Platelet factor 4 induces cell apoptosis by inhibition of STAT3 via up-regulation of SOCS3 expression in multiple myeloma

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Online Supplementary Design and Methods

Cell cultures and reagents

Multiple myeloma (MM) cell lines U266, NCI-H929 and OPM2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A complete medium of RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine (Invitrogen, Carlsbad, CA, USA) was used to maintain these cell lines at 37°C in a 5% CO_2 atmosphere. Cells were treated with recombinant human platelet factor 4 (PF4; Peprotech, Rocky Hill, NJ, USA) at a concentration of 4 μM in most experiments unless otherwise specified. Cycloheximide was purchased from Enzo (Ann Arbor, MI, USA).

Magnetic cell sorting of CD138$^+$ plasma cells and CD31$^+$ endothelial cells

Approval for this study involving human subjects was obtained from the Chinese University of Hong Kong institutional review board. Informed consent was provided according to the Declaration of Helsinki. Bone marrow mononuclear cells from MM patients or healthy donors were separated using Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences, Piscataway, NJ, USA). Immunomagnetic separation of plasma cells or endothelial cells was performed using CD138 or CD31 microbeads and miniMACS (MiltenyiBiotec, Auburn, CA, USA) according to manufacturer's protocol. The purities of plasma cells and endothelial cells obtained were >95%, as confirmed by flow cytometry.

Cell growth and proliferation assay

MM cell lines were seeded in 96-well plates at a density of $1x10^4$ cells per well and treated with PF4. At the end of incubation, cell growth was determined by WST-1 (Roche, Germany) assay and cell numbers were determined by the trypan blue dye exclusion method according to the manufacturer's protocol.

Apoptosis assay

Untreated or PF4-treated cells were stained with annexin V-phycoerythrin and 7-amino-actinomycin D (7-AAD) according to the manufacturer's directions (Apoptosis Detection Kit; BD Biosciences) and the percentage of apoptotic cells was determined by flow cytometry (BD Biosciences). After cells had been treated with phosphate-buffered saline (PBS) or 8 μM PF4, apoptosis was also determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche), caspase-3 activity assay (Chemicon, Temecula, CA, USA) and immunoblotting with cleaved PARP according to the manufacturers' instructions.

In vitro tube formation assay

Unpolymerized Matrigel (BD Biosciences) was placed (50 μL/well) in a 96-well microtiter plate (0.32 cm^2/well) and polymerized for 1 h at 37°C. MM bone marrow endothelial cells ($1x10^4$ per well) in 100 μL medium in the presence or absence of PF4 were layered onto the Matrigel surface. After 6 h of incubation in a 5% CO_2 humidified atmosphere at 37°C, tube formation was observed using a reverted phase-contrast light microscope (Olympus, Japan).

Protein/DNA arrays and electrophoretic mobility shift assay

U266 and OPM2 cells were treated with PBS or PF4 for 8 h. Nuclear extracts from cells were prepared with a nuclear extraction kit (Panomics, Santa Clara, CA, USA). Protein/DNA arrays and the electrophoretic mobility shift assay were performed according to the manufacturer's protocol (Panomics).

Luciferase reporter assay

U266 cells were transfected with STAT3-Luc (Stratagene, Santa Clara, CA, USA) and pRL-TK (Promega, Madison, WI, USA). After incubation for 16 h, PBS or PF4 was added and the incubation was continued for 8 h. Cells were then harvested and the activities of firefly and Renilla luciferases in cell lysates were determined with a dual luciferase reporter assay system (Promega). Normalized data were calculated as the quotient of Renilla/firefly luciferase activities. All the experiments were performed in triplicate.

