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# SUPPRESSION OF SPONTANEOUS CARCINOGENESIS IN KERATINOCYTE–SPECIFIC PTEN–DEFICIENT MICE WITH SELECTIVE INHIBITION OF PI3K ISOFORMS

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**Abstract**
PTEN is a tumor suppressor gene that is mutated in many human sporadic cancers and in heredi-
tary tumor susceptibility disorders such as Cowden disease. We have previously reported that
keratinocyte–specific heterozygous Pten–deficient mice (K5CrePten$^{flox/+}$ mice) displayed enhanced
susceptibility to carcinogenesis of the skin. Accordingly, to clarify whether the selective inhibition
of phosphoinositide 3–kinase (PI3K) isoforms rescues the phenotypes of K5CrePten$^{flox/+}$ mice, we
generated mice simultaneously lacking (PI3K) isoforms (p85$\alpha$, p110$\alpha$, p110$\delta$ or p110$\gamma$) and Pten.
Consequently, it was shown that heterozygous p110$\alpha$ deficiency (p110$\alpha^{+/-}$) and homozygous p110
deficiency (p110$\gamma^{-/-}$) significantly rescued susceptibility to spontaneous carcinogenesis of the skin
in K5CrePten$^{flox/+}$ mice. The present study sheds light on the possible role of p110$\alpha$ and p110$\gamma$ in
carcinogenesis of the skin ; thus providing new insights into a chemoprevention of squamous cell
carcinoma.

**Key words** : Pten, PI3K, p110$\alpha$, p110$\delta$, squamous cell carcinoma

## Introduction

PTEN is a multifunctional phosphatase whose major
substrate is phosphatidylinositol–3,4,5–trisphosphate
(PIP3)[1], a lipid second messenger molecule. PIP3 acti-
vates numerous downstream targets, including the ser-
ine–threonine kinase PKB/Akt, which is involved in anti-
apoptosis, proliferation, and oncogenesis[2]. By using its

lipid phosphatase activity to dephosphorylate PIP3,
PTEN negatively regulates the phosphoinositide–3–
kinase (PI3K)–PKB/Akt pathway and thus exerts tumor
suppression. PI3K family members are classified into
three groups according to their structures and substrate
specificities. Among them, class I PI3Ks produce PIP3
and are involved in receptor–mediated signaling. Class I
PI3Ks are further divided into two subclasses. Class IA
heterodimeric PI3Ks, consisting of a catalytic subunit
(p110$\alpha$, p110$\beta$, p110$\delta$) and a regulatory subunit (p85$\alpha$,
p85$\beta$, p55$\gamma$), are involved in receptor tyrosine kinase
(RTK) pathways, whereas class IB PI3K (p110$\gamma$) acts
downstream of G–protein–coupled receptors (GPCRs).
Both classes of PI3Ks can be activated by a wide range of

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growth factors[3].

Selective PI3K isoform knockouts in mice have been valuable in delineating the functional roles of each PI3K isoforms. Knockouts of p110$\alpha$[4] and p110$\beta$[5] are embryonic lethal, whereas knockouts of p85$\alpha$[6], p110$\delta$[7] and p110$\gamma$[8] are viable but show defects in components of immune or metabolic phenotypes. However because of the tissue specific expression of the isoforms, the functional information that can be derived from ubiquitous knockouts is limited[3]. In endothelial cells, null mutation of p110$\gamma$ or p85$\alpha$ partial rescues endothelial-specific (Tie2Cre) Pten$^{flox/flox}$ and Tie2CrePten$^{flox/+}$ phenotypes[9]. In natural killer T (NKT) cells, p110$\delta$ and p110$\gamma$ rescues LckCrePten$^{flox/flox}$ phenotypes[10]. In this study, we investigated the phenotype rescue in keratinocyte-specific Pten null mice. Especially we focused on roles of PI3K isoforms in skin carcinogenesis in physiological condition using aged PI3K and K5CrePten$^{flox/+}$ double mutant mice.

