**Abstract.** Background: Protein kinase C (PKC)α is distributed in almost all tissues and participates in various signaling pathways. However, the role of PKCα in carcinogenesis remains unclear. In this study, we performed complete skin carcinogenesis in PKCα knockout mice by repeated administration of 7,12-dimethylbenz[a]anthracene (DMBA).

Materials and Methods: Complete skin carcinogenesis was performed by repeated DMBA treatment using PKCα knockout mice. The number of tumors was determined weekly. Tumor types were determined by Hematoxylin and eosin (H & E) analysis. Tumor growth was assayed by proliferating cell nuclear antigen (PCNA) staining. Results: In the knockout mice, the average number of tumors was 16.6/mouse at 20 weeks. In contrast, in the wild-type (WT) mice, the tumor number was 6.9/mouse. Growth and malignant grade of tumors in PKCα knockout mice did not differ from those in WT mice. Conclusion: PKCα suppresses tumor formation, but not tumor growth and progression in skin carcinogenesis.

Protein kinase C (PKC) belongs to the phospholipid-dependent serine/threonine kinases that are activated by membrane lipids produced by various extracellular stimuli (1). Based on their structural similarities and co-factor dependence, there are 10 isotypes in three subfamilies. The conventional PKCs (cPKCα, βI, βII, and γ), the novel PKCs (nPKCδ, ε, η, and θ), and the atypical PKCs (aPKCζ and λ/ι). cPKC and nPKC isotypes are the major intracellular targets for potent mouse tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), suggesting that these PKC isotypes have an important role in tumor formation (1, 2).

Of the PKC isotypes, PKCα is distributed in almost all tissues and participates in various signaling pathways including cell-cycle regulation, differentiation, and apoptosis (3). Several lines of evidence suggest that PKCα is involved in carcinogenesis. Previously, we performed a two-stage skin carcinogenesis study in which 7,12-dimethylbenz[a]anthracene (DMBA) was used as an initiator and TPA as a promoter in PKCα knockout mice, which exhibited increased susceptibility to tumor formation, however, no squamous cell carcinoma (SCC) was observed in the study (4). PKCα knockout mice exhibit increased susceptibility to intestinal tumor formation in ApcMin/+ mice (5). These reports suggest that PKCα functions as a tumor suppressor. In contrast, PKCα promotes tumor growth and progression in certain tumors and cell lines (6-8). Using the DMBA/TPA protocol, PKCα overexpression in the epidermis was found to induce a more simplified manner skin tumor formation, compared to control mice (9). However, other studies found that PKCα overexpression in the epidermis did not affect skin tumor formation using the DMBA/TPA protocol (10, 11). Therefore, the role of PKCα in carcinogenesis is controversial. Other approaches may be necessary to elucidate PKCα function in tumor formation. In this study, in order to obtain further insight into the function of PKCα in tumor formation, we performed complete skin carcinogenesis by repeated DMBA treatment in PKCα knockout mice.

**Materials and Methods**

Mice. PKCα knockout mice with a genetic background of C57BL/6J and 129/SvJ were used as reported previously (4). All experiments were performed in accordance with the policies of the Animal Ethics Committee of The University of Tokyo.

Carcinogenesis. Eight-week-old female, wild type (WT) and PKCα knockout mice were used. The dorsal skin was shaved and one week later, DMBA (100 μg; Sigma, St Louis, MO, USA) dissolved in 0.2 ml acetone was applied to the shaved area, once weekly for 20 weeks. The number of tumors was determined weekly.

Histological analysis. The mice were killed by cervical dislocation and their dorsal skin or tumor samples were removed, and fixed.
in 4% formaldehyde in phosphate-buffered saline at room temperature and embedded in paraffin. Skin and tumor sections (4-μm-thick) were deparaffinized with xylene and ethanol, and stained with H&E. Tumor types were determined by H&E analysis according to criteria previously described (12, 13). The tumors were subdivided into five groups (Table I, A-E). Tumors with well-differentiated hyperplastic lesions without marked atypia of all layers of the epidermis, and with intact basement membranes, were classified as group A. Tumors with lesions with marked atypia and mitotic figures in all layers of the epidermis, with lesions equivalent to carcinoma in situ, or with focal invasions were classified as group B. SCCs were characterized by endophytic growth that progressively invaded the dermis and the subcutaneous tissue. SCCs with marked horny cells (terminally differentiated cells) were classified as group C. SCCs with differentiated cells but no clear horny pearls were classified as group D. SCCs with small areas of squamous differentiation, including spindle cell carcinoma, were classified as group E.

