Supplementary Methods

Animals

Prostate-specific *Pten* knockout mice with a C57BL/6 genetic background (*Pten*-deficient PCa mouse model [Pb-Cre+; *Pten*(fl/fl)]) were bred and maintained in specific pathogen-free cages at the Institute of Experimental Animal Sciences of Osaka University Medical School and were used as the PCa mouse models. Generation of the conditional knockout mice and genotyping were performed as previously described by our group (1). Littermate mice lacking the *Cre* transgene were treated as WT. Fecal samples from the large intestine of mice were collected during sacrifice and immediately stored at −80°C. Serum was prepared from the supernatant of blood samples collected by cardiac puncture; it was incubated at room temperature for 30 min and centrifuged for 15 min.

Cell culture and reagents

Cell lines were maintained in RPMI-1640 medium (Nacalai Tesque) supplemented with 10% fetal bovine serum, gentamicin (5 mg/L), and tylosin (60 mg/L) and cultured at 37°C in a humidified atmosphere containing 5% CO_2. Recombinant human IGF-1 (Thermo Fisher Scientific) was dissolved in PBS and added to serum-depleted medium.

Testosterone measurements

Serum of mice was assayed for testosterone using ELISA Kit for Testosterone (Testo) (Cloud-Clone Corp) according to the manufacturer's instructions. The absorbance was measured using an iMark Microplate Absorbance Reader (Bio-Rad). Serum for the measurements was prepared from the supernatant of blood samples which were incubated overnight at 4°C.

16S rRNA gene sequencing data processing

The primers targeting the V1-V2 region of 16S rRNA gene were 27Fmod (5′-AGRGTTTGATCMTGGCTCAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAGT-3′). A 251 bp paired-end sequencing was performed. The paired-end sequences were merged using PEAR (https://cme.h-its.org/exelixis/web/software/pear/). The processed sequences were clustered into operational taxonomic units, with a similarity cutoff of 97%, using UCLUST version 1.2.22q. Following this, representative sequences of each operational taxonomic unit were annotated using RDP Classifier version 2.2 with reference to the Greengenes 13_8 data base.

RNA extraction of tissue samples

Prostate tissues and hepatic tissues were frozen in RNAlater Tissue Collection: RNA Stabilization Solution (Thermo Fisher Scientific) and stored at -20°C until RNA extraction. Frozen prostate tissues and hepatic tissues were homogenized using gentle MACS tubes (Miltenyi Biotec), and RNA was isolated using RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's standard protocol. The concentration of RNA was measured by the spectrophotometric method using a BioSpec-nano (Shimadzu).

cDNA microarray analysis

The cDNA microarray analysis was conducted using prostate RNA samples on a GeneChip™ Mouse Gene 2.0 ST Array (Thermo Fisher Scientific). The arrays were scanned using the Affymetrix Gene Chip Scanner 3000, and the scanned data were processed using the Partek Genomics Suite software. The microarray data from HFD and HFD + Abx PCa mice was normalized and analyzed. The reported nucleotide sequence data are available in the GEO under the accession number GSE158524.

Histopathology and immunohistochemical (IHC) staining

Histopathology was examined via H&E staining. IHC staining was performed on 4-µm-thick sections of formalin-fixed, paraffin-embedded tissue. The slides were

deparaffinized after being heated at 68°C for 20 min. Antigen retrieval was performed by autoclaving the sample in citrate buffer, after which endogenous peroxidase activity was blocked by incubating in 3% H_2O_2. Primary antibodies diluted in Antibody Diluent, DAKO REAL (Agilent) were added to each section and incubated overnight at 4°C. A DAKO En Vision Kit (Agilent) was used for detection according to the manufacturer's instructions. Images were captured using BZ-X710 (KEYENCE) and the number of cells in the images was counted using BZ-X analyzer (KEYENCE).

Primary antibodies for IHC staining.

