Inhibition of endometrial cancer by n-3 polyunsaturated fatty acids (PUFAs) in preclinical models

Hang Zheng[1,5], Hongjun Tang[1,5], Miao Liu[1,5], Minhong He[1], Pinglin Lai[2], Heling Dong[2], Jun Lin[2], Chunhong Jia[2], Mei Zhong[3], Yifan Dai[4] Xiaochun Bai[2] and Liping Wang[3]

[1]Department of Oncology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China; [2]Department of Cell Biology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China; [3]Department of Obstetrics and Gynecology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, China and [4]State Key Laboratory of Reproductive Medicine and Jiangsu Key Laboratory of Xenotransplantation, Nanjing Medical University, Nanjing 210029, China

Supplementary Materials and Methods, Tables and Figures

Supplementary Material and Methods

Cell proliferation assay

Endometrial cancer cell lines were seeded in 24-well plates for *in vitro* cell proliferation assay. After cell adherence, various concentrations of DHA (12.5-50 μM) or cisplatin (DDP) (100 μM) were added in culture medium, with DHA was replaced every 24 h. Total viable cells were counted at different times. For another set, cells were transfected with recombinant adenovirus carried with or without *fat-1* gene and cell viability was assessed as described above.

Colony formation assays

Cells were seeded into six-well plates in triplicates at a density of 400 cells/well in 2 ml

medium containing 10% FBS. After cell adherence, various concentrations (12.5-50 μM) of DHA were added in culture medium or transfected with recombinant adenovirus carried with or without *fat-1* gene. Cultures were replaced with fresh medium containing 10% FBS or the same medium containing 12.5-50 μM DHA every 48 h and grown for 2 weeks. The number of clones was counted and the clone formation was calculated.

Cell viability assay

Cells were seeded in 24-well plates and grown until 80 % confluence. Cells were then treated with DHA (25-50 μM) or cisplatin (DDP) (100 μM) after serum-starved for 6 h, and digested by 0.25 % trypsin-EDTA. An aliquot of 10 μl from each single-cell suspension was mixed with 10 μl of 1% Trypan Blue. The viable and total cells were counted for quantification respectively under a microscope.

Western blot analysis

Cells were lysed in 2× SDS-PAGE sample buffer on ice. And equal amounts of whole protein extract were further analyzed by SDS-PAGE, transferred to a nitrocellulose membrane (Amersham Biosciences, Italy), probed overnight at 4 °C with primary antibodies. Membranes were washed with TBS/0.05 % Tween-20 and incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Proteins were detected by enhanced chemiluminescence substrates (PerkinElmer).

Gas chromatography analysis of fatty acids compositions

Lipids extraction from tissues was performed according to the general technique of Bligh and Dyer as described in our previous studies (1, 2). The gas chromatograph was performed on a Perkin Elmer Clarus 500. Identification of components was by comparison of retention times with

those of authentic standards (Sigma).

Reference

1 Lu Y, Nie D, Witt WT, Chen Q, Shen M, Xie H, Lai L, Dai Y, Zhang J. Expression of the fat-1 gene diminishes prostate cancer growth in vivo through enhancing apoptosis and inhibiting GSK-3 beta phosphorylation. Mol Cancer Ther. 2008 Oct;7(10):3203-11.

2 Wei D, Li J, Shen M, Jia W, Chen N, Chen T, Su D, Tian H, Zheng S, Dai Y, Zhao A. Cellular production of n-3 PUFAs and reduction of n-6-to-n-3 ratios in the pancreatic beta-cells and islets enhance insulin secretion and confer protection against cytokine-induced cell death. Diabetes. 2010 Feb;59(2):471-8.

Supplementary Tables

Supplementary Table S1. n-3 and n-6 PUFAs content in experimental diet

Type of Fatty Acids	Normal n-3 (g/kg diet)	High n-3 (g/kg diet)
C18:3 n-3, α-linoleic acid	0.50	0.36
C20:5, n-3, Eicosapentaenoic acid (EPA)	4.17	31.19
C22:5, n-3, Docosapentaenoic acid (DPA)	0.89	0.60
C22:6, n-3, Docosahexaenoic acid (DHA)	3.80	15.79
n-3, total	7.36	47.94
C18:2, n-6, Linoleic acid	9.35	6.13
C20:4, n-6, Arachidonic acid (AA)	0.58	0.36
n-6, total	9.93	6.49
n-3/ n-6	0.74	7.38

Values of n-3 and n-6 PUFAs in diet were measured by gas chromatography-mass spectroscopy.

