A Fucoxanthinol Induces Apoptosis in a Pancreatic Intraepithelial Neoplasia Cell

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Abstract. *Background/Aim: Fucoxanthinol (FxOH), a predominant metabolite from fucoxanthin (Fx), can exert potential anti-cancer effects in various cancers. However, limited data are available on the effect of FxOH or Fx on pancreatic cancer. The present study investigated the effect of FxOH on a cell line derived from pancreatic cancer tissue developed in Ptf1a^{Cre/+}; LSL-k-ras^{G12D/+} mice. Materials and Methods: Using flow-cytometric, microarrays, and western blotting analyses, alterations in FxOH-induced apoptosis-related gene expression and protein levels were evaluated in a mice pancreatic cancer cell line, KMPC44. Results: FxOH significantly arrested the cells at S phase along with suppression of many gene sets, such as cytokine–cytokine receptor interaction and cell adhesion molecule CAMS. Moreover, attenuated protein levels for cytokine receptors, adhesion, phosphatidylinositol-3 kinase/protein kinase B, and mitogen-activated protein kinase were observed. Conclusion: FxOH may prevent pancreatic cancer development in a murine cancer model.*

Fucoxanthin (Fx) is a non-provitamin A carotenoid with a

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characteristic allene and 5,6-monoepoxide and is abundant in edible brown algae such as *Undaria pinnatifida* (wakame), *Hizikia fusiforme* (hiziki), and *Sargassum horneri* (akamoku). Fx is one of the strongest carotenoids in terms of anti-proliferative function in human cancer cells (1). Toxicity studies demonstrated that Fx is a safe compound with no adverse events in rodents (2, 3). Fucoxanthinol (FxOH) is a deacetylated type of Fx, which has undergone metabolic conversion in the intestine, and is observed in the blood of humans and mice as a primal metabolite (4, 5). Fx has polyfunctional features, such as suppression of inflammation, obesity, and diabetes in humans and rodents (6-8). In addition, Fx and FxOH have been reported to exert potential anti-cancer effects in various cancer models in rodents (9-13). Of note, FxOH suppressed the growth of cells isolated from colorectal cancer more strongly than Fx (14). To date, no epidemiological data demonstrating the suppressive effects of Fx or FxOH on cancer are available.

Pancreatic cancer is the seventh leading cause of cancer-related mortality worldwide (15). In the United States, pancreatic cancer is expected to reach the second leading cause of cancer death by 2030 (16). Its overall rate of 5-year relative survival is currently 10% (17). Due to the many alteration of genes, signal transduction pathways, immune responses, and the tumor microenvironment, patients with pancreatic cancer have a poor prognosis (18, 19). Genomic and proteomic analyses demonstrated that high frequencies of gene mutations are identified in the oncogene *KRAS* and tumor suppressor genes *CDKN2A*, *TP53*, and *SMAD4* in human pancreatic cancer, with alterations in major signal transduction pathways including KRAS, TGF-β, and Wnt signals (20-22).

Few reports are available on the effect of Fx and FxOH on pancreatic cancer. One group reported that treatment with 1 mmol/l Fx significantly suppressed cell growth in a human pancreatic cancer cell line, MIA PaCa-2 cells, although the underlying mechanisms remains unknown (23). It is assumed that Fx- and FxOH-induced cell-cycle arrest, the attenuation of many signal transduction pathways and caspase activation will be involved, because such anticancer effects of Fx and FxOH have been reported in other cancer cells (24-31).

Several reports show the molecular mechanisms of carotenoid-induced apoptosis in pancreatic cancer cells. The non-polar carotenoid lycopene can induce apoptosis in human pancreatic cancer PANC-1 cells through alterations in NF-κB signaling, Bcl-2 and Bax, and activation of caspase-3 (32). The apocarotenoid crocetinic acid increased apoptosis in MIA PaCa-2 cells through attenuation of EGFR, AKT, and Bcl-2. In addition, crocetinic acid disrupts pancreatic cancer stem cell-like pancosphere by abrogating sonic Hedgehog, Smoothened, c-Myc, and Cyclin D1, and inhibits tumorigenesis in xenograft mice by suppressing EGFR, AKT, Bcl-2, and caspase-3 activation (33). It is assumed that Fx and FxOH induce the apoptosis of pancreatic cancer cells through similar mechanisms as described above.

Ptf1a[Cre/+]; *LSL-k-ras*[G12D/+] mice are a representative pancreatic cancer murine model characterized by the development of pancreatic intraepithelial neoplasia, as observed in humans (34). In the present study, we examined the effects of FxOH on a cell line derived from pancreatic cancer tissue in a *Ptf1a*[Cre/+]; *LSL-k-ras*[G12D/+] mouse.

Materials and Methods

Chemicals. All-*trans*-FxOH (purity, ≥98%) was prepared by Dr. Hayato Maeda (Hirosaki University, Japan). RPMI-1640 medium was purchased from Wako Pure Chemicals (Osaka, Japan). The mouse pancreatic cancer KMPC44 cell line is one of the KMPC cell lines established from pancreatic tumors in *Ptf1a*[Cre/+]; *LSL-k-ras*[G12D/+] mice with C57BL/6J background by Dr. Mami Takahashi (National Cancer Center Research Institute, Japan). The *Ptf1a*[Cre/+]; *LSL-k-ras*[G12D/+] mice were obtained by crossing the Ptf1a-Cre mice [STOCK ptf1a[tm1(cre)Cvw], Mutant Mouse Regional Resource Centers (MMRRC), Bar Harbor, ME, USA] (35) with the LSL K-Ras G12D mice [B6;129-*Kras2*[tm4Tyj], National Cancer Institute (NCI) Mouse Models of Human Cancers Consortium (MMHCC), Rockville, MD, USA] (36). KMPC44 cell line was derived from a well-differentiated adenocarcinoma developed in a female *Ptf1a*[Cre/+]; *LSL-K-ras*[G12D/+] mouse at 75 weeks of age. The experimental protocol was in accordance with the guidelines for Animal Experiments in the National Cancer Center and was approved by the Institutional Ethics Review Committee for Animal Experimentation. The cells were maintained in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin. Anti-CCR1, anti-CCR4, and anti-CXCR4 antibodies were obtained from BioVision (Milpitas, CA, USA). anti-NFκB p105/p50 and p100/p52 antibodies were from EnoGene Biotech (New York, NY, USA). Anti-CXCR7 and anti-PIK3R5 antibodies were obtained from Novus

Biologicals (Littleton, CO, USA). Anti-IL10R1, anti-claudin 1, anti-β-actin, anti-Akt (pan), anti-cyclin B1, anti-phosphorylated(p)FAK [pFAK(Tyr[397])], anti-integrin αM, anti-integrin α5, anti-integrin β1, anti-NRF2, anti-Smad2, anti-pSmad2(Ser[465/467]), anti-caspase-3, and anti-VAV1 antibodies were purchased from GeneTex (Irvine, CA, USA). Anti-Cyclin D1, anti-pMEK1/2(Ser[217/221]), anti-pERK1/2(Thr[202]/Tyr[204]), anti-pSTAT1(Tyr[701]), anti-pSTAT2(Tyr[690]), anti-pSTAT3(Tyr[705]), anti-pSTAT3(Ser[727]), anti-pSTAT5(Tyr[694]), anti-pSTAT6(Tyr[641]), anti-pAkt(Ser[473]), anti-pAkt(Thr[308]), and anti-PPARγ antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-CCR7, anti-NCAM2, anti-integrin β2, and anti-cyclin D2 antibodies were purchased from Bioss Antibodies (Beijing, PR China). Anti-pPaxillin (Tyr[31]) and anti-p53 antibodies were from Novex (San Diego, CA, USA) and Thermo Scientific (Waltham, MA, USA), respectively. All other reagents in the present study were of analytical grade.