## Materials and Methods

### 1　Generation of mutant mice

Pten$^{flox/flox}$ mice (C57BL6/J background), generated as previously described[11], were mated to Keratin5Cre (K5Cre) transgenic mice (C57BL6/J background)[12], in which expression of Cre is controlled by the K5 promoter. K5 is strongly activated in keratinocytes. Offspring carrying K5Cre plus one copy of the floxed Pten allele (K5CrePten$^{flox/+}$) were used in analyses as heterozygous mutant, and wild-type (Pten$^{+/+}$) mice, respectively. P85$\alpha$$^{+/-}$K5CrePten$^{flox/+}$, p110$\alpha$$^{+/-}$K5CrePten$^{flox/+}$, p110$\delta$$^{+/D910A}$K5CrePten$^{flox/+}$ and p110$\gamma$$^{-/-}$K5CrePten$^{flox/+}$ double mutant mice were generated by crossing p85$\alpha$$^{+/-}$ mice (C57BL/6J background)[6] or p110$\alpha$$^{+/-}$ mice (C57BL6/J background)[4] or p110$\delta$$^{+/D910A}$ mice (C57BL6/J background)[13] or p110$\gamma$$^{-/-}$ (C57BL6/J background)[8] with K5CrePten$^{flox/+}$ mice, respectively. The Institutional Review Board of the Akita University School of Medicine approved all animal experiments.

### 2　PCR analysis of Pten genotypes

Genomic DNA from mouse tails was isolated and amplified by PCR following a published protocol[11]. Sense primer [5-GTCACCAGGATGCTTCTGAC-3] and anti-sense primer [5-GAAACGGCCTTAACGACGTAG-3] were used to detect the floxed Pten allele ; sense primer [5-GTCACCAGGATGCTTCTGAC-3] and antisense primer 5-GTGACATCAACATGCAACACTG-3] were used to detect the wild-type Pten allele ; and sense primer [5-ATGCCAATGCCCCCTCAGTTCCT-3] and antisense primer[5-TGCCCCTTTTTATCCCTTC-CAGA-3] were used to detect the Keratin5Cre transgene.

Sense primer [5-AGG TGA GAT GAC AGG AGA TCC TGC CC-3] and antisense primer [5-TGT CTT CAG TCA CCC CGT TGA T-3] to detect the flanking p85$\alpha$ allele and [5-GGA GCC TTT ATT CAC AGT CAG TAT GT-3] to detect the wild type p85$\alpha$ allele were used. Sense primer [5-AGA GGC TAT TCG GCT ATG ACT G-3] and antisense primer [5-CTA GTC CTA CTA GAC CTG CTT-3] were used to detect the p110$\alpha$ mutant allele. Sense primer [5-CTC CCC AAT GGA ATG ATA GTG-3] and antisense primer [5-TCT TTT CTT CAC GGT TGC CT-3] were used to detect the p110$\alpha$ wild type allele. Sense primer [5-AAC GAA GCT CTC AGA GAA AGC TGG-3] and antisense primer [5-CCT GCA CAG AAA TGC ACT TCC-3] were used to detect the p110$\delta$ D910A allele. Sense primer [5-GGG GTG GGA TTA GAT AAA TG-3] and antisense primer [5-TAC CTG CAG AGG ACA GAG GAG-3] were used to detect the p110$\gamma$ mutant allele. Sense primer [5-TCA GGC TCG GAG ATT AGG TA-3] and antisense primer [5-GCC CAA TCG GTG GTA GAA CT-3] were used to detect the p110$\gamma$ wild type allele.

### 3　Histological Analysis

For histological analysis, dorsal skin samples and tumors were fixed in 4%PFA and embedded in paraffin before sectioning according to standard protocols. Sections of 5 $\mu$m were cut and stained with H & E.

