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Supplemental information

Mitochondrial complex I inhibitors suppress tumor growth through concomitant acidification of the intra- and extracellular environment

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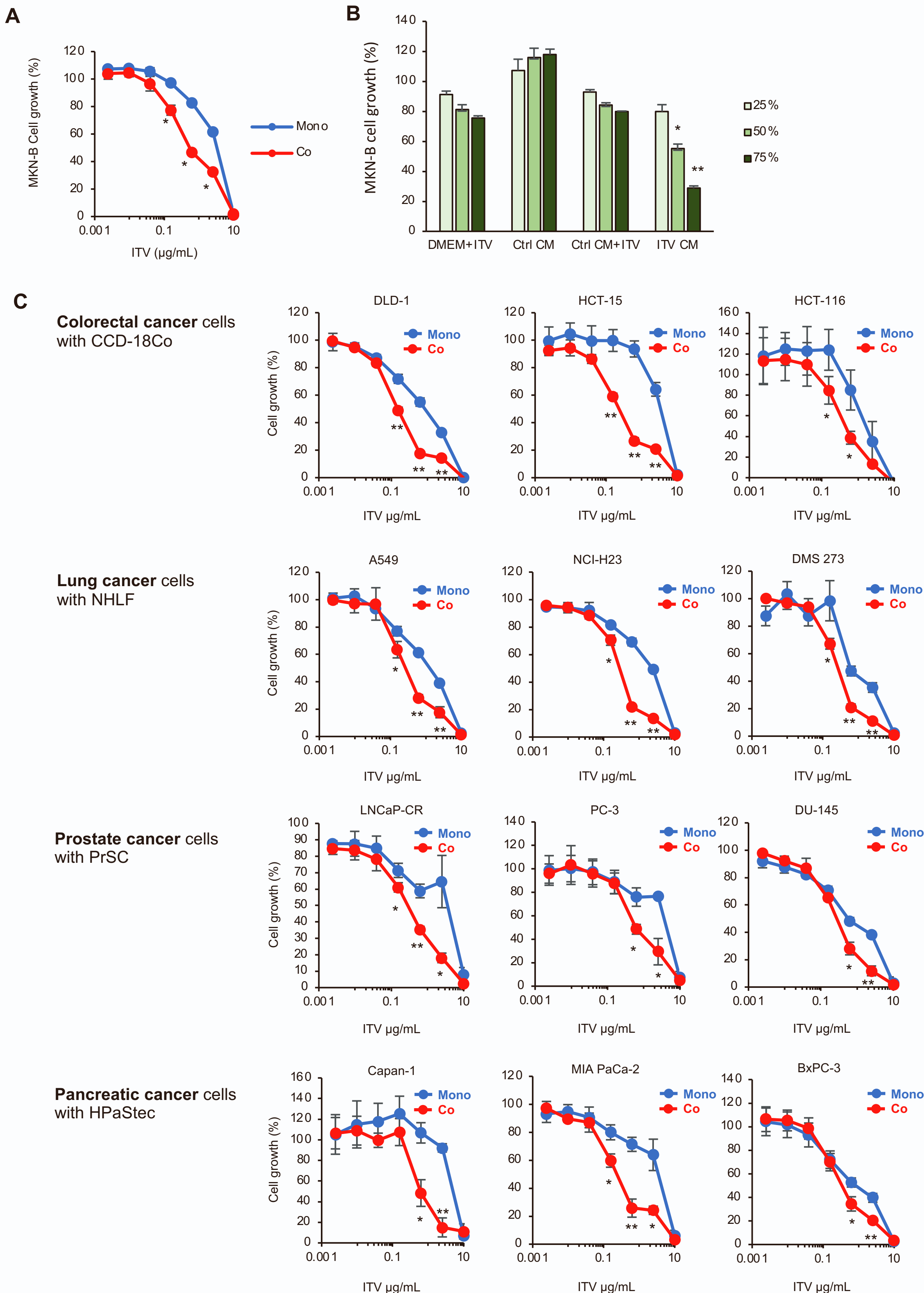

Figure S1. The anti-cancer activity of ITV was potentiated in co-culture conditions of cells from several organs. Related to Figure 1. (A) Growth of MKN-B cells cultured alone (Mono) or with Hs738 (Co) for 3 days at the indicated ITV concentrations were measured using GFP fluorescence intensity and expressed as percentage relative to that of cells without ITV treatment (n = 3; *P < 0.005, **P < 0.001 vs. mono). (B) Growth of MKN-B cells upon treatment with ITV CM, Ctrl CM, and their respective controls (DMEM+ITV and Ctrl CM+ITV) for 3 days (*P < 0.001 vs. Ctrl CM+ITV). For each condition, 1 µg/mL ITV was used. (C) Growth of colorectal, lung, prostate, and pancreatic cancer cell lines that were cultured alone (Mono) or with the indicated stromal cells from the same organ as cancer cells (Co) for 3 days with the indicated concentrations of ITV were measured using GFP fluorescence intensity and expressed as percentage relative to that of cells without ITV treatment (*P < 0.05, **P < 0.001 vs. mono). Data are presented as the mean ± s.d. and were analyzed using two-sided Student's t-test (n = 3).

