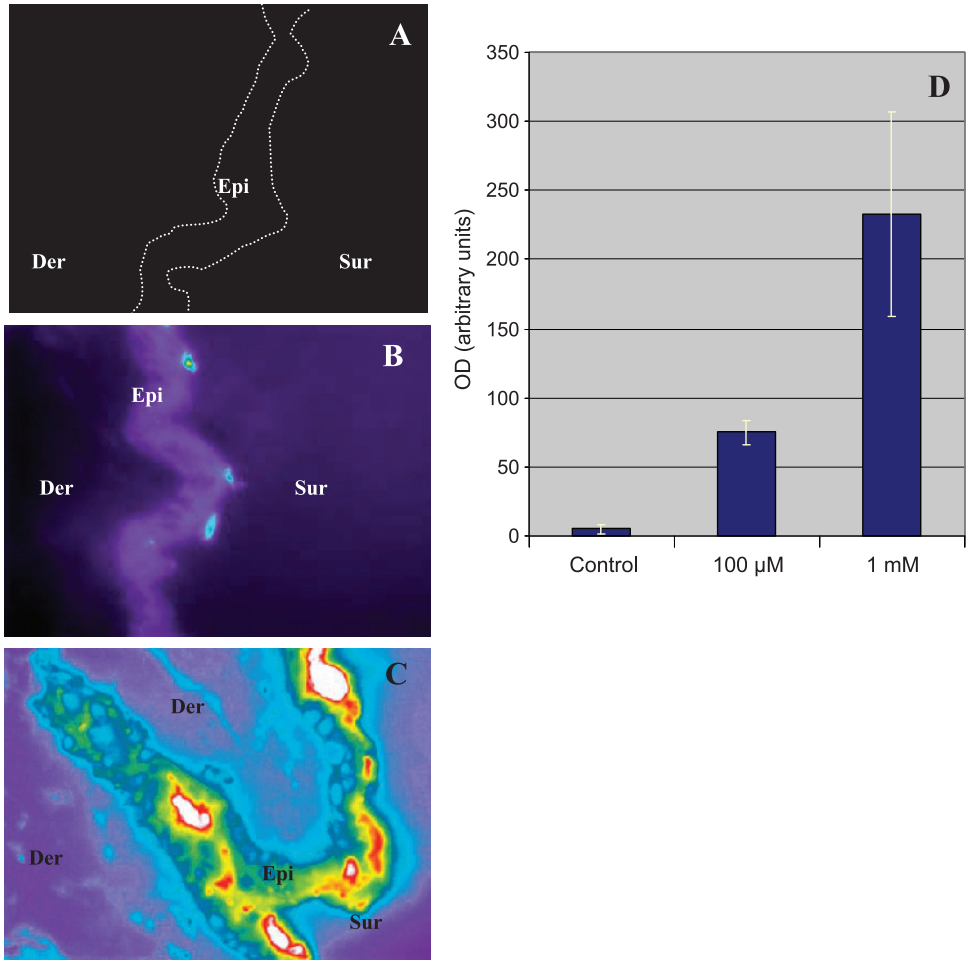


**Figure 5.** FITC-CMK skin penetration. CMK was labeled with FITC, purified, and analyzed by HPLC/MS. FITC-CMK–treated skin sections were analyzed with the Metamorph software using pseudocolor to assess the relative amount of fluorescence in the epidermis (Epi), dermis (Der), and skin surface (Sur). Sham (A), 100 $\mu$M (B), and 1 mM FITC-CMK (C) treatments, respectively. The epidermal fluorescence was subtracted from background levels and plotted in a bar diagram (D). Exposure time: 1 second, ($P = .001$).