Supplementary Table S2. Primer sequences for PCR amplification

Gene	Forward (5'→3')	Reverse (5'→3')
fat-1	GGACCTGGTGAAGAGCATCCG	GCCGTCGCAGAAGCCAAAC
SCID	GGAAAAGAATTGGTATCCAC	AGTTATAACAGCTGGGTTGGC

Supplementary Table S3. Fatty acids composition in mice tumors

Type of Fatty Acids (mol % of total fatty acid)	HEC-1-A		RL95-2	
n-3 PUFAs diet	Normal	High	Normal	High
C18:3 n-3, α-linoleic acid	0.45 ± 0.12	0.77 ± 0.22	0.34 ± 0.09	0.67 ± 0.12
C20:5, n-3, Eicosapentaenoic acid	0.11 ± 0.01	0.76 ± 0.09	0.21 ± 0.02	0.45 ± 0.01
C22:5, n-3, Docosapentaenoic acid	0.41 ± 0.08	0.61 ± 0.12	0.23 ± 0.02	0.44 ± 0.09
C22:6, n-3, Docosahexaenoic acid	2.86 ± 0.32	4.02 ± 0.31	2.76 ± 0.12	4.77 ± 0.61
n-3, total	3.83 ± 0.39	6.16 ± 0.98	3.54 ± 0.22	6.33 ± 0.98
C18:2, n-6, Linoleic acid	15.23 ± 2.22	14.38 ± 2.02	16.23 ± 2.45	16.55 ± 1.66
C20:4, n-6, Arachidonic acid (AA)	2.23 ± 0.43	1.98 ± 0.33	2.09 ± 0.34	1.44 ± 0.24
n-6, total	17.46 ± 0.87	16.36 ± 2.45	18.32 ± 2.47	17.99 ± 1.67
n-3/ n-6	0.22 ± 0.02	0.38 ± 0.06 #	0.19 ± 0.02	0.35 ± 0.03#

Values of n-3 and n-6 PUFAs in endometrial tumors (n = 8) were measured by gas chromatography-mass spectroscopy, data indicate mean ± SD. #, $p < 0.001$compared with normal n-3 PUFAs diet.

Supplementary Table S4. Fatty acids composition in SCID or *fat-1*-SCID mouse tails.

Type of Fatty Acids (mol % of total fatty acid)	SCID	*fat-1*-SCID
C18:3 n-3, α-linoleic acid	0.69 ± 0.22	1.46 ± 0.18
C20:5, n-3, Eicosapentaenoic acid (EPA)	0.44 ± 0.10	0.61 ± 0.04
C22:5, n-3, Docosapentaenoic acid (DPA)	0.51 ± 0.18	0.95 ± 0.12
C22:6, n-3, Docosahexaenoic acid (DHA)	1.98 ± 0.55	3.08 ± 0.84
n-3, total	3.62 ± 0.86	6.10 ± 1.23
C18:2, n-6, Linoleic acid	10.44 ± 2.71	7.99 ± 1.70
C20:4, n-6, Arachidonic acid (AA)	4.14 ± 0.92	2.23 ± 0.28
n-6, total	14.58 ± 2.67	10.22 ± 1.72
n-3/ n-6	0.24 ± 0.06	0.60 ± 0.10 #

Values of n-3 and n-6 in animal tails (n = 10) were measured by gas chromatography-mass spectroscopy, data indicate mean ± SD. #, $p < 0.01$ compared with SCID mice.

Supplementary Table S5. Fatty acids composition in mice uteri.