Animal experiments. Four female C57BL/6JJmsSlc mice (5 weeks old) were purchased from Sankyo Labo Service (Tokyo, Japan) and were randomly placed with 2 mice per cage. Mice were fed solid food (Grade: MF, Oriental Yeast Co. Ltd.) and water *ad libitum* and were kept at a controlled temperature, humidity, and in a 12:12 h light/dark cycle. After a week of acclimation, mice were anesthetized with isoflurane. KMPC44 cells were dissociated into a single-cell suspension and inoculated into the pancreases of all mice at 1×10^6 cells/50 µl Hank's Balanced Salt solution using a 25 gauge 1 ml disposable syringe with laparotomy. The open abdominal part in mice was immediately closed using a surgical clip. After 3 weeks, tumor onset in the pancreas tissue was observed with laparotomy. The experiments were conducted in accordance with the current guidelines for animal experiments in the Health Sciences University of Hokkaido that were reviewed by the Committee of Animal Experimentation in the Health Sciences University of Hokkaido.

Cell viability assay. KMPC 44 cells were seeded at a density of 5×10^4 cells/ml in 24-well plates in 10% FBS/RPMI-1640 medium and adhered for 3.5 h. Cells were then exposed to 1% FBS/RPMI-1640 medium with FxOH (final concentrations, 1 and 5 µM and incubated for 2 days. Unexposed control cells were treated with vehicle (DMSO) only. Cell viability was monitored at 450 nm using an ELISA microplate reader (TECAN Japan, Tokyo, Japan) by adding WST-1 reagent.

Cell cycle analysis. KMPC44 cells were seeded at a density of 5×10^4 cells/ml in 10-cm plates in 10% FBS/RPMI-1640 medium and adhered for 3.5 h. Cells were then exchanged into 1% FBS/ RPMI-1640 medium with FxOH (final concentration, 5.0 µM) or vehicle (DMSO) only and incubated for 2 days. Cells were dissociated into a single-cell suspension, washed with phosphate-buffered saline (PBS) and prepared with 70% ethanol and subsequently ribonuclease A (Nacalai Tesque, Kyoto, Japan). Cells were stained with propidium iodide (Sigma-Aldrich, St Louis, MO, USA) and mixed with 0.1% bovine serum albumin (BSA)/PBS. The percentage of apoptosis-like micronuclei (Sub-G_1) and in each phase of the cell cycle were measured using a FACSAria-III flow cytometer (BD Biosciences).

Total RNA extraction and purification. Total RNA in KMPC44 cells with or without FxOH treatment was prepared using an RNeasy Mini Kit and RNase-Free DNase Set and QIA shredder (QIAGEN, Valencia, CA, USA), in accordance with the

manufacturers' instructions. The concentration and quantification of total RNA were determined using a Nanodrop® ND-1000 (NanoDrop, Wilmington, DE, USA) and agarose gel electrophoresis, respectively.

Microarray analysis. Affymetrix GeneChip Whole Transcript Expression Arrays were used for transcriptome analyses of all samples. In brief, total RNA (500 ng) was subjected to first-strand and subsequent second-strand complementary DNA (cDNA) syntheses. The single-strand cRNA transcripts were prepared from cDNA libraries using an *in vitro* transcription protocol. Single-strand cDNA templates were synthesized from the cRNA and labeled with biotin using a GeneChip hybridization, wash, and stain kit (Applied Biosystems, Foster City, CA). The biotin-labeled cDNA was hybridized to a Clariom™ S mouse array (Thermo Fisher Scientific, Carlsbad, CA, USA). The microarrays were then scanned using an Affymetrix GeneChip Scanner 3000 7G system. Data were analyzed using Transcriptome Analysis Console (TAC) software version 4.0.2 (Applied Biosystems, Foster City, CA). The differentially expressed genes between KMPC44 cells with or without FxOH were identified using ≥2.0 and ≤-2.0-fold with cutoff *p*-value [one-way analysis of variance (ANOVA), *p*<0.05]. Gene expression profiles were shown using principal coordinate analysis (PCoA), volcano-plots, and hierarchy clustering heatmap. Functional interpretation of pathway-based gene sets was performed using gene set enrichment analysis (GSEA) software ver. 4.0.3 (Broad Institute of Harvard University and Massachusetts Institute of Technology, MA, USA) (37, 38). The databases of the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used in bioinformatics analyses (Bioinformatics Center, Institute for Chemical Research, Kyoto University, Japan; Human Genome Center, Institute of Medical Science, University of Tokyo, Japan).

Western blotting. KMPC44 cells with or without FxOH treatment were collected and lysed, and the resulting protein concentrations were measured using the Bradford assay. The isolated proteins (10 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) and then electroblotted onto Hybond PVDF membranes (Amersham Bioscience, Little Chalfont, UK). The PVDF membrane was blocked using Tris-buffer saline containing 0.1% Tween 20 with 1% BSA (1% BSA/TBS-T) at room temperature for 1 h, followed by incubation with each primary antibody in 1% BSA/TBS-T at 4°C overnight. The membranes were probed with HRP-conjugated anti-mouse or anti-rabbit secondary antibodies in 1% BSA/TBS-T at room temperature for 1 h. Protein bands were visualized using a luminol-enhanced chemiluminescence assay (Millipore, Billerica, MA, USA).

Statistics analysis. Mean±standard error (SE) are presented for all values. Statistical tests were performed using the Student's *t*-test between two groups, and ANOVA on differentially gene expressions, and one-way ANOVA with Tukey–Kramer post hoc tests on multiple comparisons. Differences were considered statistically significant at *p<0.05, **p<0.01 or exact *p*-value.

Results

Effect of FxOH on cell growth in pancreatic cancer KMPC44 cells. After C57BL/6JJmsSlc mice were inoculated with a cell suspension of KMPC44 cells (1×10^6 cells), 100%

of the mice developed pancreatic tumors within a month (Figure 1A). Five μM FxOH treatments for 2 days resulted in a significant decrease in cell growth and cell elongation of KMPC44 cells (Figure 1B and C). Their growth (×10^4 cells/well) after incubation for 2 days was as follows: control, 36.8±1.5; 1.0 μM FxOH, 34.5±2.1; 5.0 μM FxOH, 18.2±2.8. Treatment with 5.0 μM FxOH significantly increased apoptosis-like Sub-G_1 cells (control cells, 11.1±0.3% and FxOH-treated cells, 16.8±1.2%) and decreased S phase cells (control cells, 29.9±0.4% and FxOH-treated cells, 24.7±0.4%) in KMPC44 cells (Figure 1D).

