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# *Pem* renders tumor cells resistant to apoptotic cell death induced by a CD8$^+$ T cell-mediated immune response or anticancer drug treatment

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ABSTRACT

*Pem*, a member of homeobox genes, is an oncofetal gene which is preferentially expressed in reproductive tissues and in multiple tumor cell lines. However, the function of *Pem* in tumor cell lines has not been elucidated. Herein we report that the ectopic expression of *Pem* in TC-1, a human papillomavirus type 16 (HPV-16) E7-expressing surrogate cervical tumor cell line, demonstrated a significant increase in extracellular signal-regulated kinase (ERK) activity and multiple resistance to various apoptotic pressures from an E7-specific CD8$^+$ T cell-mediated immune response and anticancer drug treatment. The observed resistance to apoptotic death of the *Pem*-over-expressing TC-1 tumor cells (TC-1/*Pem*) was associated with the down-regulation of a pro-apoptotic molecule, such as BIM, and up-regulation of an anti-apoptotic molecule, such as Bcl-2 protein, which mediated ERK activation. We also observed that the intratumoral injection of an ERK inhibitor enhanced the therapeutic efficacy of E7-specific CD8$^+$ T cell adoptive transfer or anticancer drug treatment against the resistant TC-1/*Pem* tumor. This is the first evidence demonstrating an association between *Pem* and a signaling pathway, namely the ERK-mediated survival signal transduction pathway. Thus, our data indicate that activation of the ERK pathway represents a new mechanism of *Pem*-mediated multiple resistances and the present research will contribute to the development of a novel strategy in cancer therapy against *Pem*-over-expressing tumor cells.



## 1. Introduction

The *Pem* homeobox gene is an oncofetal gene which is preferentially expressed in reproductive tissues and multiple tumor cell lines [1]. Originally, the *Pem* gene was isolated from a murine T lymphoma library by differential screening [1]. The mouse *Pem* gene encodes a protein consisting of 210 amino acid residues, which is distantly related to paired-like homeodomain-containing proteins [2]. The *Pem* gene is expressed in a specific stage of embryogenesis [2]. Recently, it was found that *Pem* is the founding member of the PEPP homeobox subfamily, a small group of homeobox genes on the X chromosome that are normally expressed in reproductive tissues, such as the ovary and testis [3,4]. Despite its reproductive tissue-specific expression, *Pem* is aberrantly expressed in a wide range of tumor cell types regardless of their origin [1]. The molecular function of *Pem* in *Pem*-expressing tumor cells has not been addressed. Recently, it was reported that *Pem* physically interacts with the tumor suppressor protein, menin, and the cell division cycle 37 homolog [5,6]. Interestingly, the ectopic expression of *Pem* in reproductive

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tissues causes an increase in DNA breaks of adjacent germ cells, although *Pem* protein exhibits intracellular properties [4]. As a result of these findings, we hypothesize that *Pem* may play a role in tumor cells.

In an effort to understand the molecular function of *Pem*, we used cancer chemotherapeutic and immunotherapeutic approaches routinely established in our laboratory with TC-1, a human papillomavirus type 16 (HPV-16) E7-expressing mouse tumor cell line, surrogate cervical cancer model. In this study, we found the ectopic expression of *Pem* in TC-1 (TC-1/*Pem*) induces resistances of tumor cells to cancer therapeutic agents, such as an anticancer drug and E7-specific $CD8^+$ cytotoxic T lymphocytes (CTLs), both *in vitro* and *in vivo*. Remarkably, we found that ERK activation in TC-1/*Pem* results in a change in the balance of apoptosis-regulating molecules. This change could make TC-1/*Pem* resistant to apoptotic cell death induced by anticancer drug treatment or E7-specific CTL immune response *in vitro* and *in vivo*. Thus, our data indicate that activation of the ERK pathway represents a new mechanism of *Pem*-mediated multiple resistance of tumor cells and provides a fundamental groundwork for the development of a novel strategy in cancer therapy against the refractory tumors caused by the aberrant expression of *Pem*.