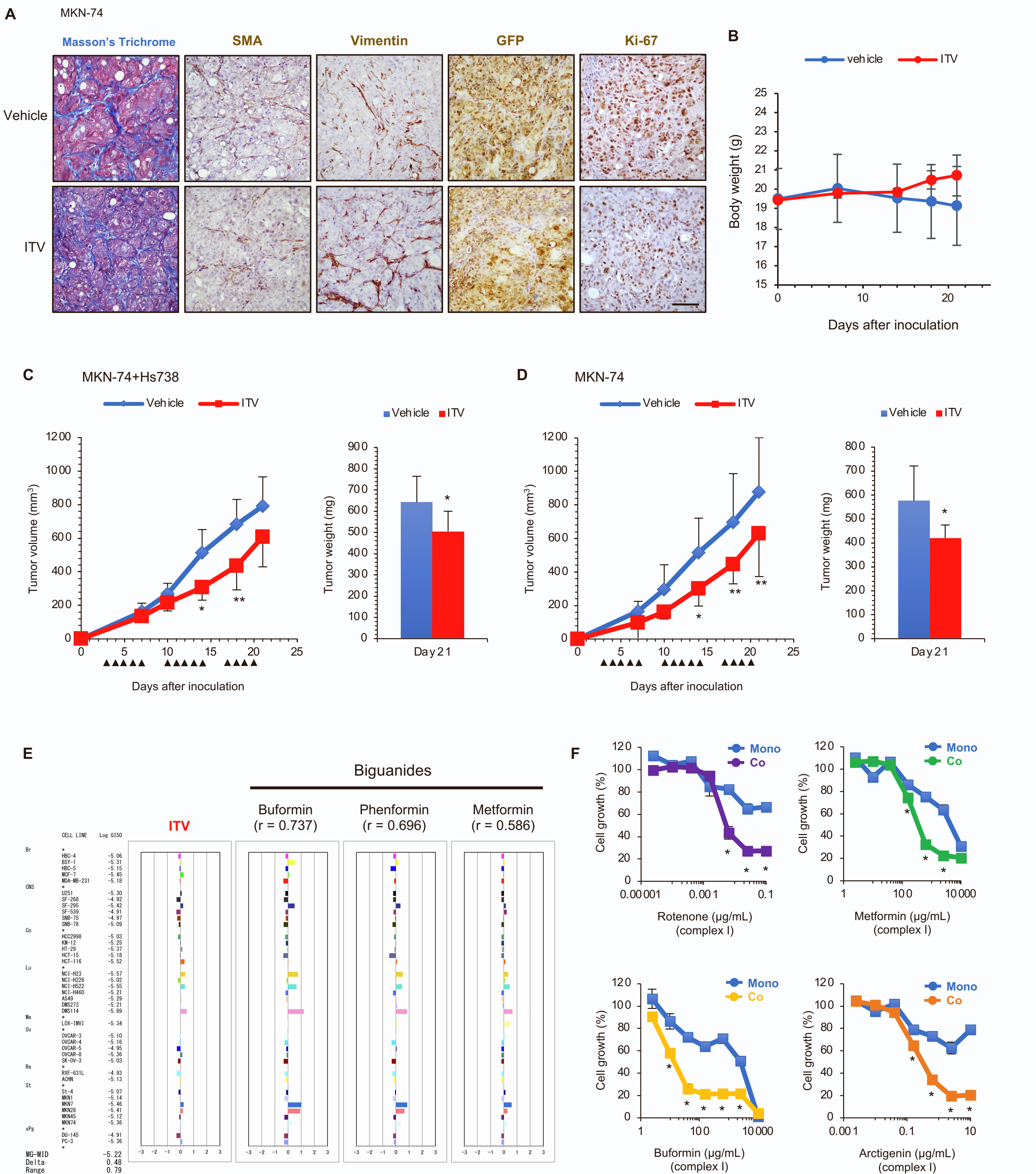

Figure S2. ITV suppressed tumor growth of mono-inoculated and co-inoculated MKN-74 cells with Hs738 cells. Related to Figure 1. (A) Formalin-fixed, paraffin-embedded tumor sections from MKN-74-injected mice treated with the vehicle or 12.5 mg/kg ITV. Scale bar, 100 μm. (B) ITV-treated mice did not exhibit any weight loss compared to vehicle-treated mice (n = 5). (C and D) Tumor volumes in mice mono-inoculated and co-inoculated with MKN-74 and Hs738 cells treated with the vehicle or 12.5 mg/kg ITV injected on the days marked with black triangles (n = 5; *P < 0.05 vs. vehicle). Tumor volumes at day 21 after ITV injection are shown as a bar graph. These figures were modified based on previous results (Abe et al., 2013). (E) Fingerprint patterns are shown as a result of ITV evaluation against JFFCR39 cancer cell lines. Details have been described elsewhere (Dan et al., 2002; Paull et al., 1989; Yamori, 2003). (F) Growth of MKN-B cells that were cultured alone (Mono) or with Hs738 (Co) for 3 days with the indicated concentrations of rotenone, metformin, buformin, or arctigenin (n = 3; *P < 0.005 vs. mono). Data are presented as the mean ± s.d. and were analyzed using a two-sided Student's t-test.