Effect of FxOH on the transcriptome in KMPC44 cells. Transcriptome changes were evaluated in KMPC44 cells treated with 5 μM FxOH for 1 day. As a result, the PCoA plot indicated that a distinct genetic distribution existed between the KMPC44 cells with and without FxOH treatment (Figure 2A). Hierarchical clustering heatmaps on 4,000 of 8,515 genes showed differences in the genes between the two groups (Figure 2B). Volcano plots revealed that both the gene fold-change and *p*-values of up-regulated genes were higher than down-regulated genes (Figure 2C). There were 5,708 up-regulated and 2,807 down-regulated genes (total 8,515 genes) in FxOH-treated KMPC44 cells compared to control cells (Figure 2D). Pathway analysis showed that 26 of the top 50 pathways were gene groups associated with growth and inflammation as follows: adhesion, adipogenesis, apoptosis, immune response, chemokine, cytoskeleton, epidermal growth factor receptor (EGFR), G-protein-coupled receptor (GPCR), integrin, interleukin, mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), phosphatidylinositol-3 kinase/protein kinase B (PI3K/AKT), signal transducers and activators of transcription (STAT), transforming growth factor beta (TGF-β), Toll-like receptor, and wingless/integrated (Wnt) signals (Figure 2E, black circle).

In total, 78 up-regulated and 7 down-regulated genes in KMPC44 cells after 5 μM FxOH treatment were involved in more than 5 gene sets based on TAC analysis. Most of genes belonged to adhesion, apoptosis, immune response, cell cycle, EGFR, GPCR, integrin, Jun, MAPK, NF-κB, PI3K/AKT, protein kinase C (PKC), Ras, STAT, TGF-β, and Wnt signals (Tables I and II).

Among 8,515 genes significantly altered in KMPC44 cells with 5 μM FxOH treatment, GSEA revealed that 5 signal pathways (cancer cell growth, cytokine–cytokine receptor interaction, cell adhesion molecules CAMS, ECM receptor interaction, calcium signaling pathway, and chemokine signaling pathway) were significantly enriched compared to untreated control cells (Table III and Figure 3). However, no significant enrichment in cancer cell growth was observed in FxOH-treated KMPC44 cells. Furthermore, genes belonging to the cytokine–cytokine receptor interaction and cell adhesion molecules CAMS in GSEA were plotted using
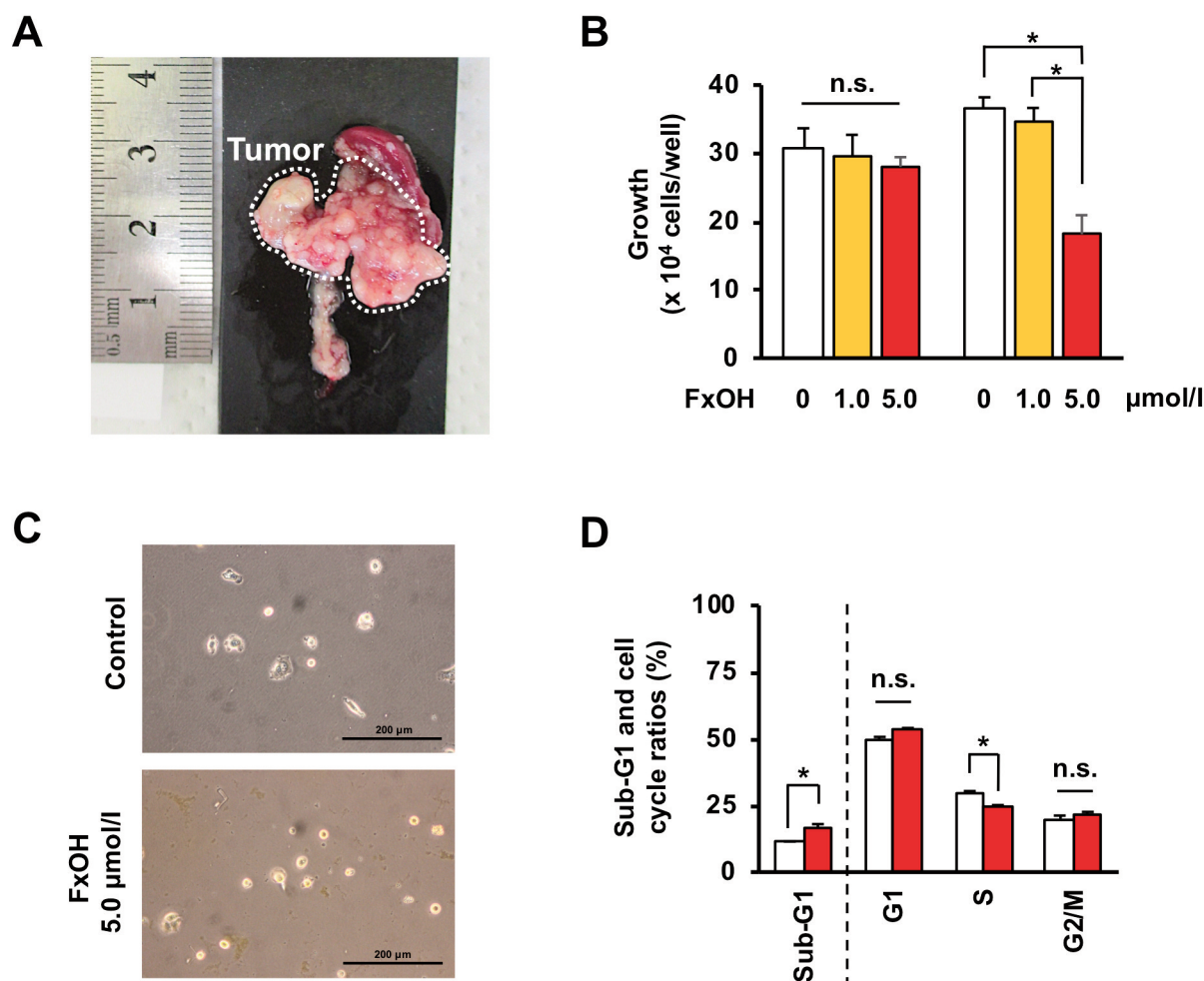
Figure. 1. *Effects of FxOH on tumorigenicity, growth, and apoptosis induction in pancreatic cancer cells derived from Ptfla$^{Cre/+}$: LSL-K-ras$^{G12D/+}$ mice. (A) A representative pancreatic tumor produced by injection of KMPC44 cells. KMPC44 cells were dissociated into a single-cell suspension and injected into the pancreases of C57BL/6JJmsSlc mice at 1×10^6 cells/50 μl Hanks's balanced salt solution. After 1 month, the mice were sacrificed and the tumors were photographed. (B-D) KMPC44 cells were treated with (B) 1.0 and 5.0 μM of fucoxanthinol (FxOH) for 1 day (left panel) and 2 days (right panel). (B) Cell viability was measured using the absorbance from a WST-1 assay. Means±SE (n=6). (C and D) 5.0 μM of FxOH for 2 days. (C) Images of KMPC44 cells were taken using a phase-contrast microscope. Bars are 200 μm. (D) Apoptotic-like cells (sub-G$_1$) and cell cycle profiles (G$_1$, S and G$_2$/M) in FxOH-treated and control KMPC44 cells are shown. The data were obtained using a FACSaria-III flow cytometer. Means±SE (n=3). *p<0.05. n.s., No significance.*

databases of the Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis (Figure 4).