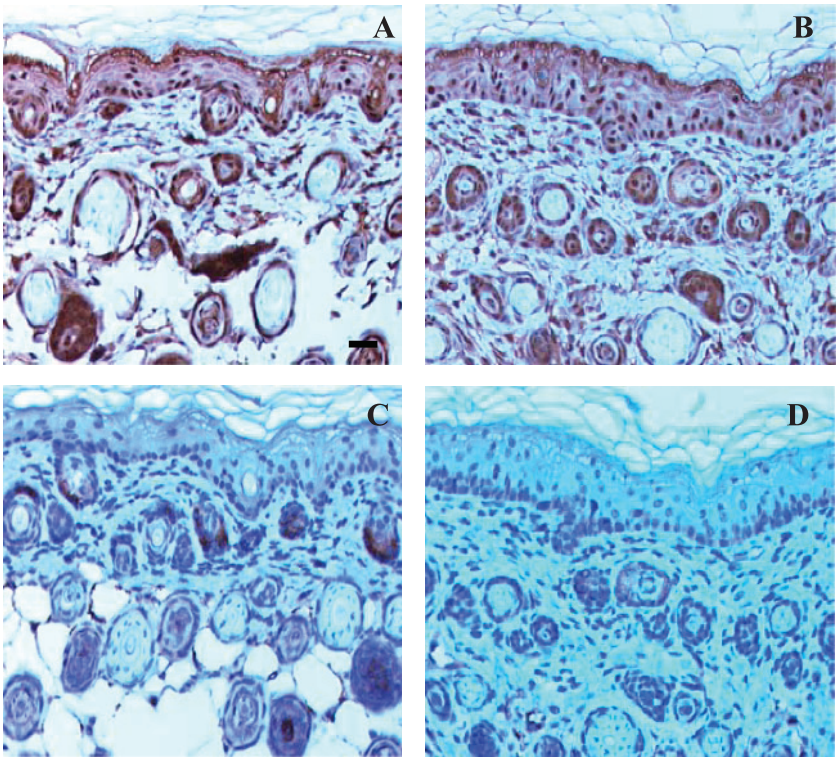


**Figure 6.** CMK-dependent inhibition of TPA-induced epidermal proliferation. The skin of K5-PACE4 transgenic mice was treated with vehicle (A and C) or CMK (300 $\mu$m; B and D) for 2 days before TPA treatment. Tissues were collected and fixed, and the relative expression of total and phosphorylated IGF-1R was assessed by immunohistochemistry. Note the decrease in IGF-1R phosphorylation in the hair follicles in the treated epidermis despite the relative constant IGF-1R expression. pIGF-1R immunohistochemistry and hematoxylin counterstain.

allowing a premature interaction between stromal components and the epithelial cells, resulting in increased proliferation and accelerated stromal invasion [15]. Blockage of PACE4 activity also resulted in decreased invasiveness, as evidenced by a reduction in lymph nodes metastasis and especially by a significantly lower incidence in pulmonary metastases in CMK-treated animals.

Interestingly, both WT and K5-PACE4 transgenic mice subjected to a two-stage carcinogenesis protocol showed a reduction in SCC multiplicity and metastasis after CMK treatment. Nevertheless, cancer reduction after CMK application in WT mice was less marked and significant than in K5-PACE4 animals. Although this effect in WT mice indicate that CMK is able to block endogenous PCs present in the skin, such

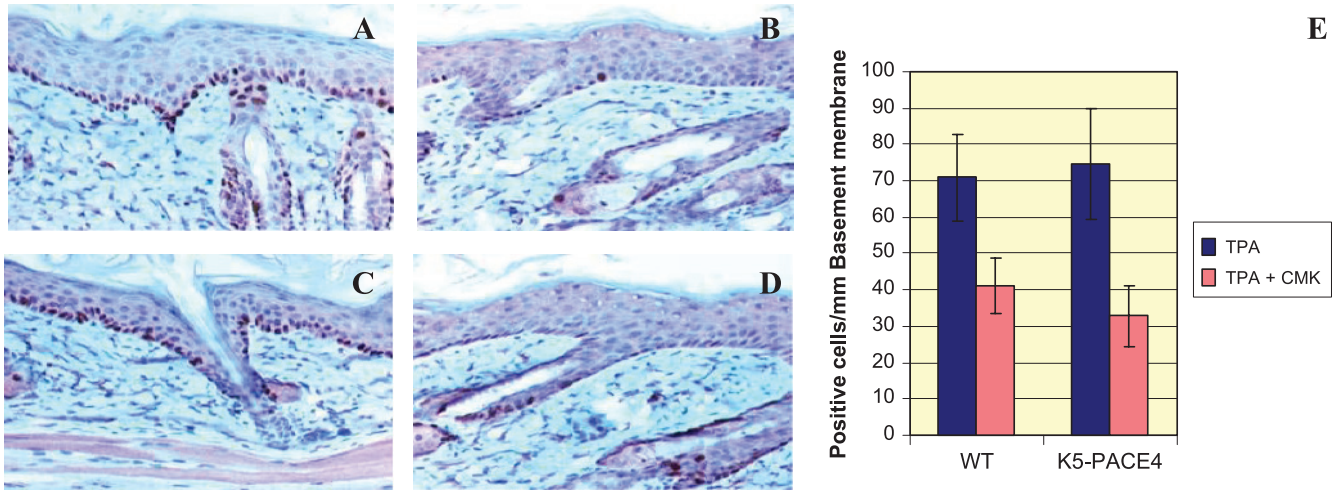


**Figure 7.** Skin from wild-type (A and B) or Tg (K5-PACE4; C and D) mice were treated with TPA (twice weekly) to increase the low basal level of cell proliferation and then treated either with vehicle alone (A and C) or with CMK 100 $\mu$M (B and D) daily for 2 weeks. Fragments of dorsal skin were fixed and embedded in paraffin; sections were stained with anti–Ki-67 antibody. The number of labeled basal epidermal keratinocytes was measured per length of interfollicular epidermal basement membrane and expressed as positive cells per millimeter of basement membrane (E) $P < .001$. Original magnification, $\times 100$.