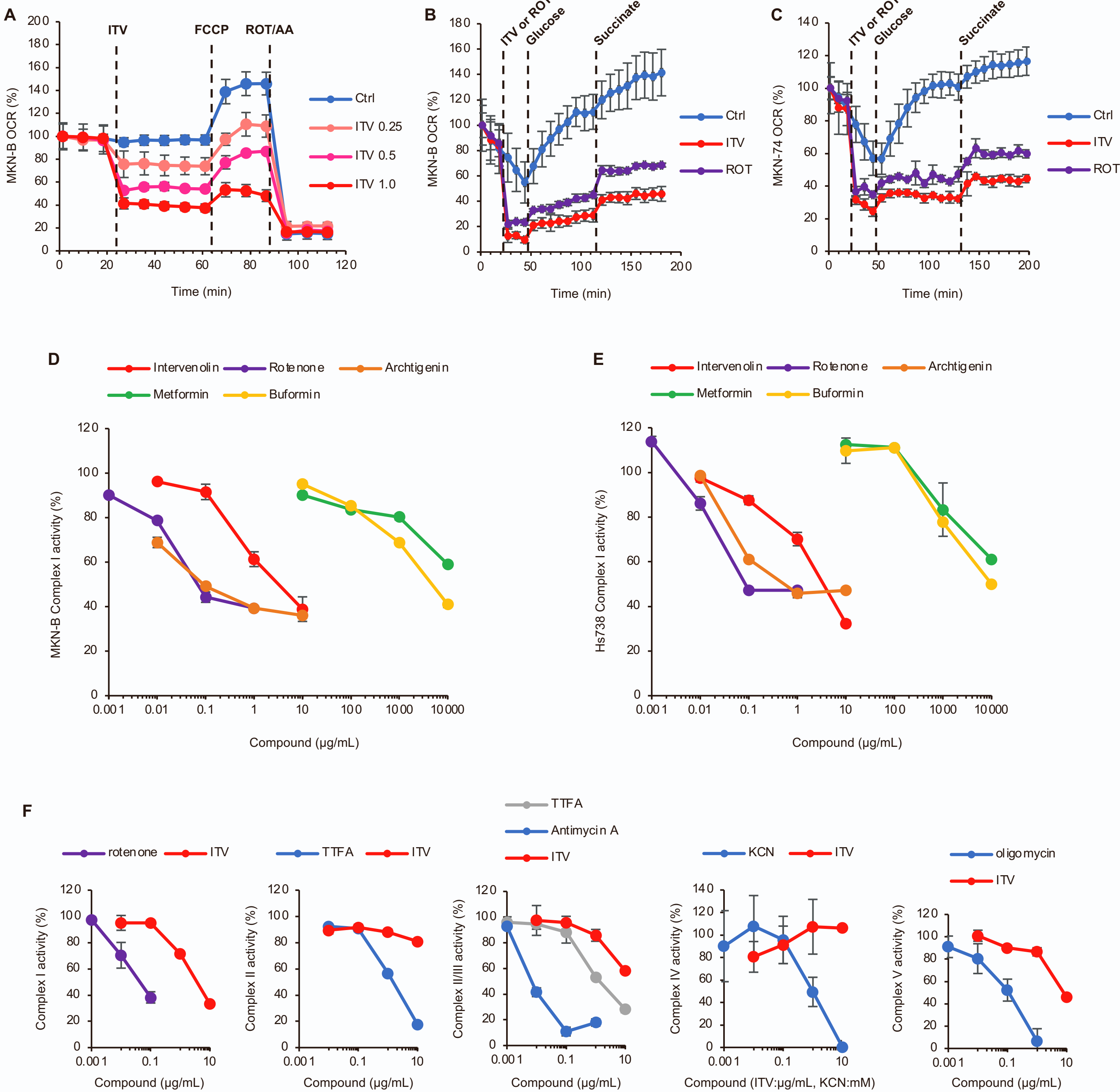

Figure S3. Intervenolin (ITV) inhibits mitochondrial complex I activity. Related to Figure 2. (A) Results of seahorse flux analyzer assays showing the OCR of MKN-B cells. Assay buffer (Ctrl) or the indicated concentrations of ITV were introduced at 24 min; FCCP, 64 min; and rotenone (ROT) and antimycin A (AA), 88 min. (B and C) OCR of MKN-B and MKN-74 cells were measured by flux analyzer assays followed by 60 min glucose/pyruvate depletion. Assay buffer (Ctrl), ITV, or ROT were introduced at 24 min; glucose, 48 min; and succinate, 128 min. (D and E) Activity of the mitochondrial complex I was measured using a NADH assay. Reduction rate of NADH that normalized by non-treated value was shown as complex I activity. The activity of mitochondria without compounds was defined as 100%. (F) Inhibitory activity of ITV against mitochondrial complexes I, II, II/III, IV, and V compared with each specific inhibitor. Data are presented as the mean ± s.d. (n = 3) and were normalized according to the baseline.

