PACE4 Expression in Mouse Basal Keratinocytes Results in Basement Membrane Disruption and Acceleration of Tumor Progression

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Abstract

Collagen type IV degradation results in disruption and breakdown of the normal basement membrane architecture, a key process in the initiation of tumor microinvasion into the connective tissue. PACE4, a proprotein convertase, activates membrane type matrix metalloproteinases (MT-MMPs) that in turn process collagenase type IV. Because PACE4 is overexpressed in skin carcinomas and in vitro overexpression of PACE4 resulted in enhanced invasiveness, we investigated whether or not in vivo PACE4 expression leads to the acquisition of invasiveness and increased tumorigenesis. Two transgenic mouse lines were designed by targeting PACE4 to the epidermal basal keratinocytes. Transgenic keratinocytes showed increased processing of MT1-MMP and MT2-MMP in collagenase IV activation and collagen type IV degradation. Higher collagenolytic activity partially disrupted normal basement membrane architecture favoring epithelial endophytic growth into the dermis and accelerating invasion and metastasis after chemical carcinogenesis. PACE4 overexpression resulted in enhanced susceptibility to carcinogenesis and tumor progression pointing to a new target for blocking tumor cell invasiveness. (Cancer Res 2005; 65(16): 7310-9)

Introduction

Actively proliferating cells have a higher probability of accumulating mutations leading to cancer. Because of their high rate of renewal or turnover, stratified epithelia are more likely to undergo malignant transformation. Epidermal basal cells constitute a proliferation-proficient cell layer that, after successive steps in differentiation, will replenish the more superficial layers that are constantly shed. Basal cells can also initiate a burst of cell division in response to injuries or chemical stimuli. Among the latter, environmental carcinogens are able to produce mutations that together with enhanced cell division result in neoplastic development. The majority of non–melanoma skin cancers arise from the basal keratinocytes. Basal cells express keratin 5 (K5). The K5 promoter is silenced as soon as they commit to differentiation. This specific localization of K5 expression in the basal layer of the epidermis and outer root sheath (1) has been used for targeting different cancer-related genes to epidermal basal keratinocytes (2) and studying the in vivo effects after overexpressing genes, such as transforming growth factor-β (3), epidermal growth factor receptor (4), and insulin-like growth factor (5).

Proprotein convertases constitute a family of serine proteases that activate their cognate substrates by limited proteolysis at consensus sequence RXR/KR (6). Many of these, such as insulin-like growth factor-I (7) and its receptor (8), membrane-type metalloproteinase (MT-MMP; refs. 9, 10), and integrins (11–14) have direct roles in tumor progression and metastasis. Although proprotein convertases recognize and cleave a specific sequence, variations in the amino acid sequences that surround this motif, as well as preferences in K or R preceding the COOH-terminal R, account for differences in the efficiency of substrate cleavage (15). Furin, a well-studied member of the proprotein convertase family, has been associated with enhanced invasion and proliferation in head and neck (16), breast (17), and lung cancers (18). Conversely, inhibition of its activity by the inhibitor PDX, resulted in reduced proliferation and invasion in human squamous cell carcinomas (SCC; ref. 19), colon adenocarcinoma (20), and astrocytoma cell lines (21). PACE4, a secreted extracellular proprotein convertase, shares many biochemical properties with furin (22, 23). PACE4 is overexpressed in aggressive mouse skin spindle cell carcinomas but not in low-grade SCC (24). Moreover, in vitro experiments showed that the sole overexpression of PACE4 conferred tumorigenic properties to papilloma-derived murine cells lines (25).

In order to study the role of PACE4 in tumor progression in vivo, we developed transgenic mice that express PACE4 in the epidermal basal layer. Although transgenic mice did not seem to have gross abnormalities, a more in-depth analysis revealed a weakened and less prominent epithelial basement membrane. In agreement with these observations, MT1-MMP and MT2-MMP were processed with increased efficiency in transgenic epithelial keratinocytes. Acute topical applications with 12-O-tetradecanoylphorbol-13-acetate (TPA) led to increased proliferative response. Moreover, these animals developed more chemically induced SCC than their wild-type counterparts.

Materials and Methods

Materials. TPA and 7,12-dimethylbenz(a)anthracene (DMBA) were purchased from Sigma-Aldrich (St. Louis, MO). FVB/N mice, 6 to 8 weeks of age, were purchased from Taconic (Germantown, NY).