Western blotting

The western blots were performed as previously described.[1] Immunoblots were probed with antibodies against phospho-STAT3 (1:2000), STAT3 (1:2000), cleaved poly (ADP-ribose) polymerase (cleaved PARP; 1:1000), Mcl-1 (1:1000), β-actin (1:3000) (Cell Signaling, Beverly, MA, USA), SOCS3 (1: 800)

(Abcam, Cambridge, MA, USA), Bcl-2 (1:1000), Bcl-XL (1:1000) (BD Biosciences), and VEGF (1:1000) (Santa Cruz, Biotechnology, Santa Cruz, CA, USA).

RNA extraction and real-time polymerase chain reaction

First-strand cDNA was synthesized from one microgram of total RNA using MuLV reverse transcriptase, random hexamers and RNase inhibitor (Applied Biosystems, Carlsbad, CA, USA). To assess mRNA expression of putative genes, a real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR master-mix according to the manufacturer's protocol (HT7300 system, Applied Biosystems). PCR primer sequences are listed in *Online Supplementary Table S1*.

Cell transfections

NCI-H929 cells were transfected with constitutively active STAT3 plasmid (Stat3-C Flag pRc/CMV) kindly provided by Dr. Jim Darnell (Addgene plasmid 8722),[2] or empty vector (pcDNA3.1) using Nucleofector Kit T solution (Lonza, Germany). Stable clones were selected by G418 (Invitrogen). For knockdown of SOCS3, CXCR3B and LRP1, U266 cells were transfected with SOCS3 (Cell Signaling), CXCR3B or LRP1 (Applied Biosystems) and scrambled short-interfering RNA using Nucleofactor Kit C solution.

Subcutanous Matrigel xenograft and severe combined immunodeficient-rab mouse models

Six- to 8-week-old male non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were housed and monitored in our Animal Research Facility. All experimental procedures and protocols had been approved by the Animal Experimentation Ethics Committee (The Chinese University of Hong Kong). In the subcutaneous Matrigel xenograft model, mice were inoculated subcutaneously with $3x10^7$ OPM2 cells in 100 μL of RPMI 1640 medium, together with 100 μL of Matrigel (BD Biosciences). When the tumor was measurable, mice were treated by tail vein injection with PF4 (200 ng) or PBS three times/week. For the severe combined immunodeficiency-rabbit (SCID-rab) mouse model, femora and tibiae from 4-week old New Zealand rabbit bone grafts were subcutaneously implanted into SCID mice, as previously described.[3] Eight weeks after bone implantation, $1x10^6$ U266 cells were injected directly into the rabbit bone implant. Mouse sera were monitored serially for the levels of circulating immunoglobulin (hIg) of the M-protein isotype (lambda light chain) by an enzyme-linked immunosorbent assay according to the manufacturer's protocols (Bethyl, Montgomery, TX, USA). Mice were injected via the tail vein with PBS or PF4 (200 ng or 20 ng per mouse three times/week) for 12 consecutive weeks.

Immunohistochemistry

Twelve weeks after treatment with PF4, rabbit bones were fixed and decalcified with 8% formic acid (Sigma-Aldrich, St Louis, MO, USA). The bones were embedded in paraffin for sectioning. Sections were probed with CD138(1:50) (Abcam), CD31(1:30), VEGF (1:100) (DAKO, Denmark), cleaved caspase-3 (1:1600), and STAT3 (1:100) (Cell Signaling). Immunocomplexes were visualized using diaminobenzidinetetrahydrochloride (DAB) substrate (BD Biosciences) and counterstained with hematoxylin.

Statistical analysis

Whether differences observed in PF4-treated *versus* control cultures were statistically significant was determined using the Student's t-test. Values are represented by the mean ± standard deviation (SD) or standard error of the mean (SEM) of triplicate experiments. A P value <0.05 was considered statistically significant. Statistical significance in animal studies was determined using Student's t-test. Survival of mice was evaluated using Kaplan-Meier analysis.