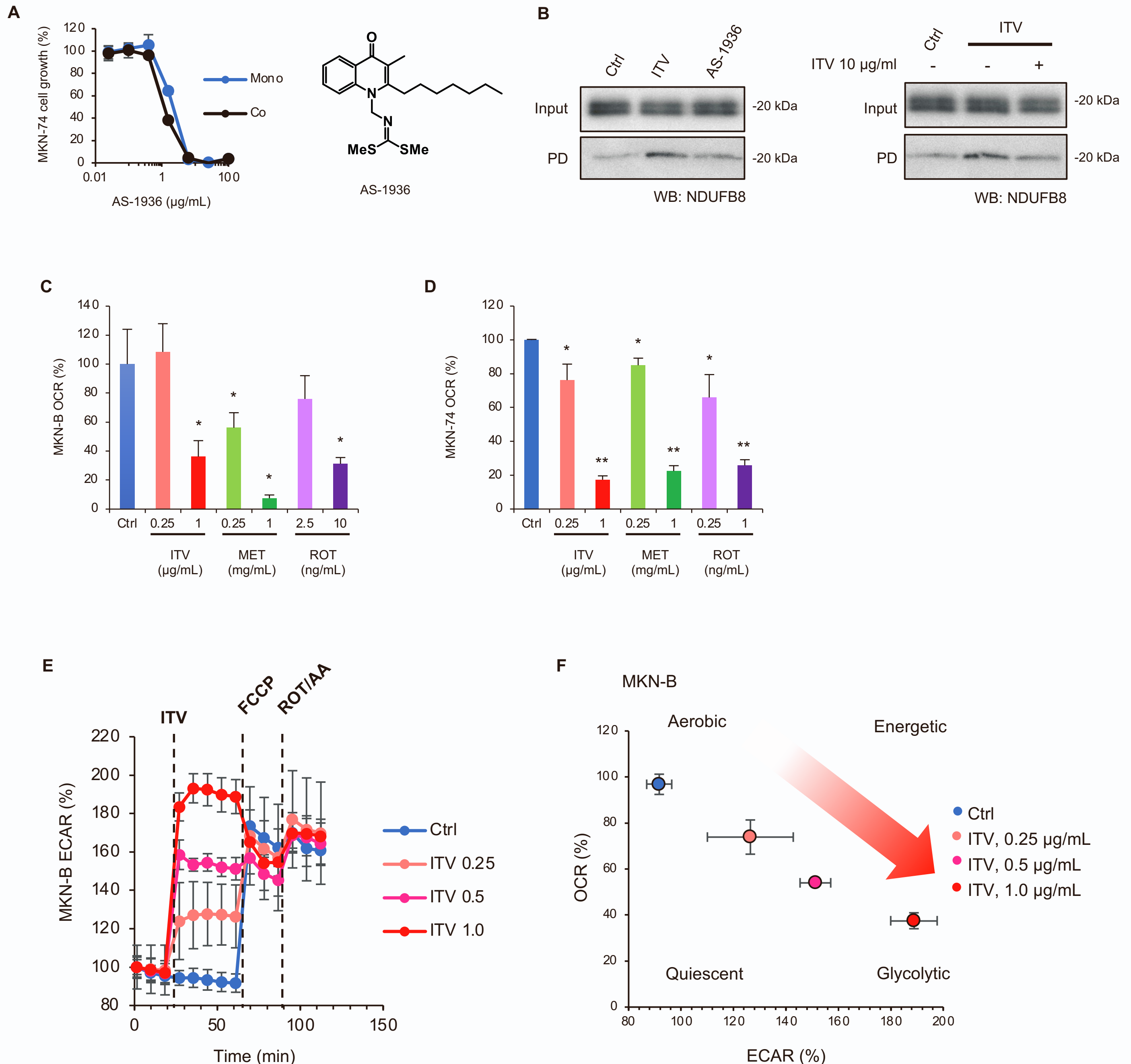

Figure S4. Intervenolin (ITV) induces metabolic shift in cancer cells by complex I inhibition. Related to Figure 2. (A) Growth of MKN-74 cells that were cultured alone (Mono) or with Hs738 (Co) for 3 days and treated with the indicated concentrations of AS-1936. Structure of AS-1036 is shown. (B) The results of pull-down (PD) followed by western blotting using NDUFB8 antibodies. Mitochondria isolated from Hs738 cells were treated with compound-conjugated beads. The competition of ITV is shown (right). ITV, ITV-conjugated beads; AS-1936, AS-1936-conjugated beads. (C and D) OCR of MKN-B and MKN-74 cells as measured using MitoXpress assay after treatment with the indicated concentrations of ITV, metformin (MET), or rotenone (ROT) for 180 min. The OCR of cells without compounds was defined as 100% (n = 3; *P < 0.05, **P < 0.00001 vs. Ctrl). Data were analyzed using two-sided Student's t-test. (E) Seahorse flux analyzer assay results showing the extracellular acidification rate (ECAR) in MKN-B and MKN-74 cells as in Fig. 2a. (F) Energy map of MKN-B cells based on OCR and ECAR values. The status of the metabolic pathways is classified as aerobic, glycolytic, energetic, or quiescent. Data are presented as the mean ± s.d. (n = 3) and normalized according to the baseline.

Ctrl (DMSO)

ITV

Pentose Phosphate Pathway

Purine Metabolism

Glycolysis

Choline Metabolism

Lipid Metabolism

Lys & Trp Metabolism

Nicotinamides

BCAA Metabolism

Urea Cycle

TCA Cycle

Glutathione Metabolism

Met Cycle

Polyamines & Creatine

Figure S5. Effect of Intervenolin (ITV) on intracellular metabolites involved in central energy metabolic pathways. Related to Figure 2. MKN-74 cells were treated with DMSO or ITV (1 μg/mL) for 3 h.