Generation and identification of K5.PACE4 mice. The 4 kb full-length wild-type rat PACE4 cDNA (26) was excised from its parental pcNeo vector using SalI and EcoRI. The fragment was blunt-ended by treatment with the Klenow fragment of DNA polymerase and ligated into the SmsI site between the rabbit β-globin intron and polyadenylation sequences from a vector described previously and inserted into the K5 expression vector (2). Orientation and integrity of the insert were confirmed by restriction analysis and sequencing. The K5.PACE4 transgene was microinjected into...
the pronuclei of mouse embryos obtained from either ICR female mice mated to FVB male mice or FVB female mice mated with FVB male mice. DNA was extracted from clipped tails as described (27). Mice were genotyped by PCR analysis of tail DNA utilizing primers specific for an internal fragment rat PACE4 and the junction 3'-PACE4/SV40 polyadenylation signal (see Table 1). The sequences and the expected size of the amplicon are shown in Table 1. Throughout this study, all transgenic mice used were homozygous.

Southern blot and determination of copy number. Ten micrograms per lane of spleen or liver DNA was digested with KpnI and HIndIII, and then subjected to electrophoresis on 0.8% agarose and transferred to Hybond N+ (Amersham, Buckinghamshire, United Kingdom). The blot was hybridized with random primed-32P-dCTP-labeled probe from the transgene. The transgene band to be digested was a ~7 kb DNA fragment generated by HIndIII digestion of chromosomal DNA. As a standard for determining the copy number, we digested the K5-PACE4 plasmid used for hybridization of the 32P-dCTP-labeled probe with DNA extracted from densitometry, comparing the intensity of the signals generated by the corresponding 7 kb band was excised and purified. The amount of controls were calculated as described in http://www.med.umich.edu/tamc/ spike.html.

Estimation of the copy number of the transgene was done by densitometry, comparing the intensity of the signals generated by hybridization of the 32P-dCTP-labeled probe with DNA extracted from transgenic animals with those generated by controls containing 0, 1, 2, 5 and 10 copies of the 7 kb band per genome.

**Tumor induction experiments.** A single 100-nmol initiating dose of DMBA in 0.2 mL acetone was applied topically to the shaved dorsal skin of 6- to 8-week-old female mice. One week after DMBA treatment, TPA (4 nmol) in 0.2 mL of acetone or acetone alone was applied twice weekly to the skin for the duration of the experiment (30 weeks). Tumor incidence and multiplicity were observed weekly starting at 8 weeks of TPA promotion. The number of mice per group was as follows: DMBA + TPA, 25 wild-type mice, 24 transgenic mice (line #1) and 35 PACE4 transgenic (line #2); DMBA + TPA, 100 nmol, 24 transgenic mice and 25 wild-type mice, 20 wild-type mice, 20 transgenic mice from each animal line. Carcinomas were recorded by gross observation as infiltrating and/or ulcerating lesions and confirmed by histologic analysis (see below). Autopsies of carcinoma-bearing mice were done and metastasis in axial lymph nodes, lung, liver, and spleen were recorded. All tumors were analyzed histologically. Papillomas were classified either as regular papillomas or as papillomas that had little or no dysplastic changes without an endophytic component (type A) or as papillomas with an endophytic component characterized by moderate or advanced dysplastic changes (type B; ref. 28) as well as by areas of epithelial down-growth into the papillary dermis (endophytic component). SCC were classified according to histopathologic grade (29). Most SCCs were endophytic growths that invaded the dermis and s.c. tissue. The differentiation patterns defining the histopathologic grade were: (a) grade 1 SCC, very well differentiated with keratinizing cells (c) grade 3 SCC or poorly differentiated tumors, containing <25% tumor mass showing evidence of keratinization; and (d) grade 4 SCCs, very poorly differentiated tumors or spindles cell carcinomas containing very little or no histologic evidence of keratinization. Papilloma and carcinoma were photographed at a magnification of 2.5× (NA 0.08) and 10× (NA 0.45), respectively.

