Radio-sensitization of the Mouse Osteosarcoma Cell Line LM8 with Parthenolide, A Natural Inhibitor of NF-κB.

Introduction
The transcription factor nuclear factor κB (NF-κB) is constitutively active in the murine osteosarcoma cell line, LM8. Parthenolide (Par), a sesquiterpene lactone, has been reported to show antitumor activity through inhibition of the NF-κB DNA binding and other mechanisms. In this study, we investigated the radio-sensitizing activity of Par in vitro and in animal models by s.c. inoculation of Luc-LM8, a stable transfectant of NF-κB reporter construct into LM8.

Methods

Cell culture
Murine osteosarcoma cell line LM8 was cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin mixture in 5% CO2 at 37°C. We established Luc-LM8, a transfectant with pNF-κB-Luc (Stratagene, La Jolla, CA) into LM8, for evaluation of NF-κB activity by luciferase reporter assay in vitro and in vivo.

Animals
C3H male mice, age 5 weeks, were purchased from Japan Oriental Yeast Co., Ltd. for in vivo tumor growth assay and survival assay.

Tumorigenicity and Metastatic Potential
Luc-LM8 cells (1x10⁶) were suspended in 100 µL PBS and inoculated s.c. into the right thigh of mice. We investigated whether Luc-LM8 have an ability to form a tumor in vivo and metastatic potential to the lung.

Luciferase Assay
Luc-LM8 cells (1x10⁵) were incubated in 6-well plates with various concentrations (0, 0.5, 1.0, 2.0 µg/mL) of Par (Sigma-Aldrich, St. Louis, MO) for 24 h and luciferase activities were quantified using the Single-Luciferase Assay System (Promega, Madison, WI).

Cell Proliferation Assay
Luc-LM8 cells (1x10⁵, 96-well plates) were incubated with Par (0 and 1.0 µg/mL) for 24h, and then irradiated with 0.2,4,6 Gy, 180 kVp X rays. At 72h after irradiation, cell viability was assessed with the Premix WST-1 Cell Proliferation Assay System (TAKARA BIO, Otsu, Japan).

Apoptosis Detection Assay
Luc-LM8 cells (1x10⁵, 12-well plate) were incubated with Par (0 and 1.0 µg/mL) for 24h, and then irradiated with 0.2, 4, 6 Gy, 180 kVp X rays. At 48h after irradiation, apoptosis detection assay was done with TACS Annexin V-FITC Apoptosis Detection Kit (R&D, Minneapolis, MN).

Tumor Growth Assay
Luc-LM8 cells (1x10⁵) were inoculated s.c. into the right thigh of mice. To investigate whether Par enhances radio-sensitivity of tumor, mice were divided into four groups; Par (Par alone), RT (irradiation alone), Par+RT, and control (n= 4-5). The control group was injected i.p. with a vehicle every day starting from day 7, when tumor establishment was usually identified. Par was injected i.p. at a dosage of 2 mg/kg daily from day 7. RT with 4 Gy was given to primary tumors on day 14. All mice were sacrificed at day 28, and primary tumors were collected for tumor size evaluation and histological study.

Survival Assay
To investigate whether the combination of the continuous administration of Par and RT improve the prognosis, mice were inoculated s.c. with Luc-LM8 cells (1x10⁵) and divided into 2 groups (n=10, each). Both groups received RT (4Gy) for primary tumors on day 14 and experimental group received Par injection from day 7 (Fig. 4a).

Statistics
Data were presented as mean ± SD. For in vitro studies and tumor growth model, groups were compared by one-way ANOVA and individual groups were compared using the two-tailed Student’s t test. For the survival model, a Kaplan-Meier survival curves were used and the log-rank test was used to compare the individual groups. All analyses used a P value with a 95% confidence interval.

Results
Luc-LM8 exhibited local tumor growth ability and metastatic potential to the lung (Fig.1a). The primary tumors could be recognized by day 5 in all mice (n=24), and metastases were found in 6 mice histologically evaluated. These mean that keeps malignancy as LM8. The NF-κB transcription activity in Luc-LM8 cells was inhibited by Par in a dose-dependent manner (Fig. 1b).

Discussion
In the current study, Par suppressed Luc-LM8 cell growth, induced apoptosis in vitro, and inhibited tumor growth in vivo synergistically with irradiation treatment, suggesting that Par sensitized Luc-LM8 to irradiation. It is conceivable that the mechanism of radio-sensitization might be the inhibition of NF-κB activity because NF-κB has been shown to be associated with cancer resistance to RT. In addition, Par improved the prognosis of the mice bearing osteosarcoma treated with local RT, although the mechanism of this observation needs to be elucidated. Par might be a hopeful candidate for a potent radio-sensitizing drug for cancer radiotherapy.