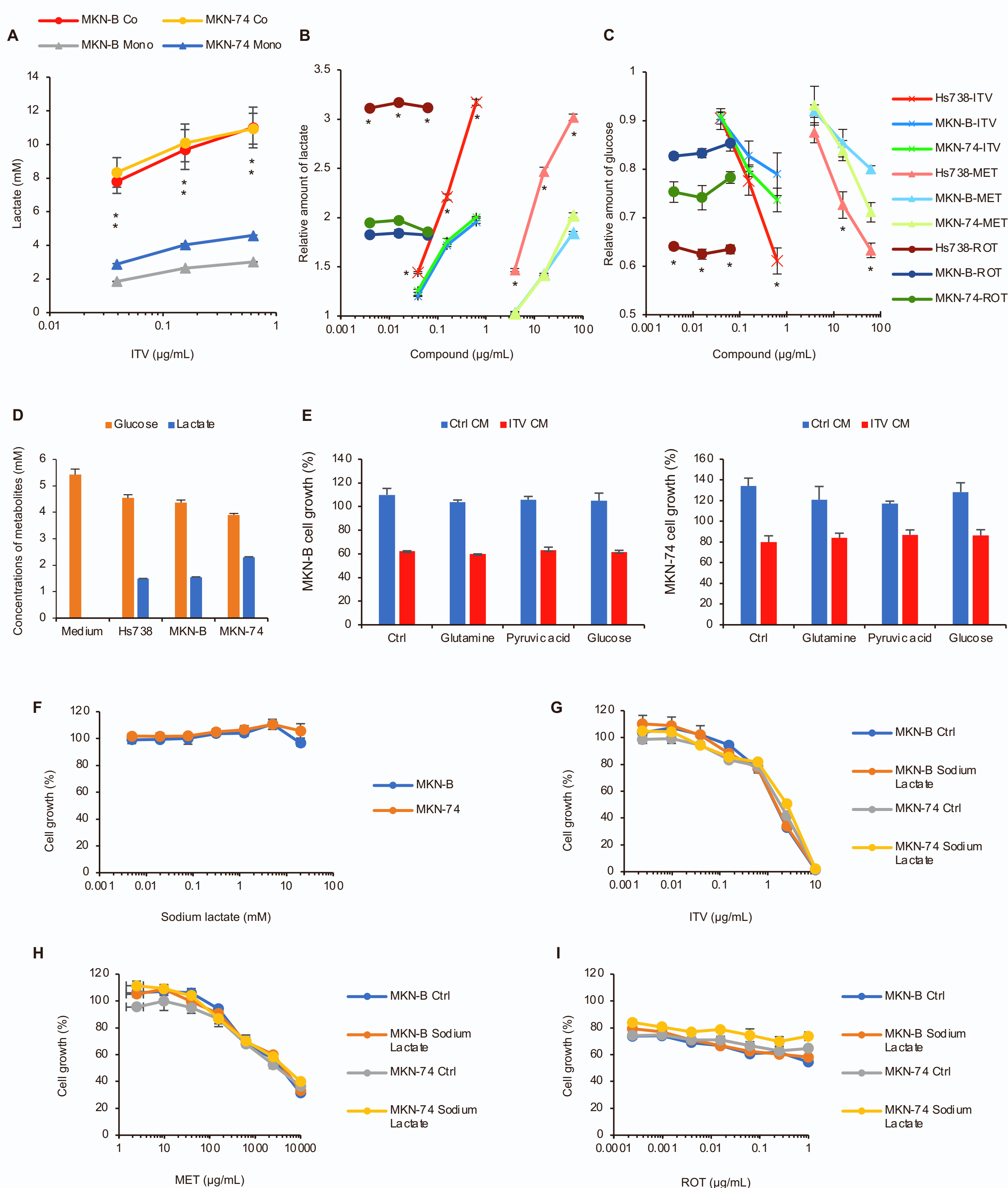

Figure S6. Complex I inhibition altered the amounts of extracellular metabolites. Related to Figure 2 and 3. (A) Lactate concentration in the monoculture (Mono) medium and the co-culture medium of MKN-B or MKN-74 cells with Hs738 (Co) with the indicated concentrations of ITV (n = 3; *P < 0.005 vs. mono). (B and C) Concentrations of lactate and glucose in the conditioned medium of MKN-B, MKN-74, and Hs738 cells relative to those in the conditioned medium of non-treated cells (D). Cells were treated with the indicated concentrations of ITV, metformin (MET), or rotenone (ROT) for 3 days (n = 3; *P < 0.01 vs. both MKN-B and MKN-74). (D) Concentrations of glucose and lactate in the conditioned medium of MKN-B, MKN-74, and Hs738 cells. (E) Growth of MKN-B and MKN-74 cells cultured in Ctrl CM or ITV CM (prepared by 1 μg/mL ITV) supplemented with extra glutamine (4 mM), pyruvate (2 mM), or glucose (4 g/L) for 3 days (n = 3). (F) Growth of MKN-B and MKN-74 cells cultured with the indicated concentrations of sodium lactate for 3 days (n = 3). (G-I) Growth of MKN-B and MKN-74 cells cultured in DMEM supplemented with 1% D-FBS (Ctrl) or the medium containing 10 mM sodium lactate in the presence of the indicated concentrations of ITV, MET, or ROT for 3 days were measured using GFP fluorescence intensity (n = 3). Data are presented as the mean ± s.d. and were analyzed using two-sided Student's t-test.