**Culture of newborn keratinocytes.** Primary epidermal keratinocytes from newborn mice were used to determine expression levels of PACE4 and related proteases because of their suitability for in vitro growth and further molecular analyses. Primary epidermal keratinocytes were established in vitro as described (30). Briefly, 2- to 3-day-old mice were killed, then washed in a 1:10 solution of betadine, rinsed twice in sterile distilled water and twice in 70% alcohol. The skin was removed and floated overnight on 2 mL of dispase (Dispe II, 25 units/mL; BD Biosciences, Bedford, MA). The epidermis was separated from the dermis, miniced and incubated with 2 mL of 0.05% trypsin and 0.01% EDTA for 20 minutes at 37°C. DNase I (100 units) was added and the cells were mechanically dissociated by vigorous pipetting followed by filtration through a 40 μm cell strainer (BD Biosciences). The cells were washed in DMEM containing 10% FCS and plated in low-Ca2+-free medium (KGM/Ca2⁻/free KGM 1:2, Cambrex, Walkersville, MD).

**Protein analysis and zymography.** Samples of skin or tumors were homogenized in PBS, centrifuged, and resuspended in RIPA lysis buffer (1× PBS solution, 0.1% SDS, 0.5% Na deoxycholate, and 1% Nonidet P40) with protease inhibitors (aprotinin, 100 mmol/L phenylmethylsulfonyl fluoride, and 100 mmol/L Na3VO4). The suspension was incubated at 4°C for 30 minutes to extract the proteins. The preparation was centrifuged and the supernatants were used as cell lysates. The protein concentration was measured with a Bio-Rad protein assay system (Bio-Rad Laboratories, Richmond, CA). These lysates were fractionated by SDS PAGE, transferred to a nitrocellulose membrane and immunoblotted with specific primary antibodies. For PACE4 detection, the primary antibody was JH1475, a rabbit polyclonal antibody against amino acids 570 to 656 of PACE4 (kindly provided by Dr. R. Mains, University of Connecticut Health Center, Farmington, CT; ref. 31). For MT1-MMP and MT2-MMP detection, the monoclonal antibodies RP1MMP14 (Triple Point, Forest Grove, OR) MAB3320 (Chemicon International, Temecula, CA) were used as the primary antibody. The membranes were incubated with the appropriate secondary antibody (anti-rabbit or anti-mouse linked horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes were washed and developed using Amersham enhanced chemiluminescence reagent. A monoclonal antibody directed against α-actin (I-19, Santa Cruz, Santa Cruz, CA) was used as loading control. Cell lysates from the fibrosarcoma cell line HT-1080 were used as MT1-MMP-positive controls (32).

Zymography was done in 10% polyacrylamide gels that had been cast into the presence of gelatin (Invitrogen, Carlsbad, CA). Briefly, samples containing 10 μg proteins were mixed with 2× loading buffer, and without prior denaturation were run on a 10% SDS-polyacrylamide gel containing 0.5 mg/mL gelatin. After electrophoresis, gels were washed to remove SDS.

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<th>Table 1. Sequences of the primers used to amplify PACE4 or GAPDH</th>
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*The size of the amplicon is indicated.
and incubated for 18 hours at 37°C in a rehydrating buffer (50 mmol/L Tris, 5 mmol/L CaCl₂, 0.02% NaN₃, 1% Triton X-100). Gels were subsequently stained with Coomassie brilliant blue G-250 and destained in 30% methanol and 10% acetic acid (v/v).

**RNA extraction and RT-PCR.** Total RNA from epidermis or cultured newborn keratinocytes was extracted using TRIZOL Reagent (Life Technologies/Invitrogen) and reverse-transcribed by Superscript One-Step RT-PCR (Invitrogen). Rat PACE4 and murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene used as a control for the RT-PCR reaction) were amplified with the primers described in Table 1.

**Analysis of epidermal thickness and cell proliferation following treatment with TPA.** Groups of mice (n = 5) were treated with two weekly applications of TPA (5 mmol) or the acetone vehicle and sacrificed 48 hours after the last treatment. Paraffin sections were stained with H&E and the skin thickness was measured with a micrometer. For analysis of cell proliferation, mice treated as described above were injected i.p. with bromodeoxyuridine (BrdUrd, 100 µg/kg body weight; Sigma-Aldrich) in PBS, 2 hours prior to sacrifice. Treated skin was then fixed in formalin, embedded in paraffin, sectioned at 4 µm, stained with H&E and anti-BrdUrd antibody (Roche), and then treated with biotinylated anti-mouse IgG and horseradish peroxidase-conjugated ABC reagent (Vector Laboratories, Inc., Burlingame, CA). Epidermal cell proliferation (presented as the labeling index) was determined as follows: a minimum of 500 basal cells from five mice per group were counted, and the percentage of BrdUrd-positive cells was calculated. Slides were mounted and observed with a Nikon Optiphot microscope using a magnification of 60×/NA = 1.4). Samples were mounted in fluorescent mounting media (Prolong Gold antifade reagent, Molecular Probes, Eugene OR) and visualized with Hoechst H 33342 (Calbiochem, La Jolla, CA) for nuclei staining and FITC (collagen IV) by excitation at 364 and 520 nm, respectively. Pictures, representing a stack of three-dimensionally reconstructed using tilt angles between 0 and 520 nm, respectively. Pictures, representing a stack of Z planes, were obtained with a Plan/Apo objective 20×, NA, 0.75, Nikon eyepiece ×10, final magnification ×200. Micrographs were obtained with a Magnafire Optronics digital camera.

