Leukemia cells remodel marrow adipocytes *via* TRPV4-dependent lipolysis

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Supplementary method

Cell culture and reagents

The leukemia cell lines THP-1, K562 and HL-60 were purchased from the Chinese Academy of Sciences Cell Bank, Shanghai, China. THP-1, K562 and FBL-3 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, contains L-Glutamine). HL-60 cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Gibco, contains 3g/L D-Glucose, L-Glutamine, HEPES). Mesenchymal stem cells (MSCs) were cultured in low glucose Dulbecco’s modified Eagle’s medium (DMEM, Gibco, contains 1g/L D-Glucose, L-Glutamine, 110mg/L Sosium Pyruvate). Adipocytes were cultured in high glucose DMEM (Gibco, contains 4.5g/L D-Glucose, L-Glutamine, 110mg/L Sosium Pyruvate). All of the above media contained 10% fetal bovine serum (FBS, Gibco), 1% L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All these cells were cultured at 37°C in 5% CO_2. RN1734 (MCE，HY19975, USA) and 4αPDD (MCE, HY18739，USA) are inhibitors and agonists of TRPV4, respectively. BM adipocytes were treated with rhGDF15 (Peprotech 120–28, USA), a neutralizing anti-GDF15 antibody (R&D Systems, MAB957, USA), Repsox (MCE，HY13012,USA), ITD1 (MCE, HY12704,USA) or PI3K-IN-1(MCE, HY12068, USA) to observe the effect of GDF15.

Adipogenic induction of Mesenchymal stem cells

All BM adipocytes used *in vitro* were derived from the induction into adipogenesis by MSCs. MSCs were planted with 5×10^5 into 24-well plates. To induce adipogenic differentiation, MSCs were treated with an adipogenic medium A consisting of high glucose DMEM supplemented with 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, USA), 0.2 mM indomethacin (Sigma-Aldrich, USA), 1 mM dexamethasone (Sigma-Aldrich, USA) and 10 mM insulin (Sigma-Aldrich, USA) for three days. Subsequently, MSCs were treated with an adipogenic medium B consisting of high glucose DMEM supplemented with 10% FBS and 10 mM insulin (Sigma-Aldrich, USA) for one day. This is one cycle of the induction. Finally, three cycles were conducted and maintained the induced adipocytes in high glucose DMEM with 10% FBS.

Co-culture assay

Differentiated BM adipocytes were placed in a normal growth medium until the co-culture experiment. For co-culture experiments, leukemia cell lines (THP-1, HL-60, K562, FBL-3) were harvested, resuspended in serum medium and planted on adipocytes of the Transwell system (0.4 μm, Corning, USA) at a density of 5×10^5 per well in 1 mL RPMI 1640 medium or IMDM medium containing 10% FBS. The adipocyte/leukemia cell co-cultures were then incubated for 4 days.

Free fatty acids detection

The adipocytes were cultured in DMEM high glucose medium containing 10% FBS. Dimethyl sulfoxide (DMSO), RN1734 (5 μM), 4αPDD (0.25 μg/mL) and rhGDF15 (200 ng/mL) were added to the above medium according to the experimental design. The supernatant of BM adipocytes was collected. The free fatty acids (FFAs) in the supernatant were determined using a free fatty acid kit (FUJIFILM Wako Pure Chemical Corporation, Japan).

Lentiviral knockdown

Scrambled lentivirus was used as the lentivirus control (Control shRNA lentivirus, Ctr). TRPV4-targeted shRNA (shTRPV4), TGFβRII-targeted shRNA (shTGFβRII) or FOXC1-targeted shRNA (shFOXC1) lentivirus were purchased from Jikai Biotechnology Co., Ltd. (Shanghai, China). MSCs were plated at a density of 5×10^5 /well in 24-well plates and were induced into adipocytes. After 24 h, BM adipocytes were infected with shTRPV4, shTGFβRII and shFOXC1 lentiviral stock. RT-qPCR and Western blot were used to analyze BM adipocytes infected for 48 h.

Calcium measurement

Fluo 3-AM (excitation wavelength, 506 nm; emission wavelength, 526 nm, AAT Bioquest, USA) was used to measure the intracellular calcium levels. BM adipocytes were cultured with rhGDF15 (200 ng/mL) for 4 days and 8 days. BM adipocytes were treated with RN1734 (5 μM) and 4αPDD (0.25 μg/mL) for 4 days. Calcium levels with treatment of 1 μg/mL Fluo 3-AM were determined by flow cytometry analysis of aliquots of BM adipocytes. The cells were incubated at 37°C for 30 min with shaking, washed with Hank's Balanced Salt Solution (HBSS). Adipocytes that were not incubated with Fluo 3-AM were used as a negative control group. Ca^{2+}-dependent Fluo3-AM fluorescence intensity was measured by flow cytometry (BD, USA).