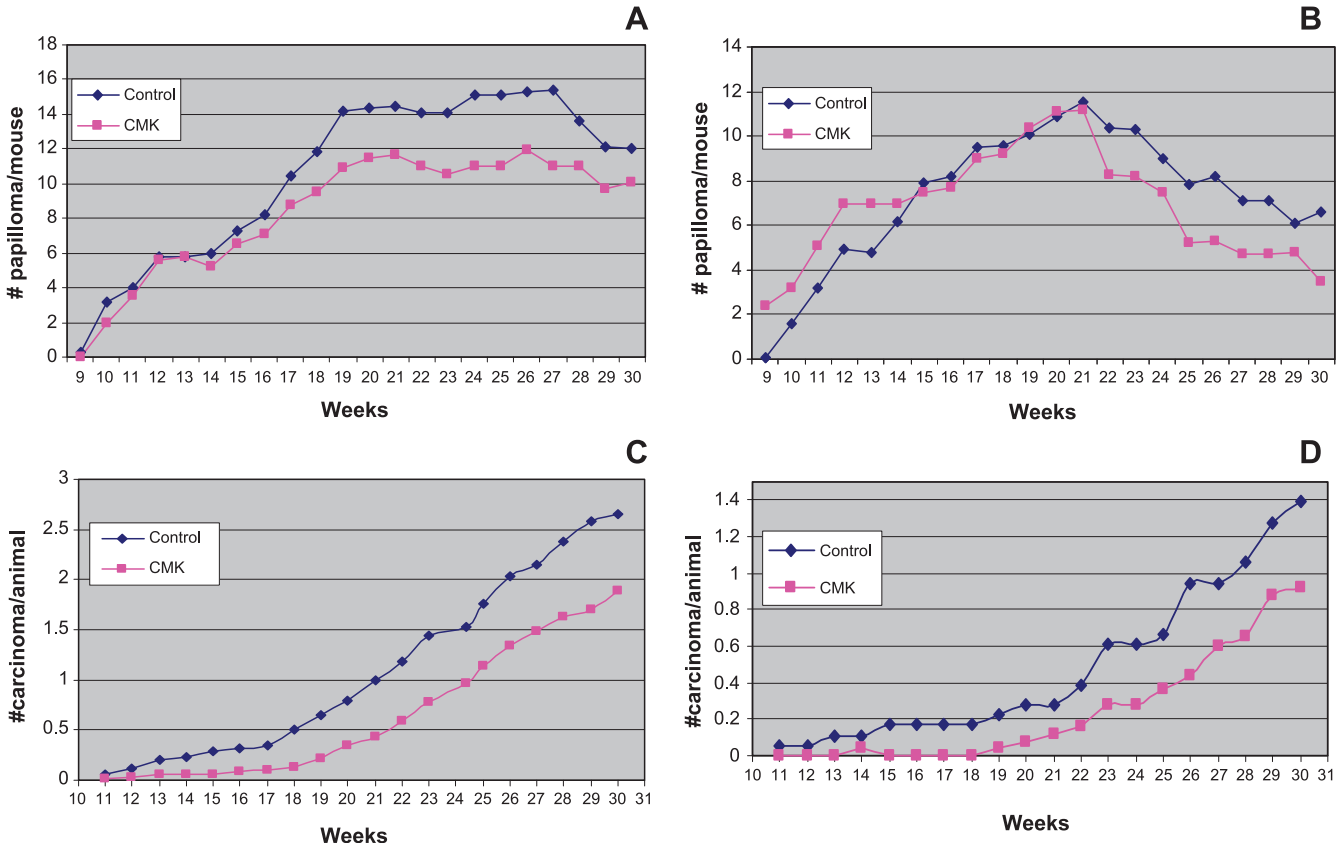


**Figure 8.** CMK inhibitory effect on tumor multiplicity. Wild-type and transgenic (Tg; K5-PACE4) mice were initiated with DMBA and treated with TPA twice a week for 30 weeks in the presence or absence of CMK (100 $\mu$M, starting at week 2). The numbers of papillomas per mouse in wild-type (A) and K-PACE4 mice (B) and SCC per mouse in wild-type (C; $P < .05$) or K5-PACE4 animals (D; $P < .01$) were determined weekly.

as epidermal furin and PC7, the transgenic overexpression of PACE4 in K5-PACE4 transgenic mice emphasized and clearly demonstrated the inhibitory effect of the drug on PACE4 activity.