**Immunofluorescence.** Frozen sections (5 µm) from skins were fixed in acetone, and incubated with an anti-mouse anticallogen IV antibody (AB756P, Chemicon International) for 1 hour at room temperature. FITC-labeled antigen IgG antibody (The Jackson Laboratory, West Grove, PA) was used as secondary antibody. Imaging was done in a Nikon Eclipse TE 2000-U microscope using a magnification of 60× (NA = 1.4). Samples were mounted in fluorescent mounting media (Prolong Gold antifade reagent, Molecular Probes, Eugene OR) and visualized with Hoechst H 33342 (Calbiochem, La Jolla, CA) for nuclei staining and FITC (collagen IV) by excitation at 364 and 520 nm, respectively. Pictures, representing a stack of Z planes, were obtained with a Roper Scientific CoolSNAP HQ camera, the images were processed using Metamorph software. Briefly, images were two-dimensionally deconvoluted, three-dimensionally reconstructed using tilt angles between 0 and 180 degrees. For quantitative measurements of collagen IV fluorescence, the ratio between the fluorescence associated with collagen IV and the stratum corneum (considered as the internal control) was calculated.

**Statistical analysis.** Statistical significance was determined by calculating P values. P values corresponding to the curves of carcinoma development were determined by Kruskal-Wallis test. The differences between the number of endophytic papillomas per animal were analyzed by comparing of frequencies using a normal approximation to the Poisson distribution. In all other studies, P values were determined using Fisher two-tailed exact test.

**Results**

**Generation and characterization of transgenic mice.** To study the effects of PACE4 expression on the highly proliferative epidermal basal cells, the full-length rat PACE4 cDNA was placed under the control of the K5 promoter targeting PACE4 expression to the basal skin keratinocytes. The construct contains the bovine K5 promoter (Fig. 1A), followed by the first intron from rabbit β-globin to enhance the efficiency of transcription, the full-length rat PACE4 cDNA and, finally the polyadenylation signal from SV40. Five founders were produced; two of them were selected to expand the animal line because they showed approximately two to four transgene copies (data not shown). Founders were genotyped by PCR of genomic DNA using two set of primers amplifying a DNA segment of either 450 bp corresponding to the PACE4-PolyA boundary or 1,000 bp internal to rat PACE4 discriminating endogenous from transgenic PACE4 genes. A representative genotyping experiment is shown in Fig. 1B, Two founders, referred to as animal lines #1 and #2 were selected to perform subsequent experiments.

Transgene copy number in the two animal lines was assessed by Southern blot analysis and densitometry. The results showed that animal line #1 contained four to five transgene copies whereas line #2 had two copies (Fig. 1C). Expression at the RNA level was assessed using RNA extracted from cultured newborn keratinocytes (Fig. 1D). Both lines showed RNA expression. The relative level of PACE4 protein expression was analyzed by Western blot. As a protein source, we used either skin lysates or conditioned medium from keratinocyte culture obtained from newborn mice. The relative amount of PACE4 expressed by the wild-type and transgenic lines was analyzed by densitometry and was normalized to β-actin expression. In agreement with the respective transgene copy number, PACE4 protein levels were twice as high in animal line #1 as in line #2 (Fig. 1F). Wild-type animals did not show expression of PACE4 in total skin or keratinocyte cultures.