References

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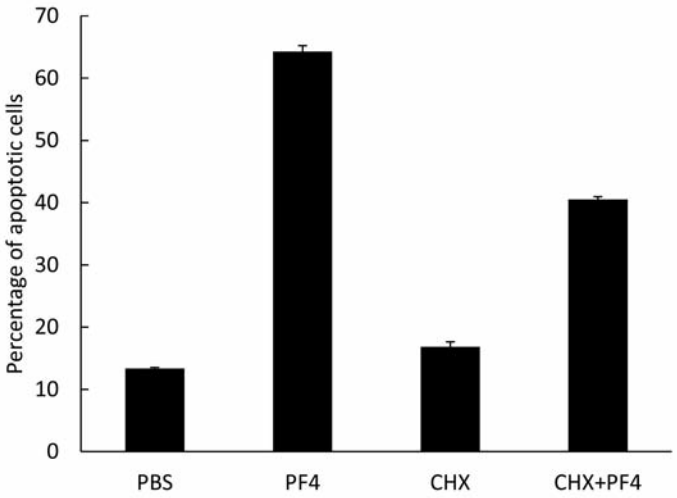
2. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, et al. Stat3 as an oncogene. Cell. 1999;98(3):295-303.
3. Yata K, Yaccoby S. The SCID-rab model: a novel in vivo system for primary human myeloma demonstrating growth of CD138-expressing malignant cells. Leukemia. 2004;18(11):1891-7.

Online Supplementary Table S1. Primers used for real-time PCR in this study.

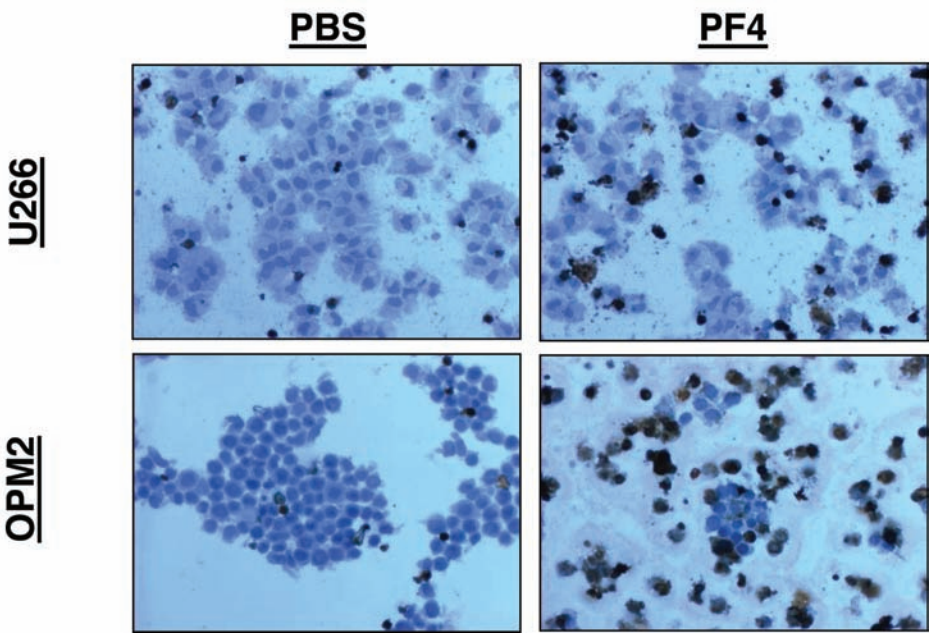
Genes	Forward primer (5'-3')	Reverse primer (5'-3')
c-Myc	AATGAAAAGGCCCCCAAGGTAGTTATCC	GTCGTTTCCGCAACAAGTCCTCTTC
Bcl-2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
Bcl-XL	GATCCCCATGGCAGCAGTAAAGCAAG	CCCCATCCCGGAAGAGTTCATTCACT
Mcl-1	TTCCAAGGCATGCTTCGGAAAC	TCTGCTAATGGTTCGATGCAG
Survivin	AGGTCATCTCGGCTGTTCCTG	TCATCCTCACTGCGGCTGTC
VEGF	ACTGCCATCCAATCGAGACC	GATGGCTTGAAGATGTACTCGATCT
SOCS3	TTCTACTGGAGCGCAGTGAC	ACTGGGTCTTGACGCTGA G
CIS	TGTGCATAGCCAAGACCTTCT	TCGGCATACTCAATGCGTACA
SHP-1	AATGCGTCCCATACTGGCCCGA	CCCGCAGTTGGTCACAGAGT