Type of Fatty Acids (mol % of total fatty acid)	Normal n-3 PUFAs diet	High n-3 PUFAs diet	WT	fat-1
C18:3 n-3, α-linoleic acid	0.37 ± 0.10	0.45 ± 0.11	0.29 ± 0.02	0.65 ± 0.11
C20:5, n-3, Eicosapentaenoic acid	0.12 ± 0.02	0.66 ± 0.06	0.14 ± 0.01	0.45 ± 0.03
C22:5, n-3, Docosapentaenoic acid	0.51 ± 0.09	0.91 ± 0.20	0.53 ± 0.06	0.88 ± 0.12
C22:6, n-3, Docosahexaenoic acid	2.56 ± 0.42	3.89 ± 0.67	2.34 ± 0.11	4.27 ± 0.61
n-3, total	3.56 ± 0.44	5.91 ± 1.22	3.30 ± 0.32	6.25 ± 0.87
C18:2, n-6, Linoleic acid	14.88 ± 3.64	13.22 ± 3.34	12.27 ± 1.70	11.09 ± 1.45
C20:4, n-6, Arachidonic acid (AA)	3.77 ± 0.53	3.58 ± 0.43	4.37 ± 0.41	2.94 ± 0.25
n-6, total	18.65 ± 3.13	16.80 ± 3.12	16.64 ± 2.47	14.03 ± 1.67
n-3/ n-6	0.19 ± 0.03	0.35 ± 0.09 #	0.20 ± 0.02	0.45 ± 0.04 #

Values of n-3 and n-6 PUFAs in uteri (n = 8) were measured by gas chromatography-mass spectroscopy, data indicate mean ± SD. #, $p < 0.001$ compared with normal n-3 PUFAs diet.

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Supplementary Figures

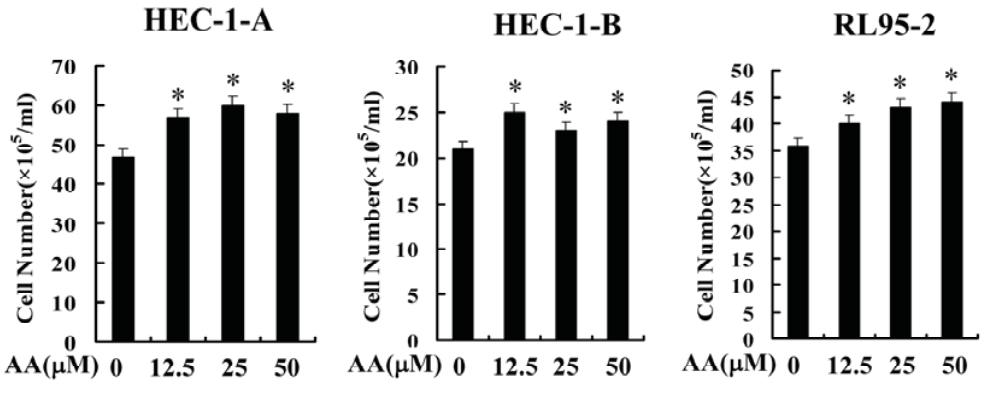

Figure S1 Arachidonic acid (AA) stimulates endometrial cancer cell growth. HEC-1-A, HEC-1-B and RL95-2 cells plated in 24-well plates were treated with serial concentrations of AA (0-50 μM) and total viable cells were counted after 96 h. *, $p < 0.05$, compared with control cells.

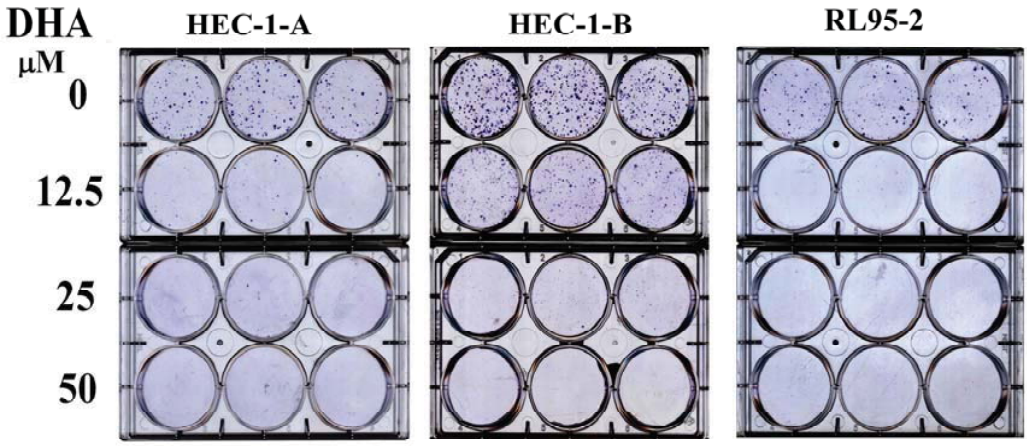

Figure S2 DHA inhibits endometrial cancer cell colony formation, HEC-1-A, HEC-1-B and RL95-2 cells plated in 6-well plates were incubated with 12.5-50 μM of DHA and subjected to colony formation assay.