Effect of FxOH on protein expression in KMPC44 cells. Based on the information obtained from the cell viability assay, cell-cycle analysis, and bioinformatics applications, the effect of FxOH on protein expression levels and activation were evaluated in KMPC44 cells. FxOH treatment attenuated CCR1, CCR4, NCAM2, pFAK(Tyr^{397}), pPaxillin(Tyr^{31}), cyclin D1, cyclin B1, pAKT(Ser^{473}), pAKT(Thr^{308}), pMEK1/2(Ser$^{217/221}$), and pERK1/2(Thr^{202}/Tyr^{204}) in KMPC44 cells. The active form of caspase-3, cleaved caspase-3 (p17/p19), was enhanced in

KMPC44 cells by FxOH treatment but not the precursor of pro-caspase-3. In addition, cytokine and chemokine (4 proteins), adhesion (5 proteins), PI3K/AKT (2 proteins), and STAT (6 proteins) signals, cyclin D2, NF-κB (4 proteins), TGF-β (2 proteins), PPARγ, p53, and VAV1, were almost unchanged or were not detected (Figure 5).

Discussion

Our results demonstrated that FxOH induced apoptosis in KMPC44 cells through alteration of the expression levels of many genes and proteins regarding cancer-associated signals.
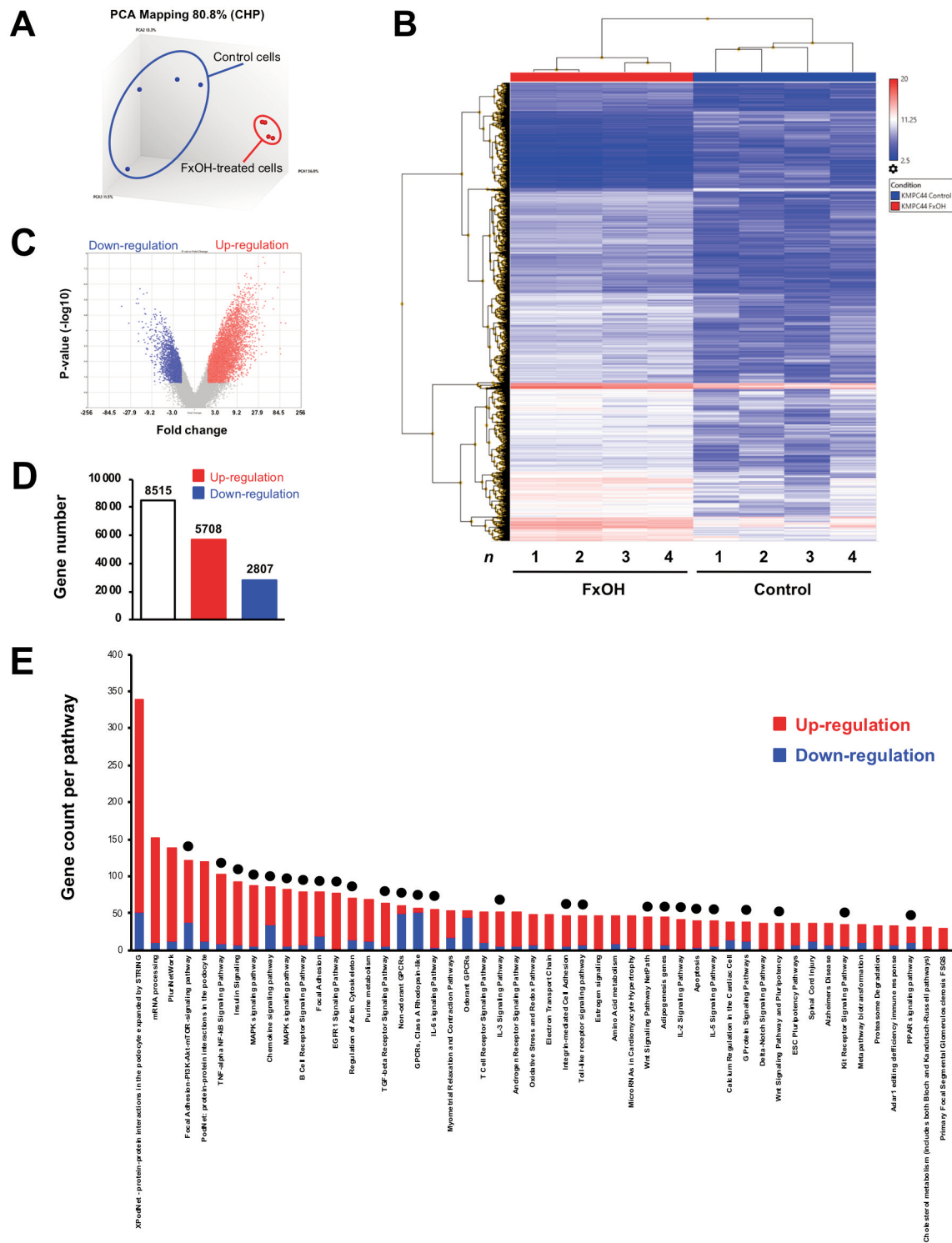
Figure 2. *Gene expression profiles in KMPC44 cells with or without fucoxanthinol (FxOH) treatment. KMPC44 cells were treated with 5 µM FxOH for 1 day. Gene expression levels between FxOH-treated KMPC44 cells and control cells were subjected to Clariom S human arrays and TAC software (n=4). Significant differences in gene expression levels, ≥2.0 and ≤-2.0 -fold with cutoff p-value (p<0.05), FxOH-treated KMPC44 cells and control cells are shown using the average value in quadruplicate. (A) PCoA plots on gene distance between the two groups. (B) Hierarchical clustering analysis for 8,515 genes between the two groups. (C) Volcano plots between the two groups. (D) Total number of up- (≥2.0-fold), and down-regulated (≤-2.0-fold) genes between the two groups. (E) The distribution of the top 50 gene sets altered between the two groups. Red, up-regulated genes. Blue, down-regulated genes. Black circle, gene sets regarding growth and inflammation.*

Table I. *Pathway-based profiles of up-regulated genes in KMPC44 cells treated with fucoxanthinol (FxOH)[a].*