Online Supplementary Table S2. Clinicopathological data of MM patients (N=26).

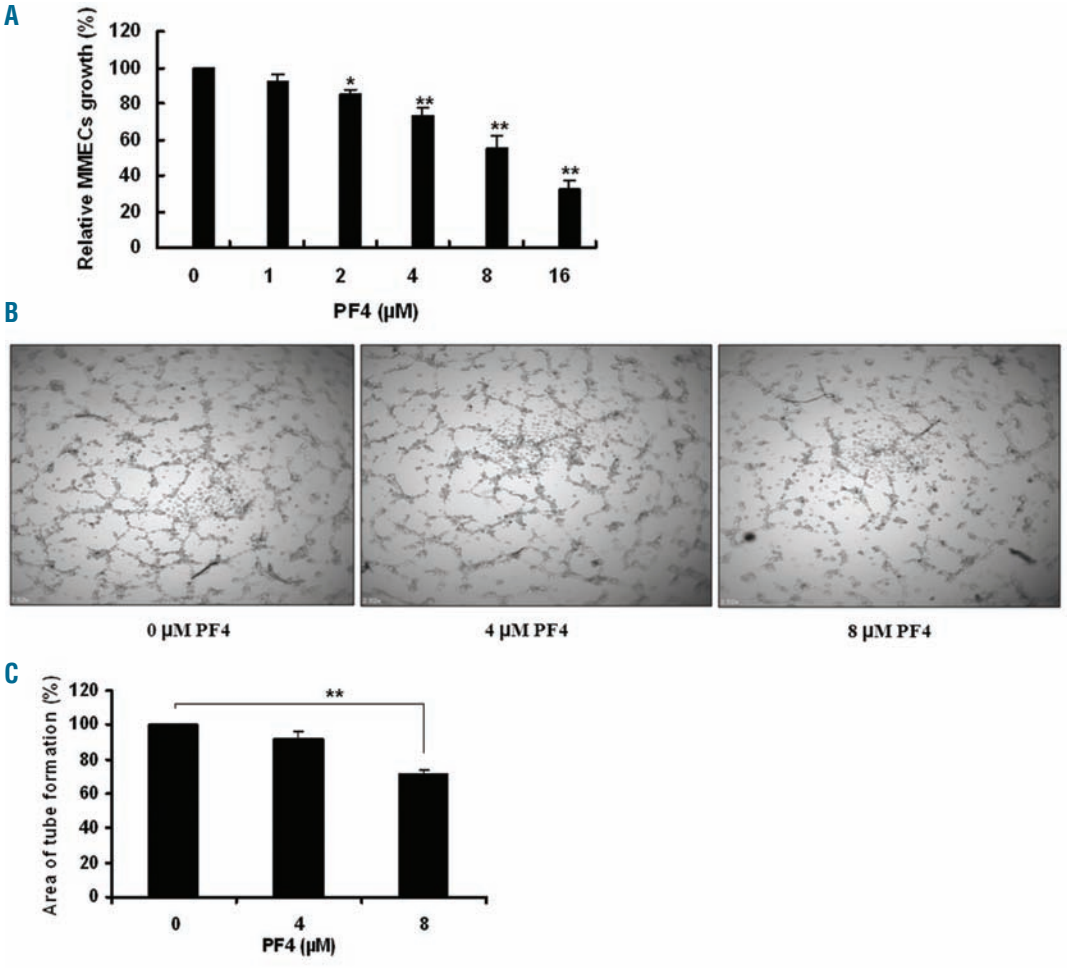
Median age, years (range)	77 (48-89)
Male : Female ratio	20:6
Isotype	
IgG: IgA: light chain	9:11:6
κ:λ	10:16
Salmon-Durie staging	No. of patients
Stage 1	9
Stage 2	12
Stage 3	5
Impaired renal function	4
Osteolytic lesion	12
Bence Jones protein	19
	Median (range)
Hemoglobin level, g/dL	8.8 (7.0-14.4)
White cell count, x 10^9/L	6.1 (2.7-21.6)
Platelet count, x10^9/L	206 (84-350)
Creatinine level, μmol/L	112 (37-656)
Calcium level, mmol/L	2.2 (1.98-2.69)
Albumin level, g/L	35 (27-45)
Bone marrow plasma cells (%)	39 (7-90)