Taken together, our *in vitro* and *in vivo* results provide evidence that PACE4 may represent a new target for cancer therapeutics. Current PC research has focused on the inhibition of furin, the prototype of the PC family. In fact, inhibition of furin has proved successful in blocking cancer cell growth and invasion in colorectal [21], breast [22], and head and neck cancer [23], among others. Furthermore, PC5 and PC7 inhibition resulted in inhibition of VEGF-C [47,48], a key factor in angiogenesis and tumor nourishment and development, showing some promise in the development of new PC inhibitors. Nevertheless, recent

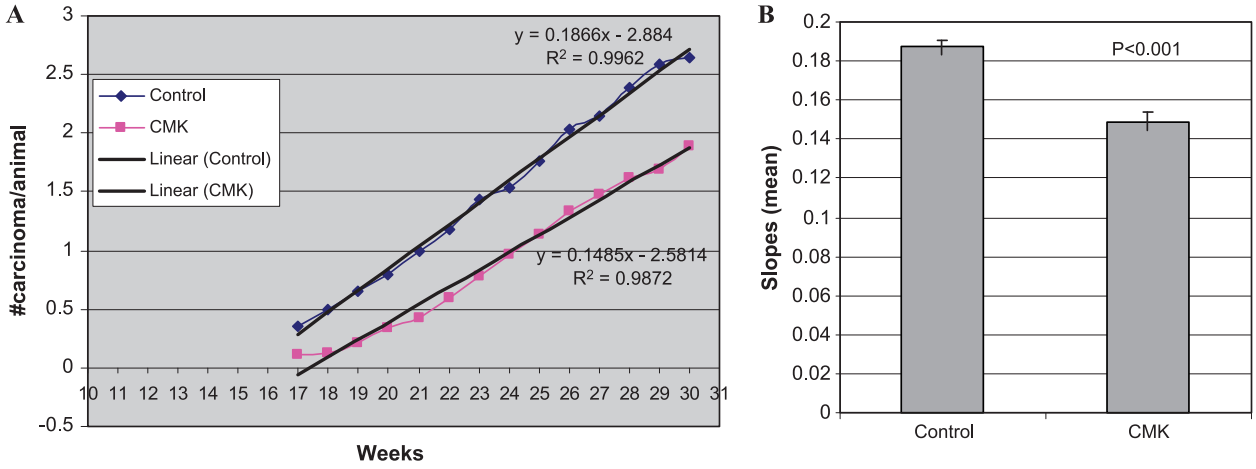


**Figure 9.** Analysis of SCC growth rate patterns in CMK-treated (100 $\mu$M) and untreated mice. Tumor multiplicity curves were adjusted to fit linear functions, and the slopes, intercept, and their SDs were calculated by regression analysis. The confidence interval of the slope and the *P* value to reject the null hypothesis were determined using a *t* distribution. (A) Linear approximation of tumor multiplicity curves *versus* time for K5-PACE animals. (B) Mean slopes with their SDs.

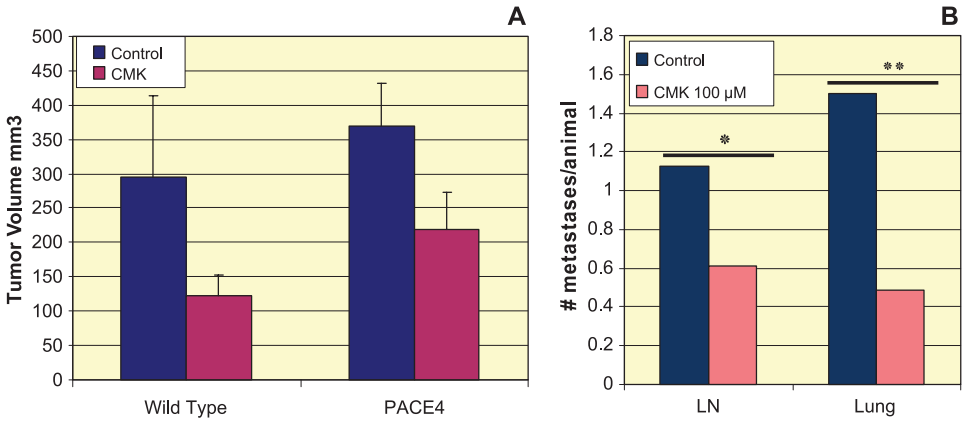


**Figure 10.** CMK inhibitory effect on tumor volume and metastatic potential. Mice were initiated with 100 nmol of DMBA and treated twice a week. (A) SCC volume was measured at 30 weeks of TPA treatment ($P < .05$). (B) Metastases to lymph nodes (LN) and lungs were measured as percentage of animals with metastasis ($*P = .055$, $**P = .045$).