## Results and Discussion

We previously reported that keratinocyte-specific Pten-null mice exhibit wrinkled skin because of epidermal hyperplasia and hyperkeratosis, as well as ruffled, shaggy, and curly hair[14]. Histological examination revealed acanthosis, sebaceous gland hyperplasia and ac-

Table 1.  Spontaneous tumors arising on PI3K isodorms and Keratinocyte-specific Pten double mutant mice

| | $n$ | spontaneous tumors (%) | tumor types of spontaneous tumors | |
| --- | --- | --- | --- | --- |
| | | | squamous cell carcinoma (%) | adenocarcinoma (%) |
| wild type | 30 | 0 (0%) | 0 (0%) | 0 (0%) |
| K5CrePten$^{flox/+}$ | 49 | 18 (37%) | 9 (18%) | 10 (20%) |
| p85$\alpha^{+/-}$ K5CrePten$^{flox/+}$ | 25 | 11 (44%) | 6 (24%) | 7 (28%) |
| p110$\alpha^{+/-}$K5CrePten$^{flox/+}$ | 17 | 5 (29%) | 0 (0%) | 5 (29%) |
| p110$\delta^{+/D910A}$K5Pten$^{flox/+}$ | 10 | 4 (40%) | 3 (30%) | 2 (20%) |
| p110$\gamma^{-/-}$K5CrePten$^{flox/+}$ | 19 | 4 (21%) | 1 (5%) | 3 (16%) |

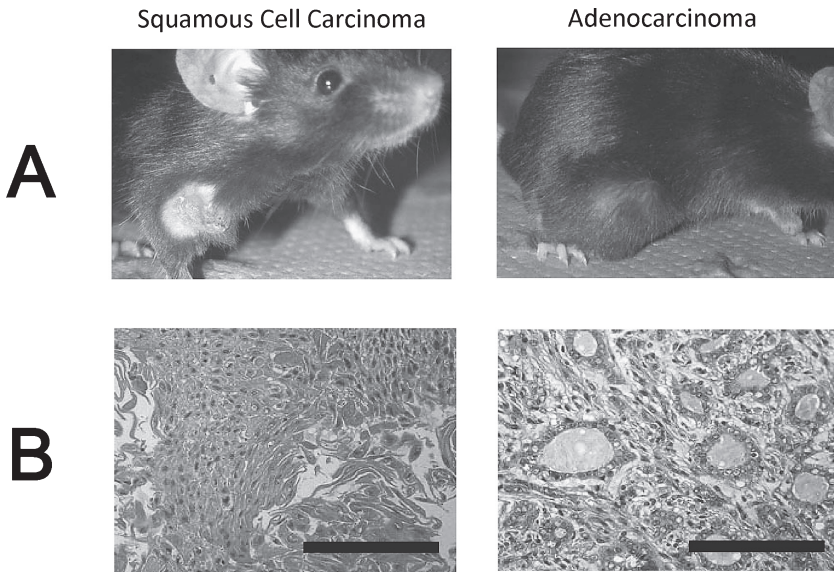Squamous Cell Carcinoma          Adenocarcinoma



Fig. 1.  (A) Spontaneous squamous cell carcinoma (left) and adenocarcinoma (right) arising on p85$\alpha^{+/-}$K5CrePten$^{flox/flox}$ mice (Arrows).  (B) H&E-stained histological sections of squamous cell carcinoma with nuclear atypia and mitosis (left) and adenocarcinoma with gland-like structure(right).  Bar, 100 $\mu$m.

celerated hair follicle morphogenesis.  Spontaneous tumors developed in 100% of K5CrePten$^{flox/flox}$ mice and 23% K5CrePten$^{flox/+}$ mice.  These tumors were papillomas, squamous cell carcinomas, and adenocarcinomas.  Tumors arising in K5CrePten$^{flox/+}$ mice arose because of a loss of heterozygosity (LOH) by PCR assays.