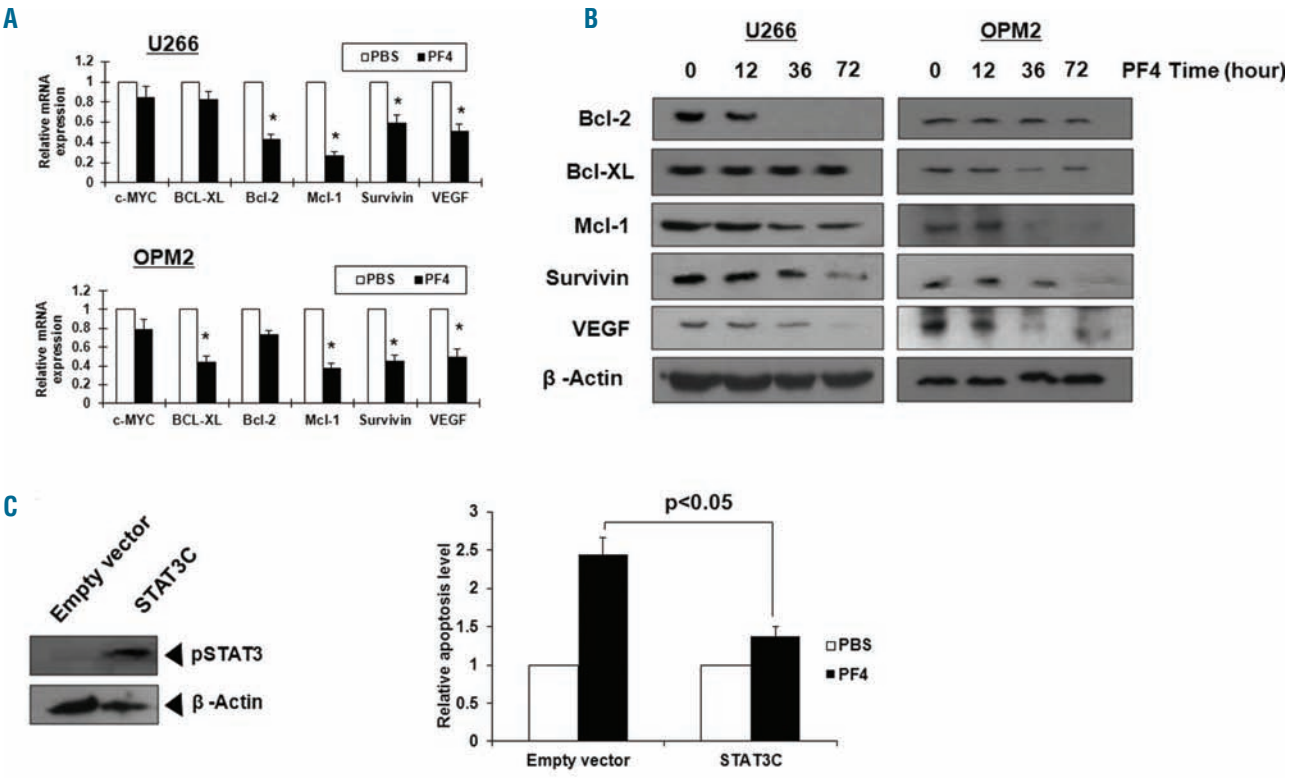
Online Supplementary Figure S1. Pretreatment with cycloheximide diminished the apoptotic effect of PF4 on MM cells. OPM2 cells were pretreated with cycloheximide (CHX) (2 μg/mL for 2 h), followed by exposure to 4 μM PF4 for 96 h. Apoptosis was measured by flow cytometry using annexin V/7-AAD staining. Results from three experiments are shown (*P*=0.001).