PACE4 enhances induced epidermal proliferation and activates matrix metalloproteinases in vivo. Both transgenic animal lines resemble the wild-type animals in major phenotypic characteristics. However, as PACE4 is a proprotein convertase associated with increased proliferation and invasive ability, we examined the biochemical and microstructural alterations of the epidermis. First, the effect of PACE4 expression on basal keratinocyte proliferation was evaluated by measuring epidermal thickness in H&E-stained specimens from wild-type and transgenic mice skin. The measurements indicated that epidermal thickness was unaltered by the expression of the transgene (Fig. 2A). However, increased susceptibility to the hyperplasigenic and proliferative effects of TPA was observed after acute topical treatment. The epidermis from transgenic mice showed a 30% to 40% increase in thickness compared with their wild-type counterparts after 2 weeks of TPA treatment (2 µg twice a week; Fig. 2A and B). Proliferation was also evaluated by BrdUrd incorporation rates. Similar to results observed by measuring epidermal thickness, control skin from wild-type or transgenic animals did not show significant differences in BrdUrd incorporation. Nevertheless, after TPA treatment, BrdUrd incorporation to both transgenic lines increased about 25%, above the wild-type levels in agreement with the changes observed in skin thickness (Fig. 2C and D).

Cell invasiveness is another biological process affected by PACE4 expression (25). In this context, the main substrates for PACE4 are MT-MMPs, metalloproteinases responsible for activation of collagenase IV (MMP-2), a major extracellular matrix–degrading enzyme. In order to evaluate whether PACE4 expression influenced the activation of MT-MMPs, and consequently, the activation of MMP-2, we evaluated MT1-MMP and MT2-MMP processing by Western blot in cell lysates from cultured keratinocytes obtained from wild-type or transgenic newborn mice. As shown in Fig. 3, both MT1-MMP (Fig. 3A) and MT2-MMP (Fig. 3F) were more efficiently processed in transgenic lines than in wild-type animals. This enhanced activation of MT-MMPs correlated with enhanced activation of MMP-2 as assessed by zymography of whole-skin lysates and conditioned medium from cultured keratinocytes. Levels of pro-MMP-2 were almost undetectable in transgenic mice. Conversely, a band corresponding to the unprocessed form of this metalloproteinase was clearly observed in wild-type animals (Fig. 3C).
PACE4 disrupts basement membrane structure. Because increased MMP-2 activation results in enhanced degradation of collagen type IV, one of the main components of the basement membrane, we hypothesized that the structure of transgenic basement membrane could be altered. In order to evaluate in vivo type IV collagen degradation, we analyzed the integrity of the basement membrane collagen component of wild-type and transgenic epidermis. Collagen type IV indirect immunofluorescence, done on frozen sections from skin derived from wild-type animals, revealed a well-defined and continuous basement membrane with a uniform thickness (Fig. 4A, top). Conversely, in line #1, many points of discontinuity and areas in which the basement membrane collagen type IV was markedly thinner than in its wild-type counterpart were seen (Fig. 4A, middle). The phenotype observed in the skin of animal line #2 was less pronounced (Fig. 4A, bottom), suggesting that this effect may be dependent on gene dosage (Fig. 4C). Collagen type IV disruption was quantified by measuring the intensity of fluorescence associated with this protein within the collagen IV component of the basement membrane (Fig. 4). Transgenic lines #1 and #2 showed 2.5 and 1.4 times the reduction relative to wild-type in the collagen type IV fluorescent intensity, respectively (Fig. 4B). In addition, the number of disruptions in the collagen type IV layer per micron of linear basement membrane was recorded. In agreement with the measurements of collagen type IV fluorescence intensity, the number of disruptions was 2 to 2.5 times higher in the transgens than in the wild-type animals. These results indicate that constitutive expression of PACE4 is associated with increased collagen type IV degradation, resulting in basement membrane alteration such as membrane thinning and localized disruptions. Collagen type IV immunofluorescence of TPA-treated skins pointed in the same direction. Transgenic animals showed increased collagen type IV disruptions and altered basement membrane structure. Nevertheless, measurements of the basement membrane’s collagen type IV component showed that both transgenic and wild-type animals exhibited similar levels of fluorescence intensity, attributable to TPA-driven stimulation of collagen type IV synthesis (ref. 33; Fig. 4F). However, transgenic animals showed increased number of collagen type IV disruptions (see Fig. 4D, insets). The number of disruptions per micron was three or two times higher in transgenic lines #1 or #2, respectively, than in wild-type epidermis (Fig. 4F). These data suggest that even after TPA treatment, transgenic animals were still able to degrade collagen type IV and exhibited significant basement membrane structural alterations. Alteration in basement membrane structure can be observed online, under supplemental materials, videos V#1, control skins, and V#2 TPA-treated skins.