Gene symbol	Description	Fold[b]	*p*-Value[c]	Pathway number[d]
Ikbkg	Inhibitor of kappaB kinase gamma	32.1	0.004	13
Araf	v-Raf murine sarcoma 3611 viral oncogene homolog	27.89	0.0123	7
Pik3r1	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	17.28	0.0061	23
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105	14.2	0.0032	28
Ppp3ca	Protein phosphatase 3, catalytic subunit, alpha isoform	13.02	0.0053	12
Rap1a	RAS-related protein-1a	12.75	3.80E-05	8
Rock2	Rho-associated coiled-coil containing protein kinase 2	11.98	0.0078	9
Rock1	Rho-associated coiled-coil containing protein kinase 1	11.91	0.0013	8
Chuk	Conserved helix-loop-helix ubiquitous kinase	11.9	0.0012	12
Mapk7	Mitogen-activated protein kinase 7	11.67	0.0006	11
Mapk14	Mitogen-activated protein kinase 14	11.42	3.59E-05	19
Rasa1	RAS p21 protein activator 1	11.35	0.0003	10
Map3k1	Mitogen-activated protein kinase kinase kinase 1	10.75	0.0004	11
Tgfb1	Transforming growth factor, beta 1	10.74	0.0367	16
Irs1	Insulin receptor substrate 1	10.64	0.0002	13
Map2k7	Mitogen-activated protein kinase kinase 7	10.4	0.0003	9
Bad	BCL2-associated agonist of cell death	10.34	0.0003	15
Pik3ca	Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide	9.97	0.0005	20
Ptpn11	Protein tyrosine phosphatase, non-receptor type 11	9.36	0.0016	18
Foxo1	Forkhead box O1	8.27	0.0004	10
Ep300	E1A binding protein p300	8.17	0.0027	13
Map3k7	Mitogen-activated protein kinase kinase kinase 7	7.86	0.0014	14
Map2k2	Mitogen-activated protein kinase kinase 2	7.8	0.007	24
Casp8	Caspase 8	7.39	0.0005	11
Crebbp	CREB binding protein	6.92	0.0063	12
Map2k6	Mitogen-activated protein kinase kinase 6	6.53	0.0141	13
Jun	Jun proto-oncogene	5.94	0.0002	26
Map2k4	Mitogen-activated protein kinase 4	5.52	0.0141	9
Prkcd	Protein kinase C, delta	5.49	0.0061	17
Bcar1	Breast cancer anti-estrogen resistance 1	5.49	0.0181	8
Akt3	Thymoma viral proto-oncogene 3	5.25	0.0121	12
Stat3	Signal transducer and activator of transcription 3	5.07	0.0007	24
Map2k1	Mitogen-activated protein kinase kinase 1	5.04	0.0035	31
Prkaca	Protein kinase, cAMP dependent, catalytic, alpha	5.01	0.0061	12
Vav2	Vav 2 oncogene	4.94	0.0009	9
Lamb2	Laminin, beta 2	4.89	0.0004	7
Ptpn6	Protein tyrosine phosphatase, non-receptor type 6	4.86	0.007	10
Csk	c-Src tyrosine kinase	4.79	0.0015	7
Pxn	Paxillin	4.79	0.0018	12
Shc1	Src homology 2 domain-containing transforming protein C1	4.63	0.0401	21
Traf6	TNF receptor-associated factor 6	4.62	0.029	12
Ikbkb	Inhibitor of kappaB kinase beta	4.6	0.0024	14
Pik3cb	Phosphatidylinositol 3-kinase, catalytic, beta polypeptide	4.5	0.0004	15
Sos1	Son of sevenless homolog 1 (Drosophila)	4.46	0.0123	23
Ptk2	PTK2 protein tyrosine kinase 2	4.35	0.0309	17
Cbl	Casitas B-lineage lymphoma	4.18	0.0161	13
Mapk1	Mitogen-activated protein kinase 1	4.15	0.0145	39
Map3k14	Mitogen-activated protein kinase 14	4	0.0037	7
Eif4e	Eukaryotic translation initiation factor 4E	3.97	0.0139	8
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha	3.92	0.0037	8
Itgb1	Integrin beta 1 (fibronectin receptor beta)	3.9	0.0097	9
Akt1	Thymoma viral proto-oncogene 1	3.89	0.0014	35
Map2k3	Mitogen-activated protein kinase 3	3.82	0.0143	9
Rela	v-Rel reticuloendotheliosis viral oncogene homolog A (avian)	3.76	0.0041	13
Arrb2	Arrestin, beta 2	3.75	0.0084	8
Tnfrsf1b	Tumor necrosis factor receptor superfamily, member 1b	3.71	0.0015	8
Stat1	Signal transducer and activator of transcription 1	3.65	0.0445	22
Hdac1	Histone deacetylase 1	3.65	0.0277	12

Table I. *Continued*

Gene symbol	Description	Fold[b]	*p*-Value[c]	Pathway number[d]
Trp53	Transformation related protein 53	3.63	0.0211	15
Rb1	Retinoblastoma 1	3.49	0.0025	12
Cdk2	Cyclin-dependent kinase 2	3.48	0.0055	9
Lyn	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog	3.4	0.0019	11
Casp3	Caspase 3	3.31	0.007	16
Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	3.01	0.0172	13
Ptk2b	PTK2 protein tyrosine kinase 2 beta	2.99	0.0023	10
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	2.96	0.0181	13
Ccnd2	Cyclin D2	2.83	0.0473	12
Fzd7	Frizzled homolog 7 (Drosophila)	2.79	0.0294	7
Jak1	Janus kinase 1	2.75	0.0176	13
Dvl1	Dishevelled, dsh homolog 1 (Drosophila)	2.74	0.0074	12
Rapgef1	Rap guanine nucleotide exchange factor (GEF) 1	2.65	0.008	8
Itgb3	Integrin beta 3	2.6	0.0478	7
Casp7	Caspase 7	2.46	0.0066	10
Bcl2	B cell leukemia/lymphoma 2	2.39	0.0088	12
Bmp4	Bone morphogenetic protein 4	2.38	0.0312	9
Atf2	Activating transcription factor 2	2.18	0.0137	12
Jak3	Janus kinase 3	2.15	0.0238	7
Egfr	Epidermal growth factor receptor	2.02	0.015	11

[a]Among all 8,515 genes with significant alterations, 78 up-regulated genes containing more than 5 gene sets classified by TAC software are exhibited. [b]Fold change of gene expression in FxOH-treated KMPC44 cells in comparison with that of control cells. [c]Significant difference between KMPC44 cells with and without FxOH treatment using one-way ANOVA (n=3). [d]Number of pathways including the gene.

Table II. *Pathway-based profiles on down-regulated genes in KMPC44 cells treated with fucoxanthinol (FxOH)[a].*

Gene symbol	Description	Fold[b]	*p*-Value[c]	Pathway number[d]
Lck	Lymphocyte protein tyrosine kinase; microRNA 8119	−2.27	0.0374	7
Prkcb	Protein kinase C, beta	−2.93	0.0103	15
Mapk4	Mitogen-activated protein kinase 4	−3.09	0.0148	9
Vav1	Vav 1 oncogene	−3.15	0.0056	12
Mapk12	Mitogen-activated protein kinase 12	−4.5	0.0325	11
Ifnb1	Interferon beta 1, fibroblast	−5.12	0.0074	7
Pik3r5	Phosphoinositide-3-kinase, regulatory subunit 5, p101	−21.44	0.0003	8

[a]Among all 8,515 genes with significant alterations, 7 down-regulated genes containing more than 5 gene sets classified by TAC software are shown. [b]Fold change in gene expression in FxOH-treated KMPC44 cells compared with control cells. [c]Significant difference between KMPC44 cells with and without FxOH treatment using one-way ANOVA (n=3). [d]Number of pathways including the gene.