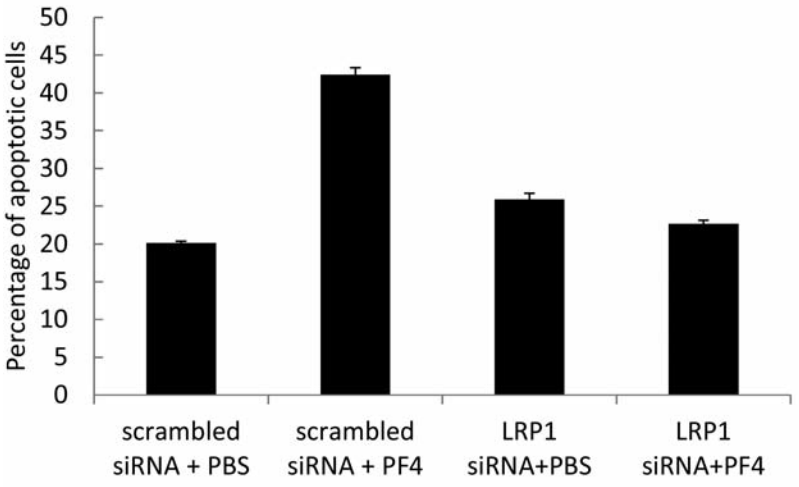
Online Supplementary Figure S2. PF4 induced apoptosis in MM cells. U266 and OPM2 were treated with PF4 for 96 h and apoptosis was determined by a TUNEL assay. The crown cells indicate apoptotic cells.



Online Supplementary Figure S3. PF4 inhibited growth and tube formation of endothelial cells isolated from bone marrow of MM patients (MMECs). (A) MMECs were treated with or without PF4 at the indicated doses for 96 h, and the extent of growth inhibition was measured by WST-1 assay. (B) MMECs were premixed with different concentrations of PF4 and added on top of the matrigel matrix. The extent of inhibition of MMEC tube formation by PF4 was assessed by a tube formation assay. Representative photographs of each treatment group from three independent experiments are shown. (C) The area of formed tubes in each field from (B) was calculated using Image J software.