Figure 1. Generation of PACE4 transgenic mice. A. K5-PACE4 wild-type construct. B, PCR from DNA extracted from tails, amplified with either primers A or B and β-globin as control. C, determination of the number of copies of the transgene by Southern blot analysis. Note the higher intensity of the band in line #1. D, RNA expression of the transgene. RNA was extracted from cultured keratinocytes and analyzed for PACE4 expression by RT-PCR. GAPDH was added as control. E, Western blot and densitometry showing differential expression of PACE4 protein in transgenic lines. Note the complete absence of PACE4 expression in the wild-type mice.
PACE4 increases susceptibility to skin cancer. In order to determine the effects of PACE4 overexpression and enhanced MMP activation on SCC development, a two-stage carcinogenesis protocol was used. During the carcinogenesis experiment, transgenic animals revealed numerous lesions resembling small papillomas. Wild-type animals also showed these lesions in reduced numbers. As this feature constituted a distinctive phenotype between wild-type and transgenic animals, we examined the lesions at the completion of the experiments (30 weeks after DMBA initiation). Histologic examination confirmed that the lesions were papillomas that could be classified into two distinct types (Fig. 5B and C). Typical exophytic cauliflower-like pedunculated papillomas (type A, Fig. 5B) and papillomas containing an endophytic component that grew towards the dermis (type B, Fig. 5C). Most of the papillomas (>70-80%) were type B in both transgenic lines. In contrast, papillomas in wild-type mice showed a higher proportion of type A papillomas. The ratio between the number of endophytic papillomas per mouse in transgenic lines to wild-type mice was recorded (Fig. 5A). Transgenic lines harbored at least three times more (P < 0.001) endophytic papillomas per animal than the wild-type animals. On the other hand, line #2 developed twice as many exophytic papillomas as line #1, highlighting the more moderate phenotype of line #2 mice expressing lower levels of PACE4 (supplemental material S1).

SCC development was remarkably enhanced in transgenic animals (Fig. 5C). The incidence of carcinoma showed a clear difference between the transgenic and wild-type animals (see supplemental material S2). Transgenic mice developed SCCs faster that the wild-type mice and the number of malignant lesions was higher at any time point in agreement with the proposed role of PACE4 as a facilitator of malignant conversion (ref. 25; Fig. 5D).

Rates of SCC formation also differed strikingly between wild-type (0.04 carcinomas per animal per week) and transgenic animals (0.092 carcinomas per animal per week; Fig. 5D).

Figure 2. Skin of wild-type, transgenic line #1, and transgenic line #2 were treated either with acetone or TPA. A, note the enhanced susceptibility to TPA-induced effects (increased thickness) on transgenic mice (P < 0.001). B, H&E staining of TPA-treated skin. Note the increase in skin thickness in transgenic animals as compared with wild-type after TPA treatment. C, proliferative response to TPA. There is a 30% to 40% increase in nuclear labeling of transgenic epidermis (P < 0.001). D, BrdUrd immunostaining of TPA-treated skin. Note the higher number of stained nuclei in transgenic tissue. A and C, data represent an average of five mice; magnification, 20×; bar, 50 μm.

Figure 3. Activation of metalloproteinases by wild-type and transgenic epidermal keratinocytes. Western blot MT1-MMP (A), MT2-MMP (B) and zymography from conditioned medium of cultured newborn keratinocytes depicting MMP-2 activity (C). Note increased MT1-MMP and MT2-MMP activation in transgenic keratinocytes as well as increased MMP-2 activity. Pro, proenzyme; i MT1-MMP, inactive MT1-MMP. As a marker to position MT1-MMP species (Pro, mature and inactive enzyme), we used a cell lysate from the fibrosarcoma cell line HT-1080.
Conversion of papillomas into SCC, measured as the ratio between the number of carcinomas and the number of papillomas, was much higher in transgenic lines than in wild-type mice (see supplemental material S3). Moreover, the number of metastases observed in transgenic mice was dependent on PACE4 expression. Although the formation of metastasis is a relatively rare event at 30 weeks of TPA treatment, the number of metastatic foci in axial lymph nodes and lung was higher in line #1 than in wild-type mice (see supplemental material S4). Line #2 showed only a marginal increment in the number of metastasis when compared with wild-type probably due to its lower expression of PACE4. Neither control untreated transgenic mice nor control wild-type mice exhibited spontaneous skin tumors during the 18-month observation period.