The following primary antibodies were used: rabbit anti-mouse Ki67 (clone: D3B5, Cell Signaling), rabbit anti-mouse and anti-human IGF-1 (ab9572, Abcam), rabbit anti-mouse phospho-IGF-1R (Tyr1161) (ab39398, Abcam), rabbit anti-mouse phospho-ERK 1/2 (Thr202/Tyr204) (clone: D13.14.4E, Cell Signaling), rabbit anti-mouse phospho-AKT (Ser473) (clone: D9E, Cell Signaling), rabbit anti-mouse phospho-MEK1/2 (Ser221) (clone: 166F8, Cell Signaling), and rabbit anti-mouse phospho-S6 Ribosomal Protein (Ser235/236) (clone: D57.2.2E, Cell Signaling).

Cell proliferation assay

PCa cell lines were seeded into 96-well plates (5000 cells/well), and reagents were added at the indicated concentrations. After culture for 48 h, the Cell Titer 96 AQueous One Solution Reagent (Promega) was added and incubated for 1 to 4 h. The absorbance was then measured with an iMark Microplate Absorbance Reader (Bio-Rad). Three independent experiments were performed, measured in triplicate.

Protein extraction for western blotting

Cultured cells were dissolved in RIPA Lysis Buffer System (Santa Cruz Biotechnology) containing Phosphatase Inhibitor Cocktail 3 DMSO solution (Sigma-Aldrich) to extract proteins. The protein concentrations of lysates were measured using the Lowry method.

The extracted proteins were stored at −80°C until western blotting was performed.

Western blotting

A total of 10 μg of protein was separated through 10% SDS-PAGE, followed by western blotting. Membranes were blocked with Blocking One (Nacalai Tesque). The membrane and primary antibodies were incubated in Tris-buffered saline overnight at 4°C. Anti-rabbit IgG, HRP-linked antibody (Cell Signaling), and Chemi-Lumi One (Nacalai Tesque) were used for detection. The ChemiDoc XRS Plus imaging system (Bio-Rad) was used to create images of the blots. Each experiment was repeated three times, independently.

Primary antibodies for western blotting.

The following primary antibodies were used: rabbit anti-mouse phospho-IGF-1R (Tyr1135/1136) (clone: 19H7, Cell Signaling), rabbit anti-mouse IGF-1R (clone: D23H3, Cell Signaling), rabbit anti-mouse phospho-ERK 1/2 (Thr202/Tyr204) (clone: D13.14.4E, Cell Signaling), rabbit anti-mouse ERK 1/2 (clone: 137F5, Cell Signaling), rabbit anti-mouse phospho-AKT (Ser473) (clone: D9E, Cell Signaling), rabbit anti-mouse AKT (clone: C67E7, Cell Signaling), and rabbit anti-mouse GAPDH (clone: 14C10, Cell Signaling).

Fecal sample preparation and LC-MS/MS settings for SCFA measurements

Fecal samples were homogenized by bead beating in 70% isopropanol (Wako), using Micro Smash MS-100 (Tomy), and were then desiccated. The fecal homogenates were diluted with ultrapure water to a concentration of 50 mg/mL and centrifuged. The clarified supernatants were stored at −80°C before being subjected to 3-nitrophenylhydrazine (TCI) derivatization using the method previously described by Liebisch G et al. (2). The derivatized samples were loaded onto an Acquity UPLC CSH C18 Column (130Å, 1.7 μm, 2.1 mm x 100 mm) (Waters) and separated using a linear

gradient with water as mobile phase A and acetonitrile as mobile phase B, both containing 0.01% formic acid. We used the following settings for the SCFAs detection: capillary voltage = 2.3kV, desolvation gas flow = 1000L/h, desolvation temperature = 400°C, source temperature = 120°C, low collision energy = 4eV, high collision energy = 40eV.

References

1. Hayashi T, Fujita K, Nojima S, Hayashi Y, Nakano K, Ishizuya Y, et al. High-fat diet-induced inflammation accelerates prostate cancer growth via IL6 signaling. *Clin Cancer Res.* 2018;24:4309–18.
2. Liebisch G, Ecker J, Roth S, Schweizer S, Öttl V, Schött HF, et al. Quantification of fecal short chain fatty acids by liquid chromatography tandem mass spectrometry—investigation of pre-analytic stability. *Biomolecules*. 2019;9:121.