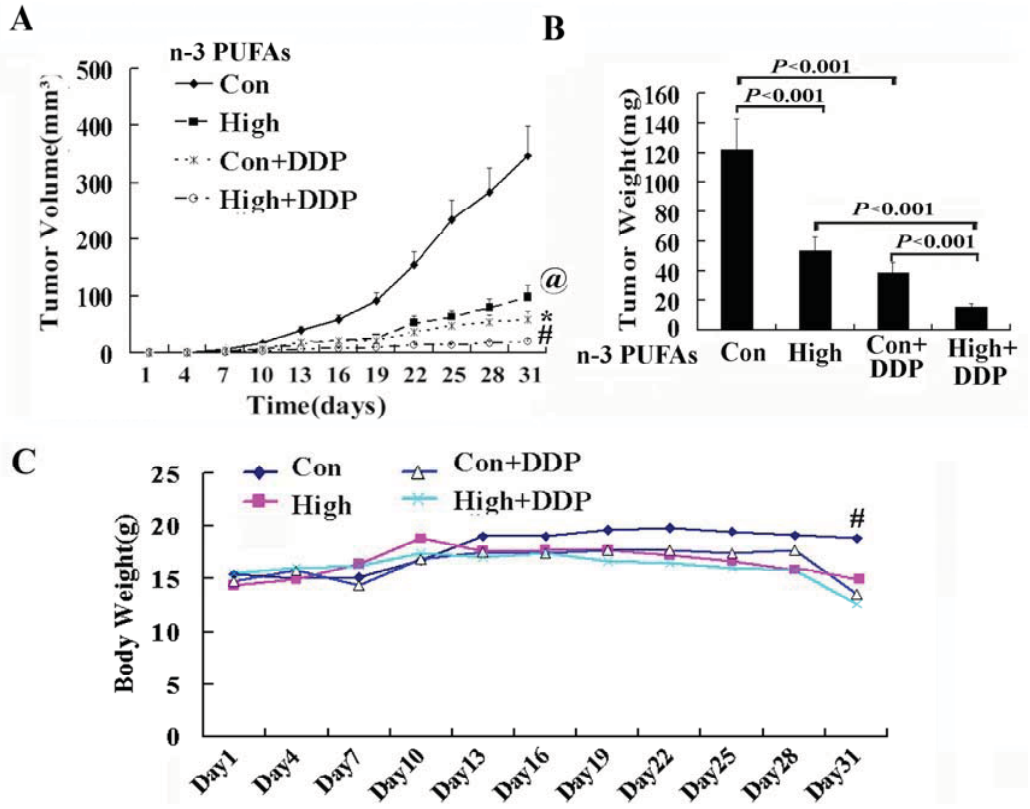

Figure S3 n-3 PUFAs promote the inhibitory effect of cisplatin on endometrial tumor growth in xenograft models. Four-week-old female nude mice were randomized into four groups (n = 6) and injected with a suspension of HEC-1-A cells. The mice were then fed with normal (Con) or high dietary ratios of n-3/n-6 PUFAs combined with or without 50 μg of cisplatin (DDP)/per mouse. Bidimensional tumor measurements were obtained as described in *Material and Methods*. **A**, the tumor growth curve and **B**, a comparison of average tumor weight on the final day between the four groups and **C**, body weight changes are shown. Bars indicate mean ± SD for three independent experiments. Con, normal diet. #, $P < 0.01$, compared with other three groups; * and @, $P < 0.01$, compared with control and high n-3 PUFAs diet combined with DDP groups.

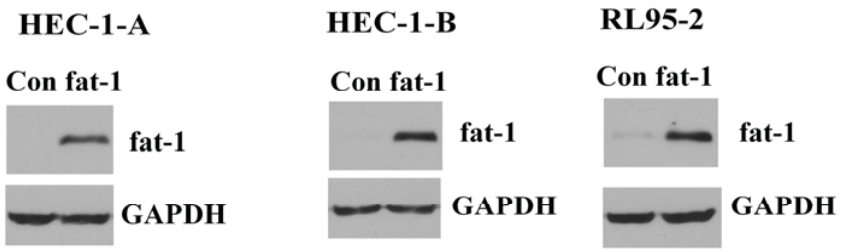

Figure S4 Ectopic expression of *fat-1* in endometrial cancer cell lines. HEC-1-A, HEC-1-B and RL95-2 cells were transfected with recombinant adenovirus carried with or without *fat-1* gene for 96 h and the expression of fat-1 RNA was confirmed by RT-PCR.