Proliferating cell nuclear antigen (PCNA) staining. Deparaffinized tumor sections were subjected to antigen retrieval with 10 mM citrate buffer (pH 6.0) at 95°C for 20 min, followed by treatment with 3% hydrogen peroxide for 10 min at room temperature. Sections were blocked with 5% normal goat serum and incubated with an anti-PCNA antibody conjugated with peroxidase (Dako Japan, Kyoto, Japan) at 37°C for 1 h. Diaminobenzidine was used as a chromogen. Hematoxylin was used for counterstaining. The number of PCNA-positive cells was determined from at least four independent fields.

Statistical analysis. Statistical analysis of the experiments was performed using the two-tailed unpaired Student’s t-test, except for histological analysis of skin tumors, which was performed using the χ² test. p-Values <0.05 were accepted as demonstrating significant differences between groups.

Results

We performed complete skin carcinogenesis by repeated DMBA treatment. The first tumors appeared after eight weeks of DMBA treatment in PKCα knockout and WT mice. The knockout mice exhibited rapid tumor development.
Figure 2. Hematoxylin and eosin (H&E) staining of tumor sections from the wild-type (WT) and knockout (KO) mice. Representative figures are shown. A: Benign tumor. B: Well-differentiated squamous cell carcinoma. Scale bar=100 μm.

Figure 3. A: Proliferating cell nuclear antigen (PCNA) staining in a representative section of skin tumors. Scale bar=30 μm. Counterstaining was performed with hematoxylin. B: Percentage of PCNA-positive cells in tumors from the wild-type (WT) and knockout (KO) mice including benign tumors and squamous cell carcinomas (n=6). There was no significant difference between WT and KO mice (p>0.1). Results are shown as mean±SE.
In this study, the proportion of tumor-bearing mice reached 72% in the KO mice, whereas it only reached 33% in the WT mice. The number of tumors in the knockout mice was 1.1±0.2/mouse (mean±SE). In contrast, the number of tumors in the WT mice was 0.4±0.2/mouse (p<0.05). At 20 weeks, all knockout and WT mice had developed tumors. The knockout mice developed 16.6±2.7 tumors/mouse. In contrast, in the WT mice, there were only 6.9±0.8 tumors/mouse (p<0.01). Repeated application of acetone as a solvent control induced no tumors. These data indicate that the knockout mice were significantly more susceptible to skin tumor formation than the WT mice regarding skin carcinogenesis.

We evaluated tumors ≥2 mm in diameter from knockout and WT mice for histopathological features at the end of the experiment (Figure 2). In the knockout mice, 56% of tumors were SCC, and 14% were moderately or poorly differentiated SCC. In the WT mice, 50% of tumors were SCC, and 16% were moderately or poorly differentiated (Table I). There were no significant differences in the malignancy grade between knockout and WT mice (Table I, p>0.1, χ² test), which indicates that PKCα was not essential for malignant progression in skin carcinogenesis.

To determine whether PKCα regulates tumor growth or not, we stained tumor sections with an antibody against PCNA, which is a proliferation marker (Figure 3). The percentage of PCNA-positive cells in the knockout mice was 63±6.1% (mean±SE), and it was 65±3.8% in WT mice. No significant difference in the percentage of PCNA-positive cells was observed in tumors between knockout and WT mice, which is consistent with our previous study (4). These data indicate that PKCα was not essential for tumor growth in skin carcinogenesis.

**Discussion**

In this study, the PKCα knockout mice were significantly more susceptible to DMBA-induced skin tumor formation. These results show that PKCα negatively regulates tumor formation, which is consistent with our previous work and other studies (4, 5, 14, 15). Our findings provide additional evidence that PKCα suppresses tumor formation. The malignant grade of tumors and tumor growth in PKCα knockout mice were no different from those in WT mice. These results indicate that PKCα is not essential for malignant progression and tumor growth. It is important to investigate the mechanisms that underlie carcinogen-induced tumor or carcinoma formation. DMBA is a polycyclic aromatic hydrocarbon and an abundant environmental contaminant (16). Thus, PKCα knockout mice could be a useful model for elucidating the underlying mechanisms that are involved in the response to environmental carcinogens linked to the development of human SCC.

The mouse skin carcinogenesis model is useful for understanding the multi-stage nature of human carcinogenesis (17). Previous studies of our group suggested that PKCα suppresses skin tumor promotion (4). It is likely that PKCα inhibits selective clonal expansion of initiated cells under the effects of TPA (17). Presently our findings suggest that PKCα may have a protective role during the initiation stage in skin tumor formation. Therefore, we propose that PKCα suppresses both tumor initiation and tumor promotion in skin tumor formation.

Based on our findings, it is possible that PKCα suppresses tumor formation, but not tumor growth and tumor progression in human. Thus, inhibition of PKCα may not be useful for cancer therapy. In fact, PKCα-specific antisense oligonucleotide, displayed no or only slight effect on advanced non-small cell lung carcinoma patients or on metastatic colorectal cancer in patients (18, 19). Additional approaches are necessary for the development of specific PKCα inhibitors or activators, which will act as anticancer drugs.

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**References**


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