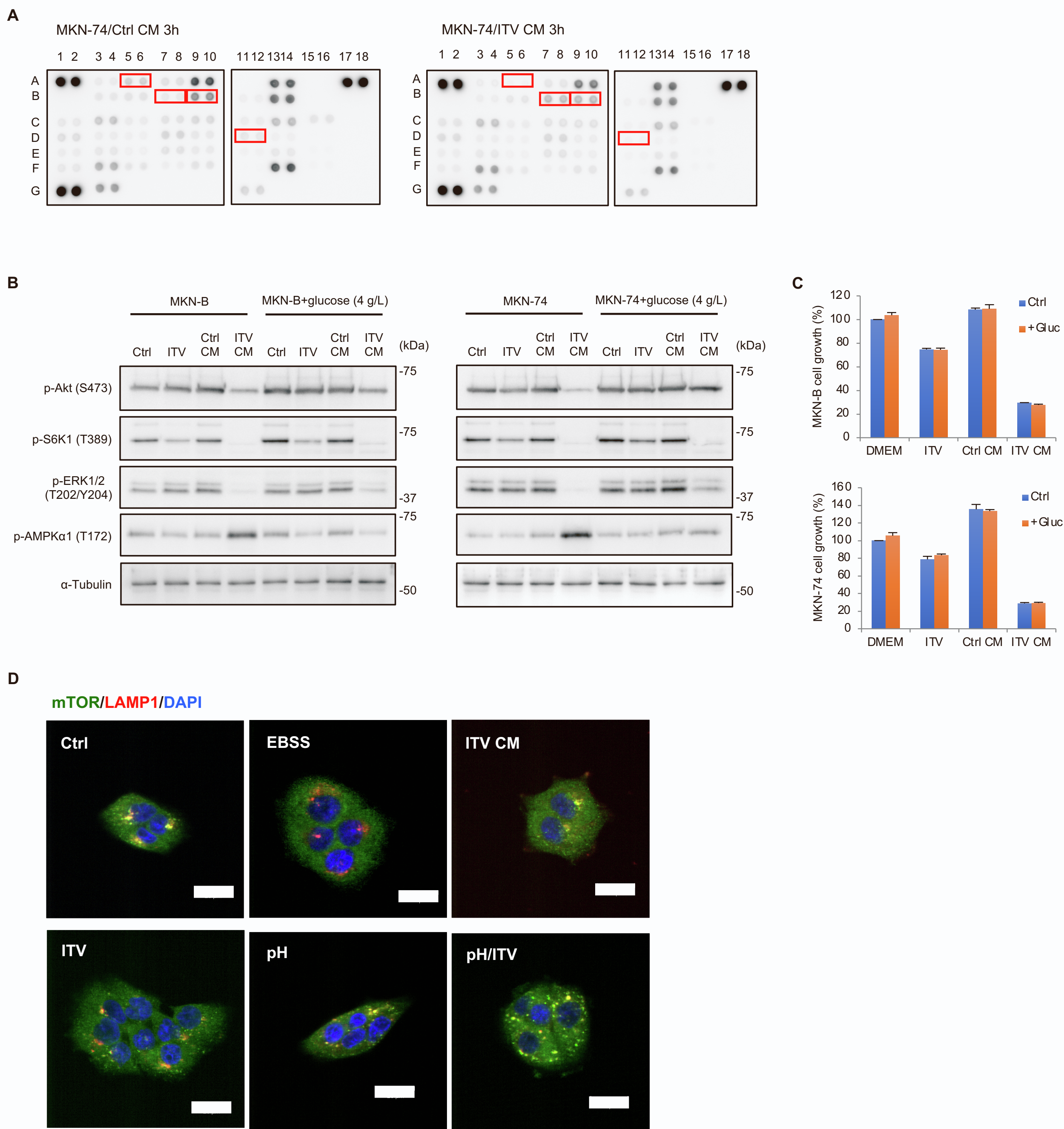

Figure S7. The phosphorylation of S6K1 in cancer cells was suppressed by ITV CM. Related to Figure 3 and 4. (A) The results of anti-phospho-kinase antibody array of MKN-74 cell lysates treated with Ctrl CM or ITV CM for 3 h. (B) Western blot analysis showing the phosphorylation levels of Akt (S473), S6K1 (T389), ERK1/2 (T202//Y204), and AMPK (T172) in MKN-B and MKN-74 cells cultured with the indicated conditions for 3 h. (C) Growth of MKN-B and MKN-74 cells cultured in the same conditions as in (B) (n = 3). (D) Signal-overlap of mTOR and LAMP1 in MKN-B cells as analyzed by immunofluorescence and confocal microscopy. Cells were cultured in the following media for 3 h: control (DMEM, 1% D-FBS), Earle's balanced salt solution (EBSS), ITV CM, with ITV (1 μg/mL; ITV), low pH (pH), and low pH with ITV (1 μg/mL; pH/ITV). EBSS treatment was used as the nutrient starved condition, a condition characterized by mTORC1 inactivation. Fixed cells were stained with mTOR antibodies (green), LAMP1 antibodies (red), and DAPI (blue). Merged images are shown. Scale bar, 20 μm. Data are presented as the mean ± s.d.