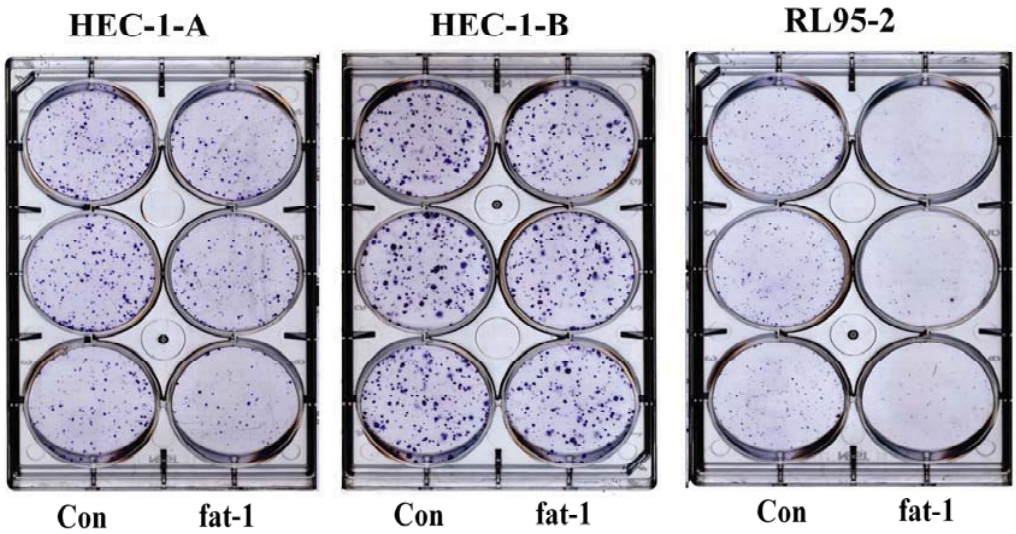

Figure S5 Endogenously produced n-3 PUFAs inhibit endometrial cancer cell colony formation. HEC-1-A, HEC-1-B and RL95-2 cells plated in 6-well plates were transfected with recombinant adenovirus carried with or without *fat-1* gene and subjected to colony formation assay