studies in Apc/Min+ mice demonstrated that expression of PC5/6 had a protective effect on intestinal cancer development, indicating that specific PC5 inhibitors may be beneficial in some malignancies [48]. PACE4 has been associated with activation of cancer molecules. However, its down-regulation in ovarian cancer [6,49], and its association with increased breast tumorigenesis [22] points to a different role of this protease in cancer. Unlike furin, PACE4 is an extracellular protease that may be more extensively regulated by extracellular signals. In this context, the breast or ovarian stroma may exert a different effect on PACE4 activity that the basement membrane, or that the strong hormonal influences may point to a different role of PACE4 in these cancers. We have previously found that increased expression of PACE4 in the basal layer of the epidermis resulted in increased disruption of the collagen component of the basement membrane and increased activation of metalloproteinases [15]. Our results point to a beneficial effect of PACE4 inhibition on the development of skin cancer as a result of decreased cell proliferation and invasion in the unique context of the basement membrane-epithelial interaction [21]. Blockage of PACE4 activity disrupts IGF-1 signaling, leading to impaired proliferation. Furthermore, continuous *in vivo* inhibition of PACE4 activity by CMK resulted in early decrease of tumor cell proliferation, delayed tumor development, as well as reduction in tumor incidence, tumor multiplicity and metastasis in a paradigmatic model of squamous carcinogenesis.

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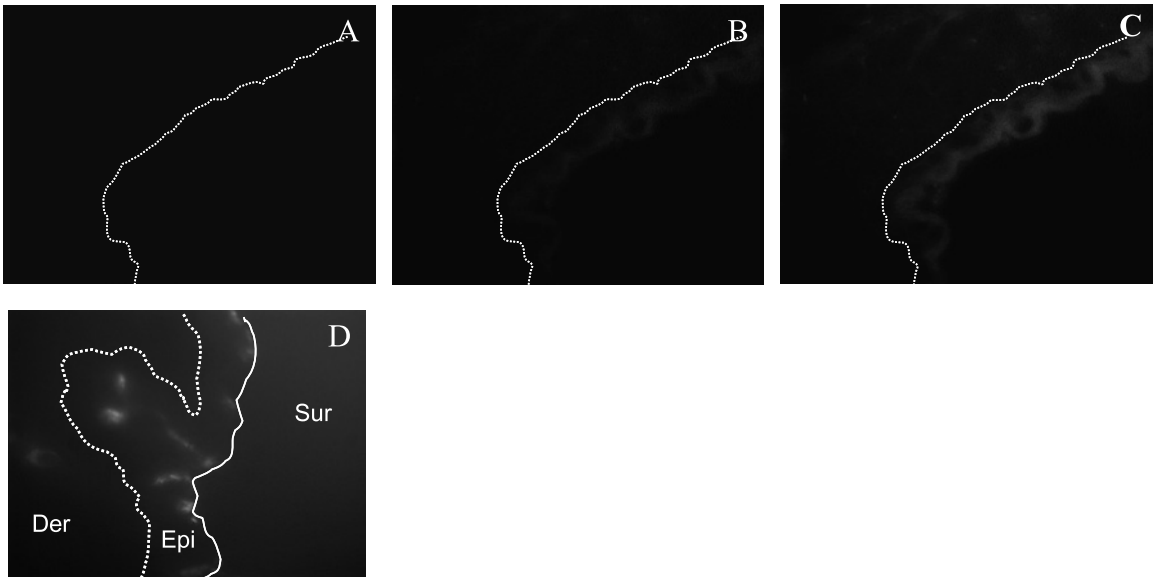


**Figure W1.** Specificity of FITC-CMK skin penetration. (A–C) Skin sections of acetone- (sham) treated skin. Pictures of the specimens were taken by exposing for 1 (A), 5 (B), or 10 (C) seconds. Note the minimal amount of fluorescence even at 10 seconds of exposure. Dotted line shows the basement membrane. (D) FITC-treated skin section. Note that FIC accumulated mostly in the superior layers of the epidermis (Epi). Der indicates dermis; Sur, skin surface.