PACE4 induces higher levels of matrix metalloproteinases and tumors of advanced malignant phenotype. In order to confirm that tumors from transgenic mice expressed PACE4 and showed increased activation of MT-MMPs and MMP-2, we analyzed cell lysates from wild-type and transgenic tumors by Western blot (Fig. 5E). All transgenic tumors exhibited PACE4 expression. In contrast, only a small fraction (~20%) of tumors from wild-type animals expressed this proprotein convertase (Fig. 5E, top). PACE4-mediated proteolysis resulted in activation of MT-MMP's, the principal proprotein convertase substrates involved in tumor cell invasiveness (34, 35). Lysates of tumors obtained from PACE4 transgenic animals showed increased ability to cleave MT1 and MT2-MMP, the active form being the only one detectable in some cases (Fig. 5E, middle). In contrast, these

Figure 4. Collagen IV immunofluorescence. Sections from acetone (A-C) or TPA (D-E)–treated animals were immunostained with a collagen IV antibody. Slides from untreated (A) and TPA-treated (D) skin are shown. Collagen IV immunofluorescence was quantified measuring the integrated absorbance using the Metamorph software. Note the decreased fluorescence associated with collagen IV in transgenic animals (Fisher, two-tailed exact test, $P < 0.05$; B). TPA treatment did not result in major differences in collagen IV immunofluorescence (E). Collagen IV disruptions were counted and the number of these disruptions per unit ($\mu$m) of basement membrane length were recorded. C, nontreated; F, TPA-treated. Note the increased number of basement membrane disruptions in the transgenic samples. Differences in the number of disruptions between wild-type and transgenic mice were highly significant (Fisher two-tailed exact test, $P < 0.001$). Magnification, 60×; bar, 10 μm.
MT-MMPs were cleaved with lower efficiency in wild-type tumors (Fig. 5E, middle). This enhanced activation of the MT-MMPs was reflected in enhanced activation of its substrate, MMP-2, as shown by zymography (Fig. 5E, bottom).

In order to confirm that PACE4 expression alters the malignant phenotype, we evaluated and graded the tumors. Histologic analysis of tumors from wild-type and transgenic mice showed notorious differences. In wild-type animals and transgenic line #2 mice (which showed lower expression of PACE4), >50% SCC were well-differentiated, grade 1 tumors (Fig. 6A and C). Grade 2 accounted for 25% and the rest (~20%) were more advanced grades 3 and 4. In contrast, histology of tumors from transgenic line #1 showed a greater number of more aggressive phenotypes (Fig. 6B). Figure 6D shows the distribution of tumors according to their grade.

**Discussion**

PACE4 is a serine protease overexpressed in high-grade murine SCC (24). Its activity results in proteolysis of substrates leading to activation of many cancer-related proteins (36). In order to show a causal relationship between PACE4 expression and increased susceptibility to carcinogenesis in vivo, we studied two PACE4 overexpressing transgenic animal lines (#1 and #2). Southern blot analyses showed that animal line #1 contained four to five copies of the transgene, while line #2 showed only two. This difference in copy number was reflected in the higher protein expression (twice as high in line #1 than in line #2).

The enhanced PACE4 expression was reflected in higher levels of activated MT1- and MT2-MMP. Increased MT1-MMP activation drives increased type IV collagenolytic activity (37, 38). Constitutive PACE4 activation may be responsible for the premature activation of MMP-2, allowing early disruption of the basement membrane collagen IV component. Sole activation of MT1-MMP may also be responsible for basement membrane degradation. MT1-MMP was recently identified as a major protease necessary for cell invasiveness of either normal or cancer cells (35). Moreover, animal models targeting the expression of this MT-MMP to the mammary gland resulted in increased induction of adenocarcinomas (39).

Collagen type IV, a major basement membrane structural protein, provides a scaffold that binds other basement membrane proteins such as laminins, entactins, and proteoglycans (40). Hence, collagen type IV degradation may disrupt the integrity of the basement membrane creating an invasion-permissive environment that contrasts with the invasion-restraining environment provided by
an intact basement membrane (41). PACE4-overexpressing mice showed disruption of the collagen IV component of the basement membrane. Moreover, the magnitude of this effect paralleled the levels of transgene expression. The intensity of basement membrane collagen type IV was reduced by 85% and 50% in lines #1 and #2, respectively. Further evidence that transgenic mice had a higher collagenolytic activity was the increased number of basement membrane collagen type IV disruptions.