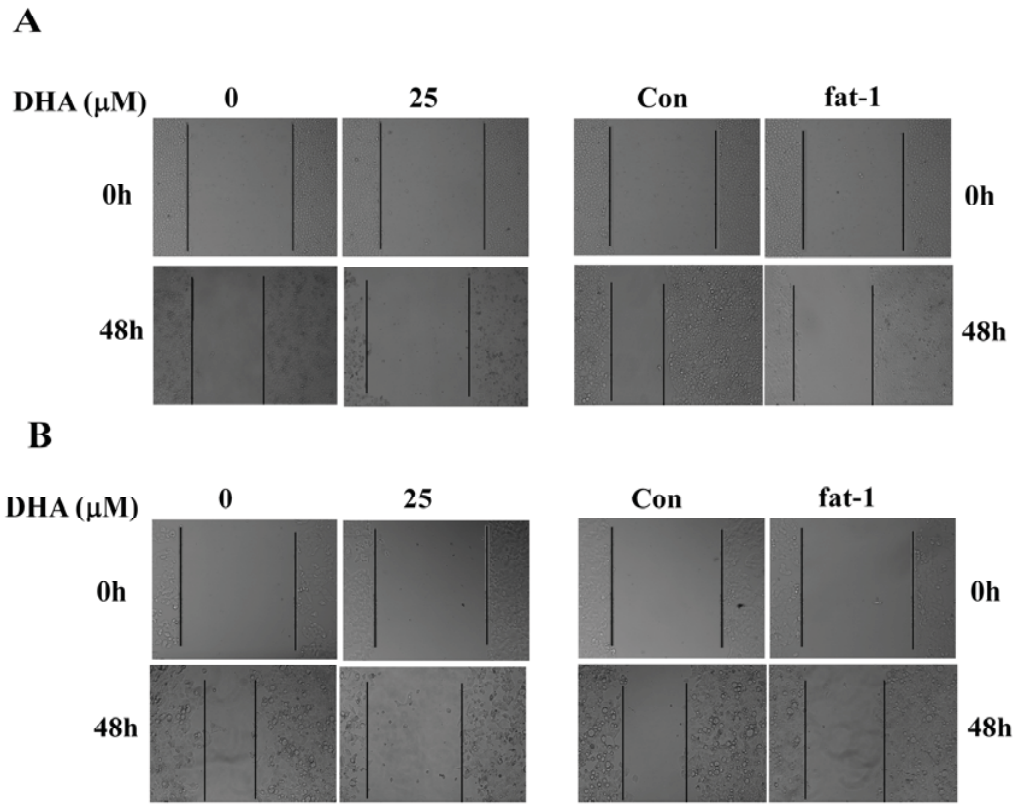

Figure S6 n-3 PUFAs prevent endometrial cancer cell migration. A, HEC-1-A, and B, HEC-1-B cells were incubated with or without 25 μM DHA and subjected to wound healing assay.

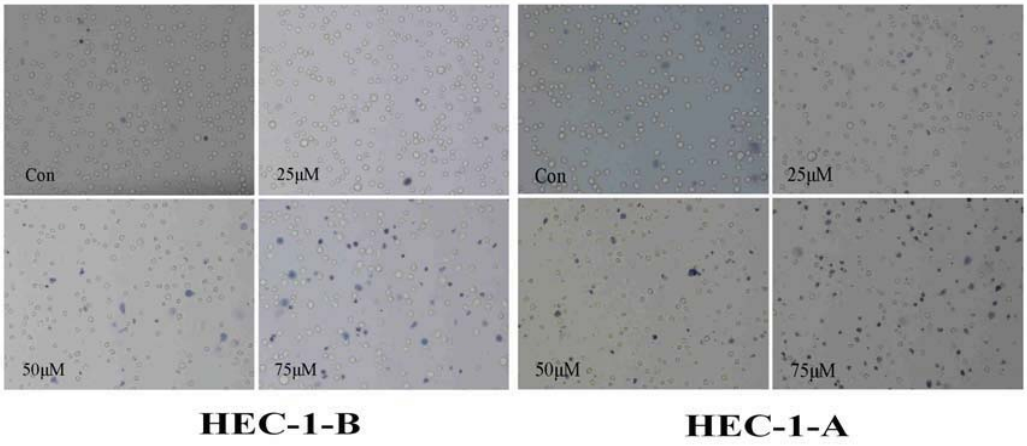

Figure S7 n-3 PUFAs promote endometrial cancer cell apoptosis. HEC-1-A and HEC-1-B cells were incubated indicated concentration of DHA in serum-free medium for 8 h and cell viability was assessed.

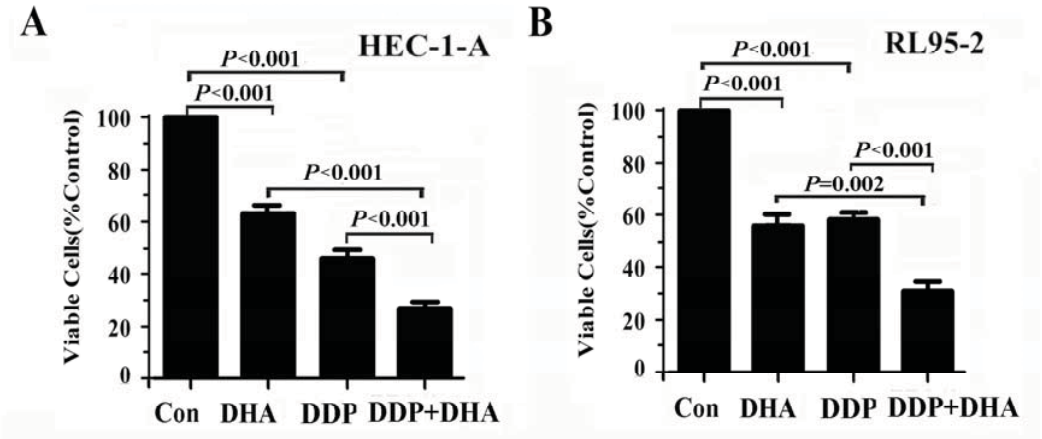

Figure S8 n-3 PUFAs promote cisplatin-induced endometrial cancer cell apoptosis. **A,** HEC-1-A and **B,** RL95-2 cells were incubated 50 μM DHA and/or 100 μM cisplatin (DDP) in serum-free medium for 8 h and cell viability was assessed.