## 2. Materials and methods

### 2.1. Mice

Female C57BL/6 and nude mice, 6–8 weeks old, were purchased from Daehan Biolink (Chungbuk, Korea). All animal procedures were done in accordance with NIH guide line for the proper use and care of laboratory animals (PHS approved animal welfare assurance number, Korea University A5806-01).

### 2.2. Cell cultures

The HPV-16 E7-expressing murine tumor model, TC-1, has been described previously [7]. In brief, HPV-16 E6, E7, and the ras oncogene were used to transform primary C57BL/6 mice lung epithelial cells to generate the TC-1 cell line. These cell lines were cultured *in vitro* in RPMI 1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 2 mM non-essential amino acids, and grown at 37° with 5% $CO_2$. Additionally, Lewis lung carcinoma (LLC), CT26, WEH164, and Neuro-2a cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cell lines were cultured *in vitro* in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin/streptomycin, and grown at 37° with 5% $CO_2$.

### 2.3. Plasmid DNA constructs

For the generation of pMSCV encoding *Pem*, total RNA was isolated from LLC mouse tumor cells with TRIzol (Gibco BRL, Carlsbad, CA, USA). cDNA was synthesized from 1 μg of the total RNA by extension with oligo (dT)18 primer and SuperScript II (Gibco BRL). The *Pem* gene was amplified with PCR using cDNA as the template and the following set of primers: 5′-GGA GATCTATGGAAGC-TGAGGGTTCCAG-3′ and 5′-GGCTCGAGTCAAAATCTCGGT-GTCGCA. The amplified product was then cloned into the BglII/XhoI sites of a pMSCV vector (Invitrogen Corp., Carlsbad, CA, USA). Plasmid constructs were confirmed by DNA sequencing.

### 2.4. Chemical reagents and cell lines

API-2 (Calbiochem Corp., San Diego, CA, USA) for Akt inhibition and PD98059 (Stressgen, Ann Arbor, MI, USA) for ERK inhibition were used for inhibition of the individual kinase pathway.

The generation of the TC-1/*Pem* cell line was performed as described previously [8]. Briefly, the constructed pMSCV/*Pem* or pMSCV/no insert DNAs were transfected into the Phoenix packaging cell line, and the virus-containing supernatant was collected 48 h after transfection. The supernatant was immediately concentrated using a centrifugal filter devices (Millipore, Bedford, MA) and used to infect target cells (TC-1) in the presence of 8 μg/ml polyethylenimine (Sigma, St. Louis, MO, USA). One day after retroviral transduction, the virus supernatant was replaced with normal culture medium, and when the cells reached 70% confluency, puromycin (5 μg/ml) was used to select for cells with integrated pMSCV/*Pem*.

### 2.5. Western blot

For each experiment, a total of $5 \times 10^5$ cells were rinsed twice with ice-cold PBS, followed by addition of 0.2 ml of protein extraction solution (RIPA, 50 mM Tris Cl [pH 8.0], 150 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 0.1% sodium dodecyl sulphate, 1% Nonidet P-40, and 0.5 mM EDTA; Elpis Biotech, Daejeon, Korea). After incubation for 30 min on ice, the cells were scraped and centrifuged. Protein concentrations were determined by the Coomassie Plus protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein were solubilized in Laemmli buffer (62.5 mM Tris/HCl [pH 6.8], 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.00625% bromophenol blue), boiled for 5 min, and then separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Bioscience, Freiburg, Germany). The membranes were probed with primary antibodies for phospho Akt (Ser473), Akt, phospho ERK (T202/Y204), ERK, p38 MAPK, Bid, Bim, Bad, phospho Bad (Ser 136), dual phosphor p38 MAPK (Stressgen, Victoria, Canada), Bcl-2, Bax, and Bak (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and β-actin (Sigma, St. Louis, MO, USA) as a internal control in Tris-buffered saline (TBS)-T containing 5% BSA (Santa Cruz Biotechnology, Inc.) at 4 °C overnight and followed by three washes in TBST. The membranes were incubated with the appropriate secondary antibodies for 1 h at room temperature and washed. The immunoreactive bands were visualized by enhanced chemiluminescence reaction (ECL; Elpis Biotech).