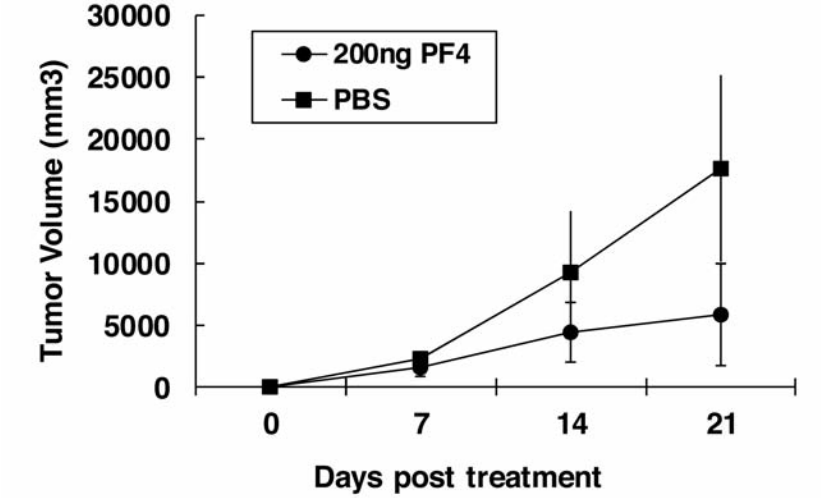


Online Supplementary Figure S4. PF4 suppressed STAT3 target genes. (A) U266 and OPM2 cells were treated with or without 4 μM PF4 for 36 h, followed by RNA extraction and quantitative RT-PCR analyses. (B) U266 cells and OPM2 cells were treated with 4 μM PF4 for the indicated periods while western blot was performed and probed using antibodies against Bcl-2, Bcl-xL, Mcl-1, survivin and VEGF. Beta-actin was used as a control. (C) NCI-H929 cells were transfected with constitutive STAT3 plasmid or empty vector. Cells were harvested and overexpression of STAT3 was confirmed by western blot analysis (left panel). NCI-H929 and constitutively active STAT3 overexpressed cells were treated with PBS or 4 μM PF4 for 96 h and then apoptosis was determined by annexin V and 7-AAD staining and analyzed by flow cytometry (right panel).

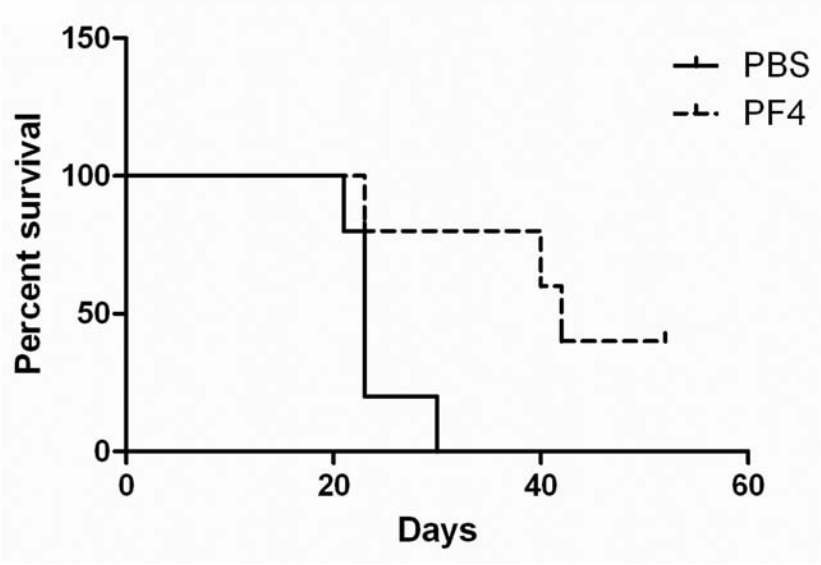


Online Supplementary Figure S5. Knockdown of LRP1 expression abrogated PF4-induced apoptosis. U266 cells were transfected with LRP1 siRNA (or scrambled siRNA), then treated with 4 μM PF4. Apoptosis was measured by flow cytometry using annexin V/7-AAD staining, and results of triplicate experiments are shown (P<0.001).

A



B



Online Supplementary Figure S6. PF4 inhibited tumor growth and prolonged survival in the subcutaneous Matrigel xenograft model. (A) OPM2 cells ($3x10^7$/mouse) mixed with Matrigel were subcutaneously implanted in the NOD-SCID mice. Three weeks later, mice were randomized to treatment groups and treated intravenously with PF4 (200 ng), three times a week for 6 weeks. Data are presented as mean tumor volume±SD (n=5/group). (B) Kaplan-Meier survival plot showed an increase in survival of mice receiving PF4 (200 ng) compared with PBS-treated controls in the subcutaneous Matrigel xenograft model in (A).