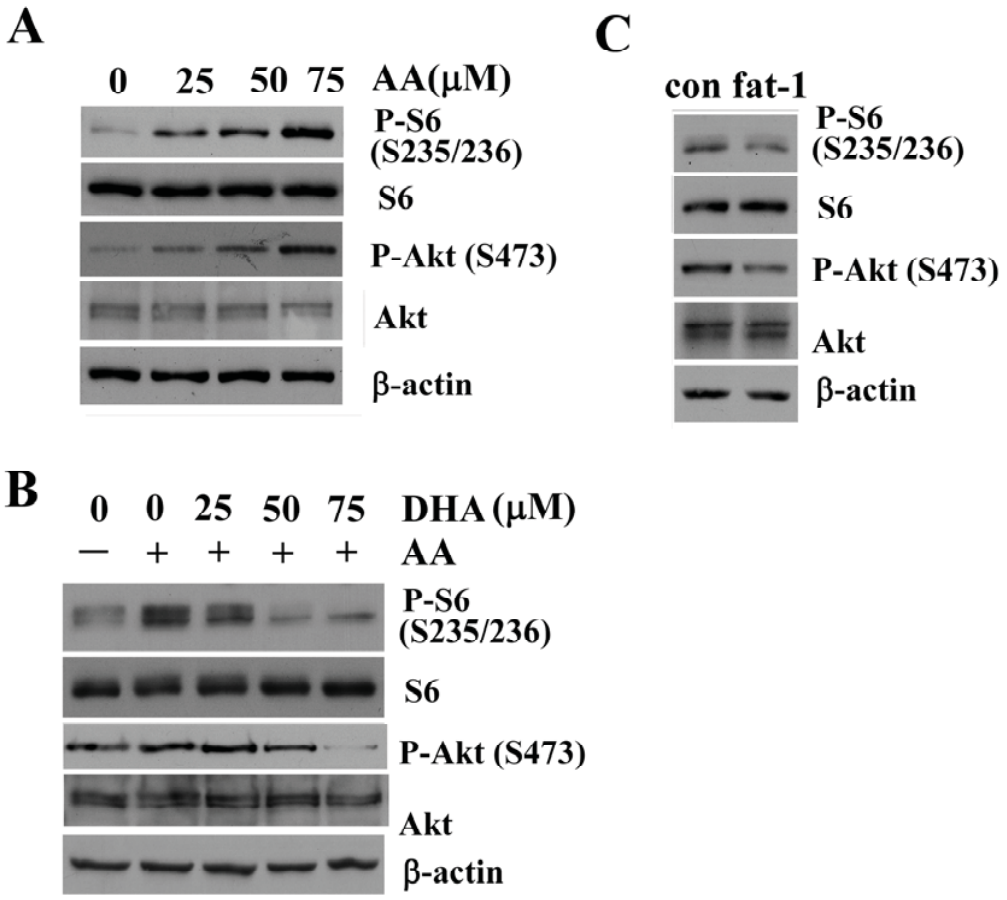

Figure S9 n-3 PUFAs inhibits mTORC1/2 in HEC-1-B cells. **A,** HEC-1-B cells were serum-starved for 12 h, followed by treatment with indicated concentrations of AA for 30 min. Proteins were extracted for western blot analysis for levels of P-S6 (S235/236) and P-Akt (S473). **B,** HEC-1-B cells were serum-starved for 12 h, followed by treatment with 50 μM of AA and indicated concentrations of DHA for 30 min. Proteins were extracted for western blot analysis for levels of P-S6 (S235/236) and P-Akt (S473). **C,** HEC-1-B cells were transfected with recombinant adenovirus carried with or without *fat-1* gene for 72 h, cell lysates were extracted for western blot analysis.