This is the first report suggesting a direct effect of FxOH in a cell line derived from a pancreatic cancer murine model. It has been demonstrated that Fx and FxOH showed a remarkable anti-cancer activity against cancer cells from various organs, *i.e.* human breast cancer, cervical cancer, colon cancer, gastric cancer, lung cancer, prostate cancer, ovarian cancer, glioblastoma, osteosarcoma, and promyelocytic leukemia cells (1, 24, 25, 27-31). It is noteworthy that FxOH significantly suppressed the growth of cells isolated from colorectal cancer specimens (14). These reports led us to speculate that FxOH can induce apoptosis in a cancer cell line derived from pancreatic cancer tissue of rodent models. Firstly, we confirmed that high tumorigenicity (4/4 mice, 100%) of KMPC44 cells derived from well-differentiated pancreatic adenocarcinoma in *Ptf1a*[Cre/+]; *LSL-k-ras*[G12D/+] mice (Figure 1A). As expected, treatment with 5 μM FxOH significantly attenuated cell growth in the S phase and induced apoptosis in KMPC44 cells (Figure 1B and D).

Gene count analyses of the top 50 pathways based on TAC analysis showed that adhesion, adipogenesis, apoptosis, immune response, chemokine, cytoskeleton, EGFR, GPCR,

Table III. *GSEA analysis in gene sets of KMPC44 cells treated with fucoxanthinol (FxOH)[a].*

KEGG pathway[b]	Size[c]	NES[d]	NOM[e] p-Value	FDR[f] q-value
Enriched gene sets in control cells				
Olfactory transduction	40	4.54	0.000	0.000
Neuroactive ligand receptor interaction	57	4.45	0.000	0.000
Systemic lupus erythematosus	22	3.53	0.000	0.000
Cytokine-cytokine receptor interaction	67	3.17	0.000	0.000
Cell adhesion molecules CAMS	34	2.66	0.000	0.000
ECM receptor interaction	24	2.53	0.000	0.000
Calcium signaling pathway	55	2.29	0.000	0.003
Chemokine signaling pathway	85	2.06	0.000	0.013
Hematopoietic cell lineage	20	2.06	0.000	0.012
Arachidonic acid metabolism	17	2.04	0.000	0.012
Antigen processing and presentation	18	2.03	0.000	0.011
Enriched gene sets in FxOH-treated cells				
Epithelial cell signaling in *Helicobacter pylori* infection	38	2.38	0.000	0.003
Amino sugar and nucleotide sugar metabolism	26	2.29	0.000	0.004
Fructose and mannose metabolism	20	2.26	0.000	0.004

[a]The gene set enrichment in significantly different 8,515 genes between FxOH-treated KMPC44 cells and the control cells were evaluated by GSEA 4.0.3 (Broad Institute of Harvard University and Massachusetts Institute of Technology, MA, USA) with a database (Human_ NCBI_Entrez_Gene_ID_MSigDB.v7.1.chip). [b]The gene sets are from a database [c2.cp.kegg.v7.1.symbols.gmt (Curated)]. [c]Number of gene. [d]NES, normalized enrichment score. [e]NOM, nominal. [f]FDR, false discovery rate.

integrin, interleukin, MAPK, NF-κB, PI3K/AKT, STAT, TGF-β, Toll-like receptor, and Wnt signals were detected as core gene sets altered in KMPC44 cells by Fx treatment (Figure 2E, black circle). Then, we defined the genes that belonged to more than 5 pathways as "higher contributory genes", and examined them. As a result, the 78 genes were up-regulated and 7 were down-regulated, and these were related to adhesion, apoptosis, immune response, cell cycle, EGFR, GPCR, integrin, Jun, MAPK, NF-κB, PI3K/AKT, PKC, Ras, STAT, TGF-β, and Wnt signals (Tables I and II). Based on the change in these gene sets, we decided to confirm the alterations of some proteins (30 molecules) related to adhesion, cell cycle, PI3K/AKT, MAPK, STAT, NF-κB, TGF-β signals, and adipogenesis, apoptosis, and immune response using western blotting analysis.

In KMPC44 cells treated with FxOH for 1 day, the number of up-regulated genes was 2-fold higher than that of down-regulated genes (Figure 2D). However, GSEA analysis showed that the five gene sets significantly altered by FxOH treatment contained many down-regulated genes (Table III, Figure 3). Moreover, the plots of cytokine–cytokine receptor interactions and cell adhesion molecular CAMS biased by the KEGG pathway exhibited suppression tendencies in their gene sets (Figure 4). Based on the findings from the KEGG map, we decided to evaluate the protein expression levels of CC motif chemokine receptor 1 (CCR1), CCR4, CCR7, CXC motif chemokine receptor type 4 (CXCR4), CXCR7, IL10R1, claudin 1, neural cell adhesion molecules 2 (NCAM2),

integrin αM and integrin β2 by western blotting analysis. Western blotting analysis showed that the CCR1, CCR4, NCAM2, pFAK(Tyr[397]), pPaxillin(Tyr[31]), cyclin D1, cyclin B1, pAKT(Ser[473]), pAKT(Thr[308]), pMEK1/2(Ser[217/221]) and pERK1/2(Thr[202]/Tyr[204]) were decreased, and cleaved caspase-3 (p17/p19) levels were increased in FxOH-treated KMPC44 cells (Figure 5).

CCR1 and CCR4, members of the CC motif chemokine receptors, are G-protein-coupled transmembrane receptors involved in cell growth and metastasis (39). CCR1 is closely associated with enhancements of AKT, MAPK, and NF-κB signals in cancer cells and in immune cells (39-41). CCR4 potentiates the recruitment of immune cells, angiogenesis, epithelial–mesenchymal transition and activate both AKT and MAPK signals in cancer cells (39, 42, 43). NCAMs are transmembrane adhesion molecules and multifunctional proteins which can activate MAPK and NF-κB signals and phosphorylate cyclic adenosine monophosphate response element-binding protein (44). NCAM2 was overexpressed in some cell lines of prostate and liver cancer (45). In the present study, it was considered that the attenuations of AKT and MAPK signals in KMPC44 cells with FxOH treatment might be induced by the suppression of CCR1, CCR4, and NCAM2. However, there is little information on the effect on CCR1, CCR4, and NCAM2 proteins in cancer cells by treatment of Fx, FxOH, and other natural compounds.