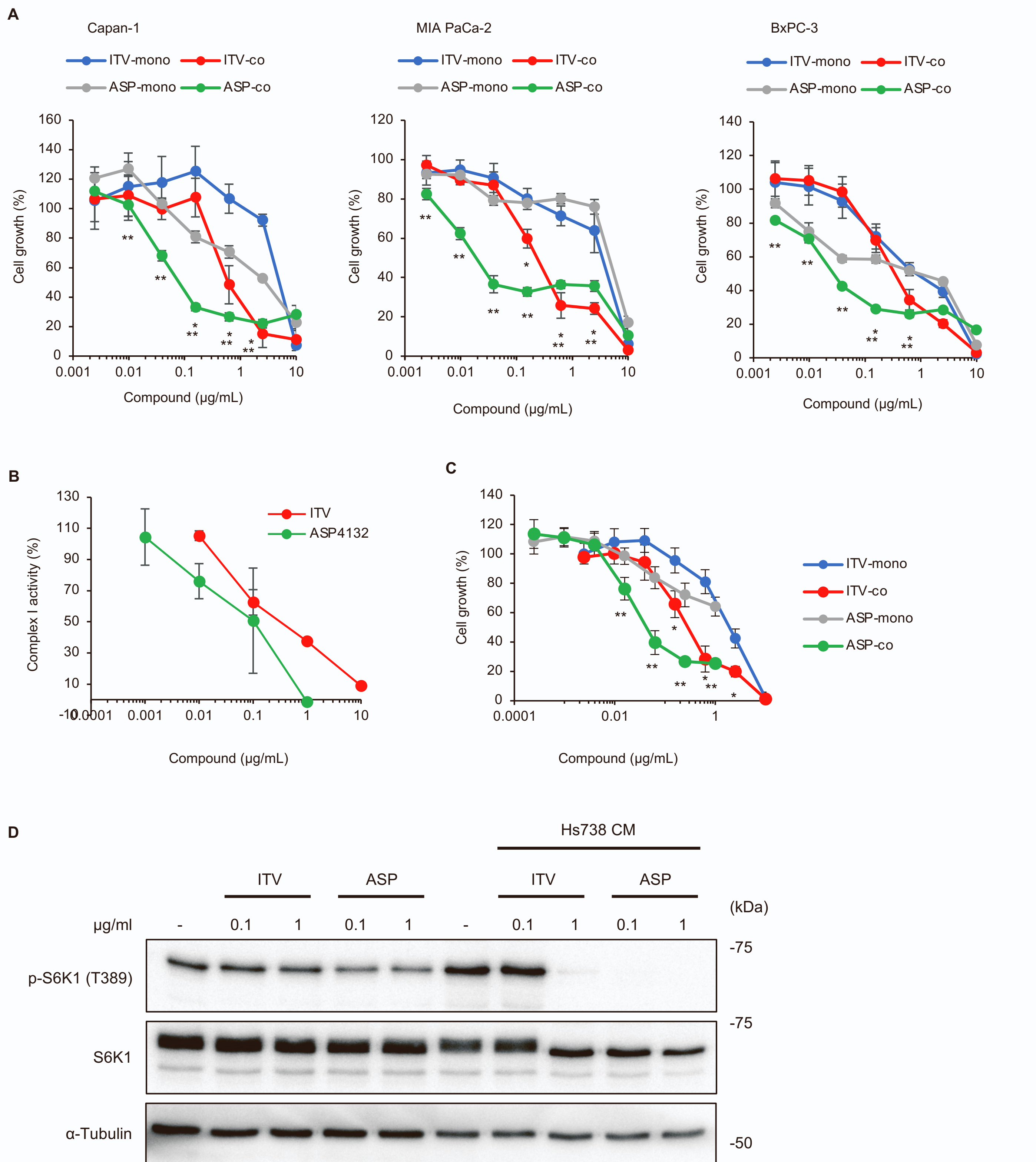

Figure S8. PDAC cells exhibited sensitivity against complex I inhibitors in co-culture conditions, and ASP4132 exhibited anti-cancer activity potentiated in acidic conditions as in the presence of ITV. Related to Figure 7. (A) Growth of Capan-1, MIA PaCa-2, and BxPC-3 cells that were cultured alone (mono) or with HPaSteC cells (co) for 3 days with ITV or ASP4132 at the indicated concentrations ($n = 3$; *$P < 0.05$ vs. ITV-mono; **$P < 0.05$ vs. ASP-mono). (B) The results of NADH assay of the mitochondria from Hs738 cells as in Figure 2B ($n = 3$). (C) Growth of MKN-74 cells cultured alone (mono) or with Hs738 cells (co) for 3 days with ITV or ASP4132 at the indicated concentrations ($n = 3$; *$P < 0.05$ vs. ITV-mono; **$P < 0.05$ vs. ASP-mono). (D) Western blot analysis showing the phosphorylation levels of S6K1 (T389) in MKN-74 cells cultured with the control medium (left) with the indicated concentrations of ITV or ASP4132 (ASP) or in the CM from Hs738 cells treated with the indicated concentrations of ITV or ASP4132 (right) for 3 h. Data are presented as the mean ± s.d. and were analyzed using a two-sided Student's *t*-test.