Degradation of collagen type IV might allow direct contact between keratinocytes and stroma, generating signals that would not take place in the absence of direct contact (42). Early degradation of collagen type IV also predisposes the epithelial cells to accelerated invasion bypassing the need for both attachment and additional degradation of the basement membrane (40, 43) by directly migrating into the dermis. In this sense, before a normal cell is initiated/promoted, PACE4 transgenic basal keratinocytes already possess the necessary conditions to start migration and invasion (Fig. 7). In summary, these results suggest that increased efficiency in MT-MMPs activation results in acceleration of tumor development and progression due, in part, to increased collagenase type IV activity. Early activation of collagen-degrading enzymes, mainly MT-MMPs and also MMP-2, produce premature changes in the biology of the extracellular matrix, i.e., basement membrane disruption (44) that may result in an early interaction between fibroblasts and epithelial cells. This early encounter may trigger a plethora of pro-proliferative effects via fibroblast growth factors, cytokines, and/or matrix proteases contributing to premature increase in epithelial proliferation and invasion (45). These features of accelerated basement membrane degradation and increased susceptibility to tumor promoters resulted in a decisively magnified response during carcinogenesis (Fig. 7).

The skin of transgenic animals showed numerous flat lesions that were not as prominent in wild-type animals. Interestingly, these lesions proved to be endophytic papillomas. As these mice showed increased collagen type IV degradation, it may be argued that due to thinning or weaker basement membrane collagen type IV component, the developing tumor cells grow unopposed directly into the dermis (Fig. 7). This feature resembles the behavior observed during complete carcinogenesis, consisting of repetitive treatments with a complete carcinogen such as benzo(a)pyrene (46). In this protocol, few exophytic (type A) papillomas develop, whereas most of the SCCs are preceded by in situ flat precursor lesions and endophytic lesions (47). This observation points to a close association between increased PACE4 expression and enhanced susceptibility to chemical carcinogens, i.e., increased PACE4 expression in animals treated with a two-stage protocol results in early aggressive tumors preceded by papillomas with endophytic growth mimicking the response vis-à-vis complete carcinogen protocols (29).

After the 30 weeks of treatment with the two-stage carcinogenesis protocol, the number of carcinomas per animal in both transgenic animal lines was twice as high as in wild-type mice. Moreover, the incidence of malignant tumors, which is defined as the percentage of animals harboring carcinomas, was consistently higher in transgenic mice: 65% to 70% of transgenic animals had tumors. In contrast, only 45% of wild-type animals developed tumors (see supplemental material S2). The rate of carcinoma growth showed remarkable differences between transgenic and mice, 0.092 versus 0.04 carcinomas/animal/wk, respectively. The

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Histology of representative SCC. Wild-type (A) and transgenic line #2 (C) developed mostly well-differentiated tumors (grades 1 and 2). Line #1 (B) generated poorly differentiated tumors (grades 3 or 4) SCC. Magnification, 10×; bar, 50 μm (C). D, relative number of grades 3 to 4 SCCs in wild-type and transgenic animals.
data pointed to the pivotal role of PACE4 in accelerating or predisposing the rate of tumor development and progression. Line #1, which expressed high levels of PACE4, developed more malignant tumors and metastasis than line #2, which expressed only modest levels of PACE4. Moreover, line #2 resembled wild-type animals in both tumor grade (mostly grades 1 or 2) and the relative low number of metastases.

In summary, PACE4 expression in vivo resulted in premature disruption of the component of the basement membrane, allowing early down-growth of carcinogen-transformed cells to the underlying structures. This early behavior resulted in an increased number of endophytic papillomas growing towards the dermis, providing an extra advantage to tumor development, and eventually invading sooner and deeper into the dermis, finally producing distant metastases (Fig. 7).

Acknowledgments

Received 4/7/2005; revised 5/26/2005; accepted 6/7/2005.

Grant support: NIH grants CA75028, CA06927, and by an appropriation from the Commonwealth of Pennsylvania.

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Figure 7. Proposed model for the mechanisms involved in PACE4-driven acceleration of tumor progression. MMP activation by PACE4 results in premature disruption of the collagen IV component of the basement membrane (BM, red). This leads to early epidermal down-growth into the stroma and development of endophytic papillomas. The latter, earlier gave rise to more advanced SCC that had the extra advantage of metastasizing.

References

32. Cagliero E, Roth T, Roy S, Maiello M, Lorenzi M. PACE4 Expression Enhances Tumor Progression.
PACE4 Expression in Mouse Basal Keratinocytes Results in Basement Membrane Disruption and Acceleration of Tumor Progression

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