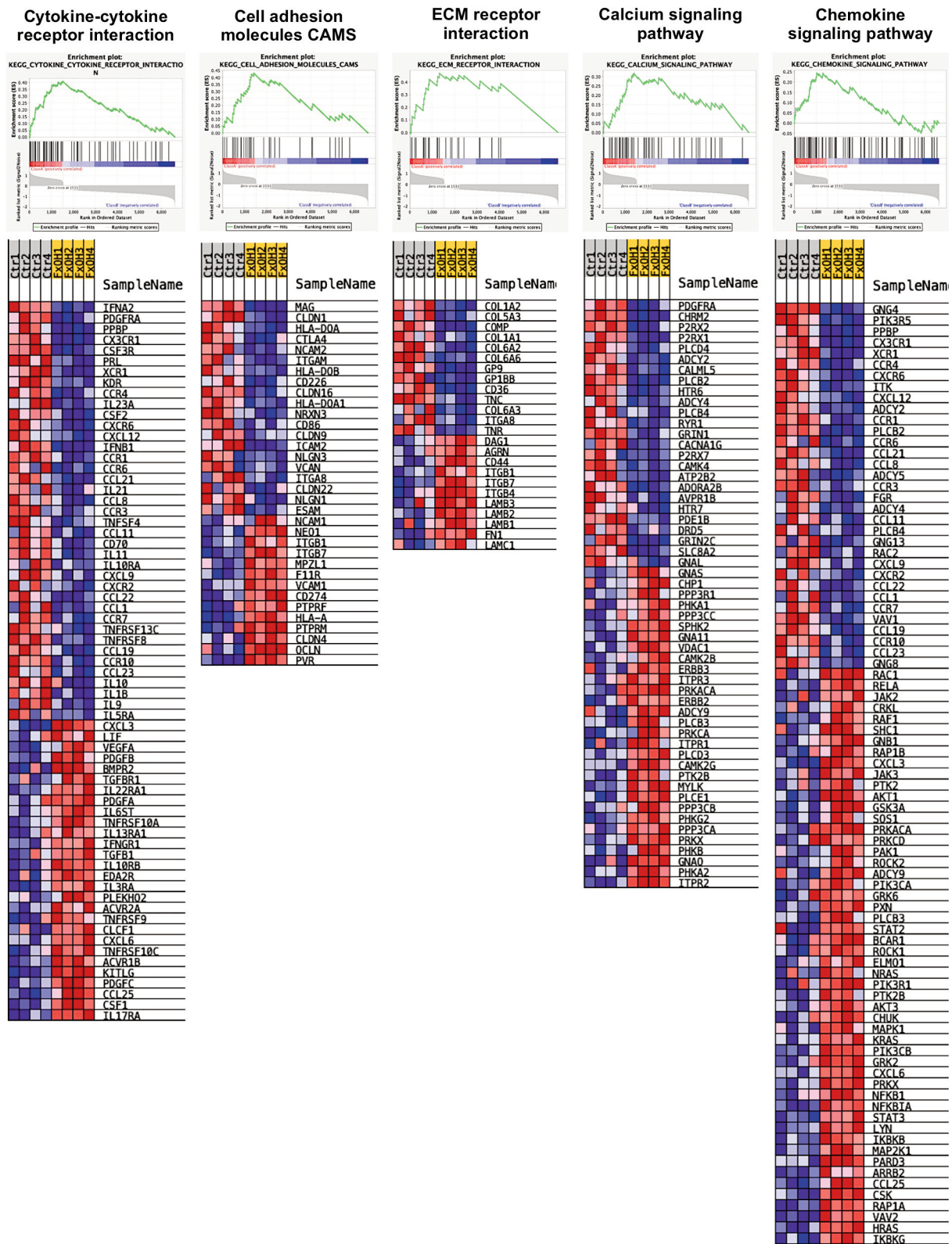pFAK(Tyr[397]) and pPaxillin(Tyr[31]), down-stream molecules of integrins, were decreased by FxOH (Figure 5).