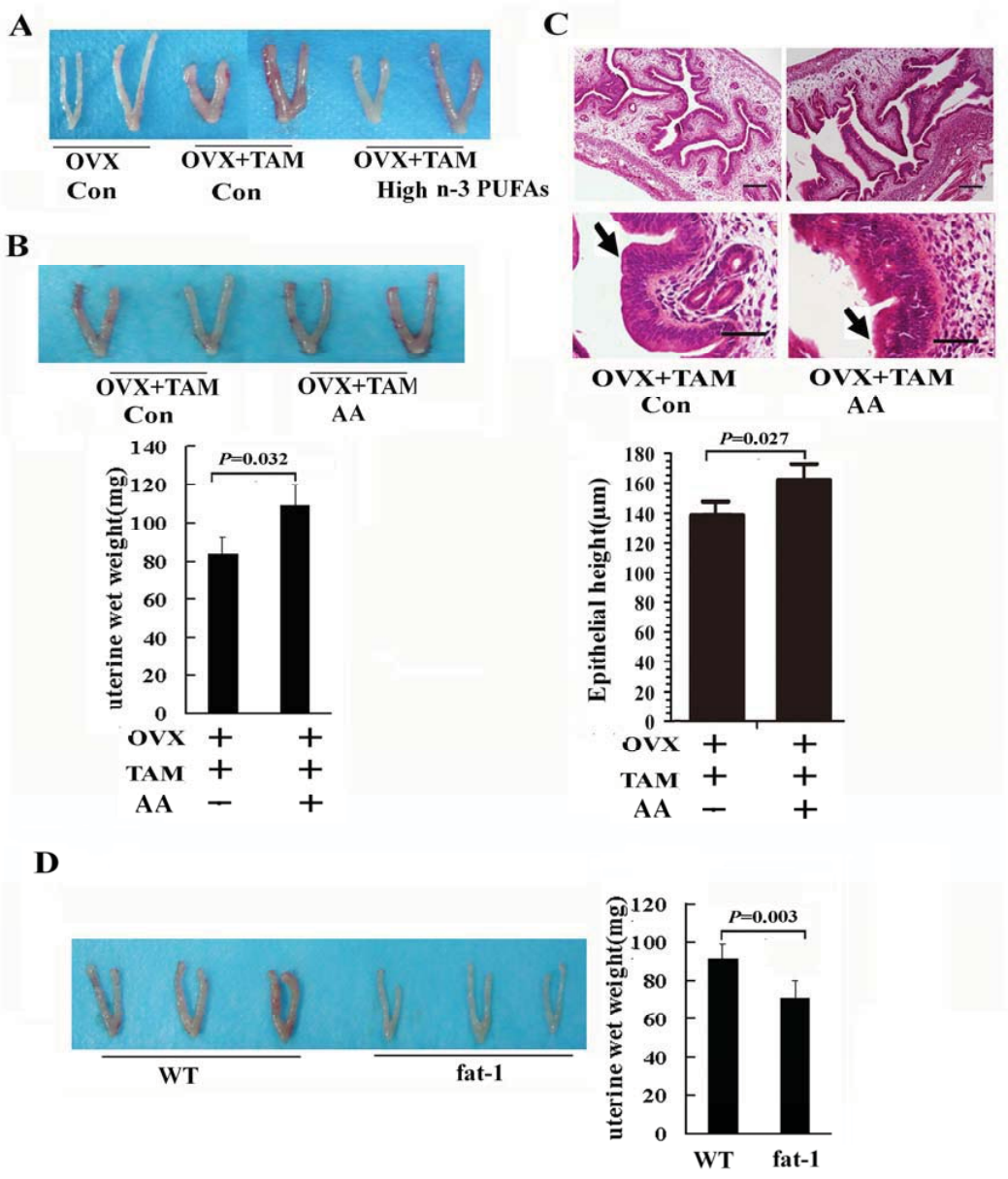

Figure S10 n-3 PUFAs prevent tamoxifen-stimulated endometrial growth in the mouse model. **A,** Balb/c mice which underwent bilateral oophorectomy were administered a normal or high n-3 PUFAs diet for 7 d, and subsequently received saline or 1 mg/kg/d tamoxifen citrate for another 3 d. The uteri were removed and global appearance of the uteri was shown. **B,** Balb/c mice which underwent bilateral oophorectomy were administered a normal or high n-6 PUFAs (6 g/kg/d AA) diet for 7 d, and subsequently received saline or 3 mg/kg/d tamoxifen citrate for another 3 d. The uteri were removed and global appearance of the uteri and uterine wet weight were shown. **C,** The

uteri were removed for HE staining and luminal epithelial cell heights were quantified. Scale bars = 400 μm (10×) or 100 μm (40×). **D,** wild type or *fat-1*mice underwent bilateral oophorectomy and 7 d later were administered 1 mg/kg/d tamoxifen citrate for 3 d. The uteri were removed and global appearance of the uteri was shown.