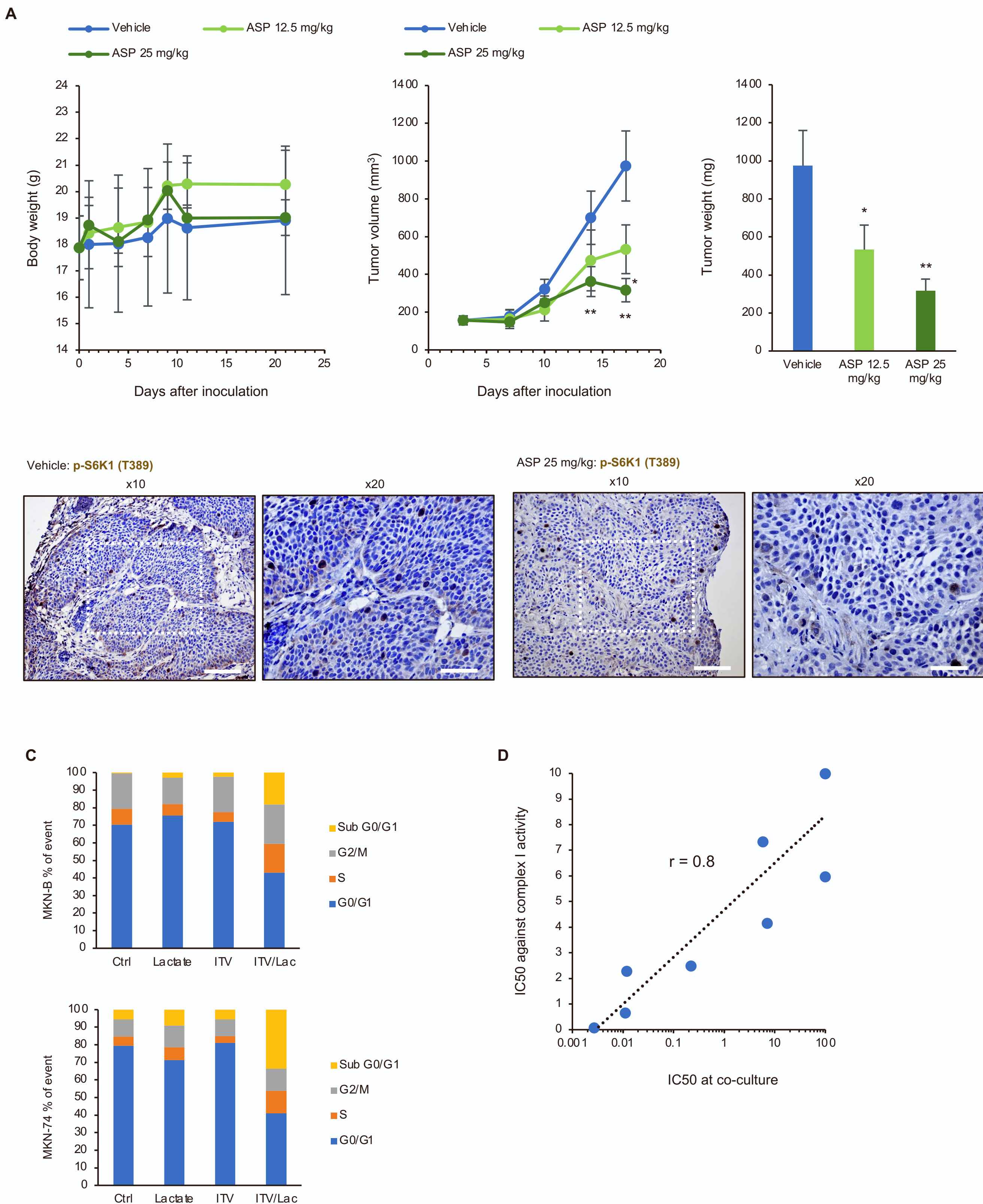

Figure S9. Complex I inhibitors suppressed the tumor growth of PDAC cells in mouse xenograft models. Related to Figure 7. (A) Mice treated with ASP4132 (ASP) did not exhibit any weight loss compared to those treated with the vehicle (n = 3). Tumor volumes in mice co-inoculated with BxPC-3 and HPaSteC cells treated with the vehicle or ASP4132 (n = 3; $^{*}P < 0.01$, $^{**}P < 0.001$ vs. vehicle). Tumor volumes at day 17 after ASP4132 injection are shown as a bar graph. (n = 3; $^{*}P < 0.01$, $^{**}P < 0.001$ vs. vehicle). (B) Immunohistochemistry of formalin-fixed, paraffin-embedded tumor sections from mice treated with the vehicle or ASP4132 using an antibody against phospho-S6K1 (T389). Scale bar, 100 µm. (C) Cell cycle analysis of MKN-B and MKN-74 cells treated with lactate (10 mM), ITV (1 µg/mL), and 1 µg/mL ITV + 10 mM lactate (ITV/Lac) for 72 h using flow cytometry. (D) IC_{50} values of ITV derivatives against gastric cancer cells in co-culture exhibit a significant positive correlation with the inhibition of complex I. r represents Pearson's correlation coefficient.

Table S1. Proteins detected using anti-phospho kinase antibody array. Related to Figure 3, 4, and S7.

	1,2	3,4	5,6	7,8	9,10	11,12	13,14	15,16	17,18
A	Reference	p38α	ERK1/2	JNK1/2/3	GSK-3α/β		p53		Reference
B		EGFR	MSK1/2	AMPKα1	Akt1/2/3	Akt1/2/3	p53		
C	TOR	CREB	HSP27	AMPKα2	β-Catenin	p70 S6 Kinase	p53	c-Jun	
D	Src	Lyn	Lck	STAT2	STAT5a	p70 S6 Kinase	RSK1/2/3	eNOS	
E	Fyn	Yes	Fgr	STAT6	STAT5b	STAT3	p27	PLC-γ1	
F	Hck	Chk-2	FAK	PDGF Rβ	STAT5a/b	STAT3	WNK1	PYK2	
G	Reference	PRAS40			Negative control	HSP90			Negative control

Table S2. IC50 values of ITV and its derivatives against the growth of MKN-74 cells cultured in mono- or co-culture conditions and against enzymatic activity of mitochondrial complex I. Related to Figure 6.

IC50 (μg/mL)	Monoculture	Co-culture	Complex I
ITV	1.16	0.22	2.49
CJ-13,136	0.10	0.003	0.08
AS-1934	10.0	7.04	4.16
AS-1664	100.0	0.01	0.66
AS-1799	100.0	0.01	2.28
SC-013	100.0	100.0	5.97
SC-015	100.0	100.0	10.0
SC-018	100.0	100.0	10.0
SC-019	100.0	5.90	7.34