Figure 3. *Gene enrichment profiles in KMPC44 cells with or without fucoxanthinol (FxOH) treatment. Significant differences in gene expression levels based on TAC software between FxOH-treated KMPC44 cells and control cells were analyzed using GSEA software. Upper panels show GSEA enrichment diagrams between the two groups. Lower panels show heatmaps of gene sets between the two groups.*
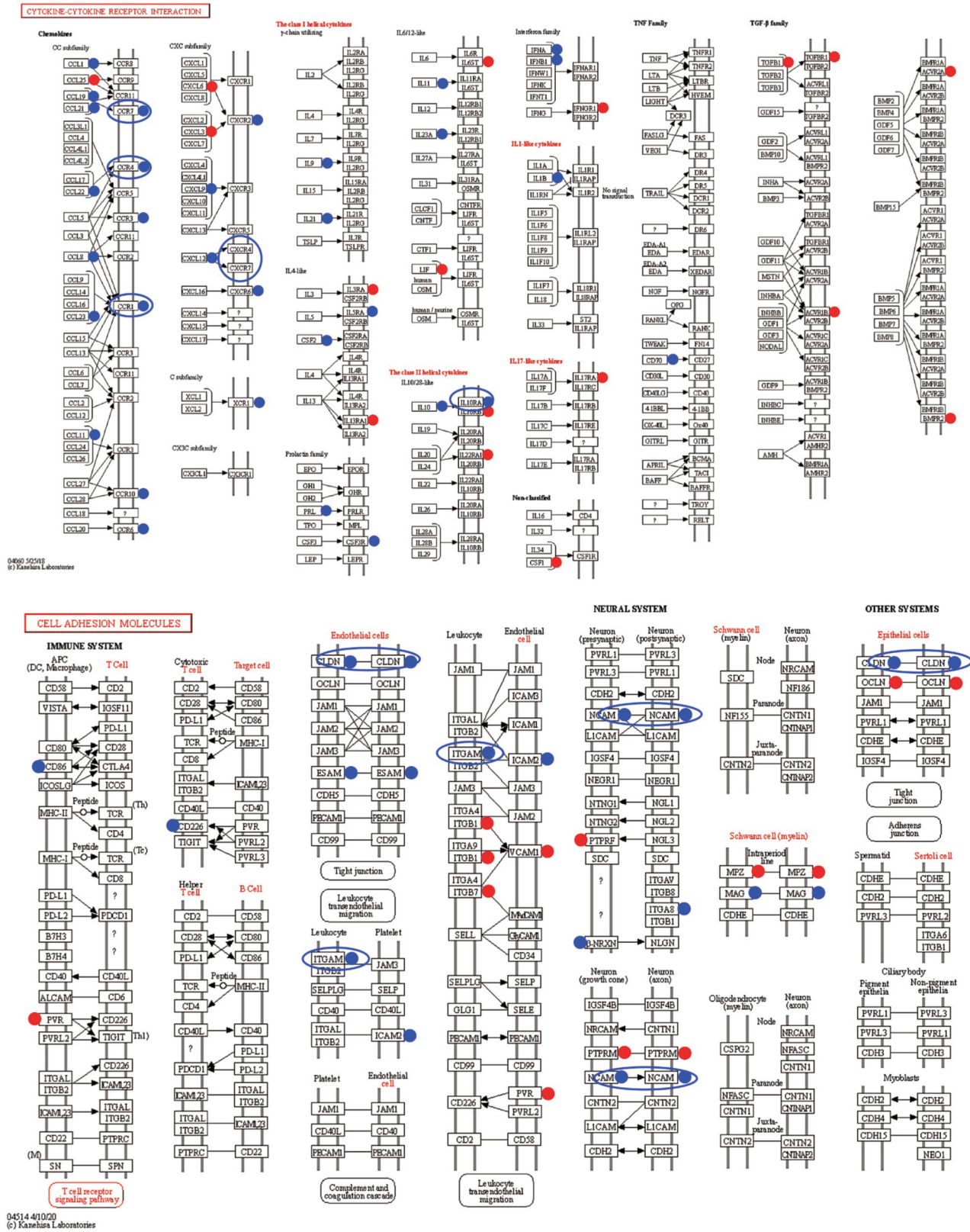
Figure 4. *KEGG pathway representing gene profiles of cytokine–cytokine receptor interaction and cell adhesion molecules based on GSEA results. Red and blue circles show up-regulated and down-regulated genes, respectively.*
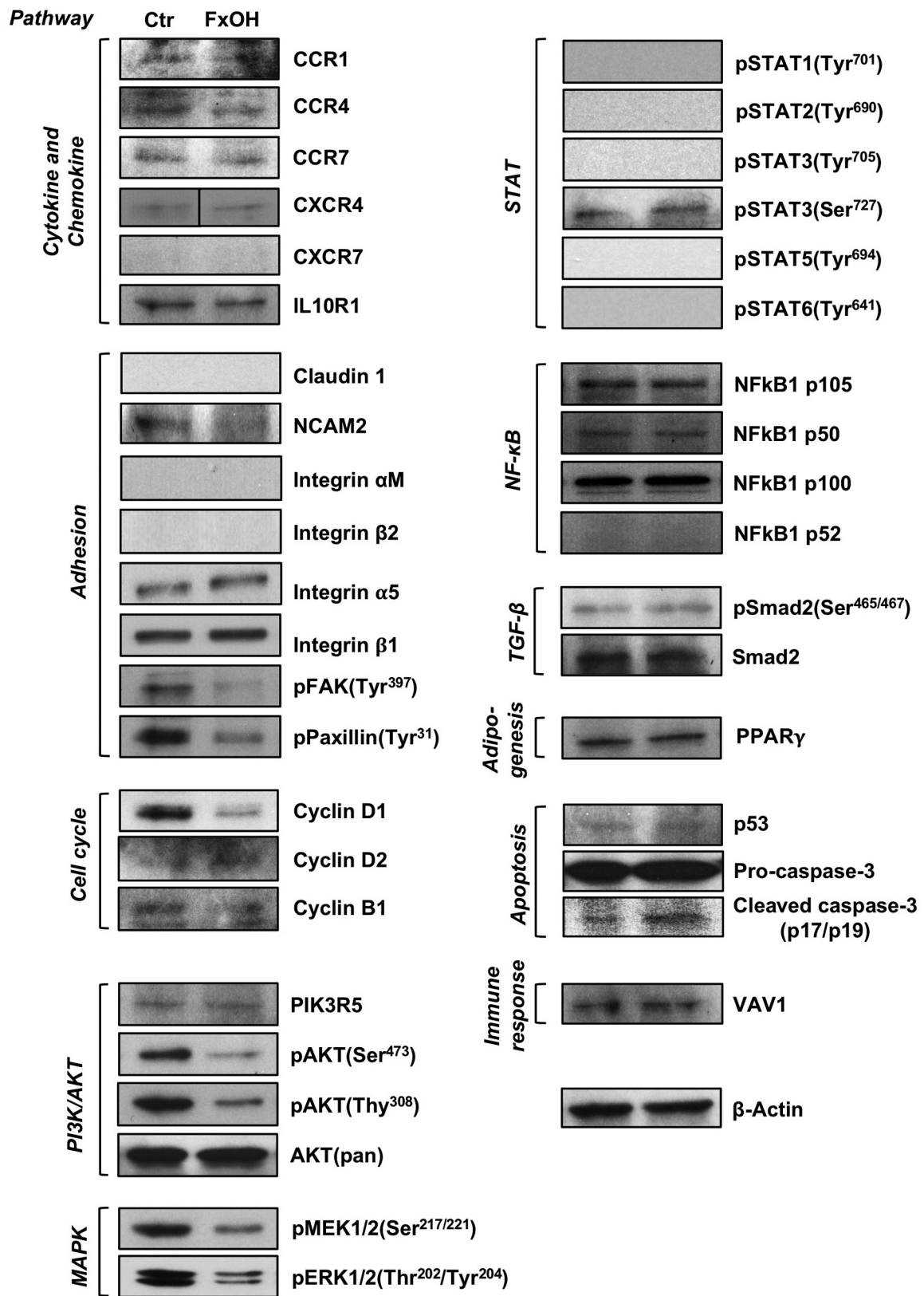
Figure 5. *Protein expression levels in KMPC44 cells with or without fucoxanthinol (FxOH) treatment. KMPC44 cells were treated with 5.0 μM of FxOH for 1 day. Protein levels were evaluated by western blotting.*

As was assumed, all integrin-relating proteins (4 proteins) were not changed or detected in KMPC44 cells with and without FxOH treatment. Our previous study showed the effect of FxOH on anoikis induction in colon cancer cells through suppression of integrin signaling (46, 47). Therefore, the adhesion capability in the cells might be reduced by FxOH. Moreover, FxOH treatment decreased cell growth not only in S phase but also the protein expression levels of cyclin D1 and B1 (Figures 1 and 5). These findings suggested that FxOH treatment induces the apoptosis in KMPC44 cells after arresting most cell cycle phases.

In summary, FxOH altered the expression levels of 8,515 genes and induced apoptosis in cancer-derived cell line KMPC44 cells derived from a pancreatic cancer $Ptf1a^{Cre/+}$; LSL-k-$ras^{G12D/+}$ murine model. The cytokine–cytokine receptor interaction, cell adhesion molecules CAMS, ECM receptor interaction, calcium signaling pathway, chemokine signaling pathway, adhesion, apoptosis, immune response, cell cycle, EGFR, GPCR, integrin, Jun, MAPK, NF-κB, PI3K/AKT, PKC, Ras, STAT, TGF-β and Wnt signals were altered by FxOH treatment in KMPC44 cells. In addition, CCR1, CCR4, NCAM2, pFAK(Tyr^{397}), pPaxillin(Tyr^{31}), cyclin D1, cyclin B1, pAKT(Ser^{473}), pAKT(Thr^{308}), pMEK1/2(Ser$^{217/221}$), and pERK1/2(Thr^{202}/Tyr^{204}), which were chosen as pivotal proteins for cancer-related signals in the gene set analyses, were significantly attenuated in KMPC44 cells after FxOH treatment. We assume that FxOH may stimulate firstly cytokine receptors and adhesion molecules on cellular membrane, and subsequently suppress the down-streams of FAK/Paxillin, PI3K/AKT, MAPK and cell cycle signals in KMPC44 cells, followed by apoptosis and anoikis. Further studies are needed to confirm the molecular mechanisms underlying apoptosis induction in various pancreatic cancer cells by FxOH. Our results suggested that FxOH may be a good candidate agent for pancreatic cancer prevention in a murine model.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

M. Terasaki conceived, designed the study and wrote the paper. M. Terasaki, T. I., W. M. and T. O. performed the experiments. A. K., H. K., M. K., H. M., Kazuo M., M. M. and M. Takahashi reviewed and edited the manuscript.

Acknowledgements

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