To characterize the effects of PI3K isoform disruption on K5CrePten$^{flox/+}$ mice carcinogenesis, PI3K and K5CrePten$^{flox/+}$ double knockout mice were monitored for spontaneous tumorigenesis.  As shown in Table 1, spontaneous tumors developed in 37% K5CrePten$^{flox/+}$ mice, 44% in p85$\alpha^{+/-}$K5CrePten$^{flox/+}$, 29% in p110$\alpha^{+/-}$K5CreP-

ten$^{flox/+}$, 40% in p110$\delta^{+/D910A}$K5CrePten$^{flox/+}$ mice, and 21% in p110$\gamma^{-/-}$K5CrePten$^{flox/+}$ mice, and there was no significant difference among these groups.  Histologically, most of these spontaneous tumors were squamous cell carcinomas with nuclear atypia and increased mitosis and adenocarcinomas with ductal structures (Fig. 1A, B).  The onset of spontaneous squamous cell carcinoma was significantly low in p110$\alpha^{+/-}$K5CrePten$^{flox/+}$ mice(0%) and p110$\gamma^{-/-}$K5CrePten$^{flox/+}$ mice(5%) compared to K5CrePten$^{flox/+}$ (18%), p85$\alpha^{+/-}$K5CrePten$^{flox/+}$(24%), and p110$\delta^{+/D910A}$K5CrePten$^{flox/+}$(30%) mice, besides there was no significant difference in adenocarconomas.  Moreover,

both squamous cell carcinoma and adenocarcinoma developed with some mice (Table 1). These results indicate that only p110$\alpha$ and p110$\gamma$ rescues spontaneous squamous cell carcinoma arising on K5CrePten$^{flox/+}$ mice, not adenocarcinomas.

PI3K subunits p85$\alpha$, p110$\alpha$, p110$\gamma$ are associated with a broad tissue distribution, whereas p110$\delta$ expression is exclusively found in leukocytes[3]. However, the role of PI3K subunits in the skin is not fully understood. Regulation of epidermal homeostasis and repair by PI3K subunits p110$\alpha$ and p110$\beta$ was reported[15]. Increased expression of the p110$\alpha$ isoform, as a consequence of gene amplification, has been reported in head and neck squamous cell carcinomas[16], as cancer-specific mutations in p110$\alpha$ have been identified in various human malignancies[17][18]. However, no mutations in any other class I isoform have been identified, but overexpression of either p110$\beta$, p110$\gamma$, and p110$\delta$ is sufficient to induce cellular transformation in chicken embryo fibroblasts, suggesting an inherent oncogenic potential of these proteins[19]. Our results indicate that heterozygous p110$\alpha$ and homozygous p110$\gamma$ deficiency sufficiently rescues development of squamous cell carcinomas but not adenocarcinomas arising on keratinocyte-specific Pten deficient mice.

This tumor type-specific difference of phenotypic rescue might be associated to the tissue specific expression of the isoforms. Because Keratin5, K6, and K14 have previously been identified in the mouse mouse mammary gland[20], the adenocarcinomas are possibly mammary glands origin. In skin keratinocytes, our results indicated the presence of class I PI3Ks p110$\alpha$ and p110$\gamma$ may confer squamous cell carcinoma tumorgenesis in Pten deficient mice. Since knockout mice for p110$\alpha$ and p110$\beta$ are early embryonic lethal, they cannot be used to further study the role of PI3K in keratinocytes[3]. However, a role of PI3K in keratinocyte biology has been suggested by previous experiments[15,21]. Our data is the first report that clearly showed the role of PI3Ks in the regulation of keratinocyte carcinogenesis using p110$\alpha^{+/-}$K5CrePten$^{flox/+}$ and p110$\gamma^{-/-}$K5CrePten$^{flox/+}$ mice.

Recently isoform-specific inhibitors have become of great interest recently because of their therapeutic potential[22]. Our data show that isoform-specific inhibitors can block the oncogenicity of class I PI3Ks. Inhibitors of p110$\alpha$, p110$\gamma$ are effective at interfering with Pten defective squamous cell carcinoma tumorgenesis and are highly selective for the targeted isoform. These observation offer promise that small, isoform-specific molecules, acting in a cellular context, will provide clinical benefits in diseases that involve deregulated PI3K.

## Acknowledgements

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