RNA sequencing

BM adipocytes that had been treated with or without recombinant human GDF15 (rhGDF15) for two days were used for this study, according to the manufacturer’s instructions. Briefly, total RNA was isolated using a RNeasy micro kit (Shanghai

Qiming Information Technology Company Limited, China), and used for double-stranded cDNA synthesis and library amplification. RNA sequencing (RNA-seq) libraries were run on an Illumina HiSeq Xten next-generation sequencer using paired-end 150bp sequencing. Red and green signals indicate high and low expression of each gene, respectively.

Western blot analysis and ELISA

Total cellular proteins were extracted with mixed RIPA lysate. Anti-TRPV4 (1:1000, Sigma SAB2104216, USA), anti-PI3K (1:1000, Abcam 40755, USA), anti-pAKT（1:1000，CST4060T, USA），anti-AKT (1:1000, Abcam 179463, USA), anti-Smad2 (1:1000, CST5339, USA), anti-pSmad2 (1:1000, CST18338, USA), anti-Smad4 (1:1000, Abcam 40759, USA), anti-pSmad4 (1:500, Invitrogen PA5-64712, USA), anti-FOXC1 (1:1000, Abcam 5079, USA), anti-HSL(1:1000, Abcam 45422, USA), anti-pHSL(1:1000, Abcam 109400, USA) and anti-β-actin (1:1000, A20120A0702, BioTNT, China) were used. Secondary antibodies were used at 1:10000 (goat anti-rabbit IgG H+L HRP, A20120A0704, BioTNT, China; goat anti-mouse IgG H+L HRP, A20120A0703, BioTNT, China). The FBL-3 cell supernatant was obtained from cells cultured in RPMI 1640 medium with 10% FBS at different cell densities (2×10^5/mL, 5×10^5/mL). The BM fluid of normal obese mice and AML mice was washed with PBS (1 mL). The ELISA analysis for mouse GDF15 was performed according to the manufacturer’s instructions (R&D, MGD150, USA).

Quantitative reverse transcription PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, UK), and RNA samples were converted into cDNA using the Primer Script TM RT reagent Kit (Perfect Real Time, TaKaRa, Japan). All real-time PCR reactions were performed using an ABI 7500 real-time PCR system (Biosystems, Foster City, CA, USA) and the SYBR Premix Ex Taq reagent kit (RR420A, TaKaRa, Japan). The primer sequences are shown in Table1. The results were analyzed using the $2^{-\Delta\Delta CT}$ method and GAPDH to normalize the results.

Cell Counting Kit-8 assay

FBL-3 cells (3×10^3) were plated into 96-well culture plates. Different concentrations of 4αPDD (0 μg/mL, 0.25 μg/mL) were added. The FBL-3 cell proliferation was tested by a Cell Counting Kit-8 assay (CCK8) kit (Dojindo, Japan). 10 μL CCK8 reagent was added to each well and incubated for 2 h at 37 °C. The measurement of absorption at 450 nm was performed using a microplate reader.

Apoptosis assay

FBL-3 cells were treated with 4αPDD (0 μg/mL, 0.25 μg/mL) for 48 h. FBL-3 cells were sorted and double labelled with Annexin V-APC and propidium iodide (PI) (Invitrogen by Thermo Fisher Scientific, USA) to analyze apoptosis, according to the manufacturer's instructions.

Oil red O staining

MSCs were planted with 5×10^5 into 24-well culture plates, followed by induction of adipogenesis. BM adipocytes were treated with dimethyl sulfoxide, RN1734 (5 μM), 4α-phorbol 12,13-didecanoate (4αPDD, 0.25 μg/mL), rhGDF15 (200 ng/mL), Repsox (25 μM) and ITD1 (1 μM). BM adipocytes were fixed in 4% formaldehyde solution for

30 min, washed with PBS for 5 min and stained with Oil red O (ORO) solution (Solarbio, China) for 30 min. We captured the images under an ordinary optical microscope (Olympus, Japan). All images were magnified for 200 times. Subsequently, isopropyl alcohol (500 μL) was added to each well and the eluted ORO was quantified through measurement of the absorption at 490 nm using a microplate reader (Thermo Fisher, USA).

Immunofluorescence

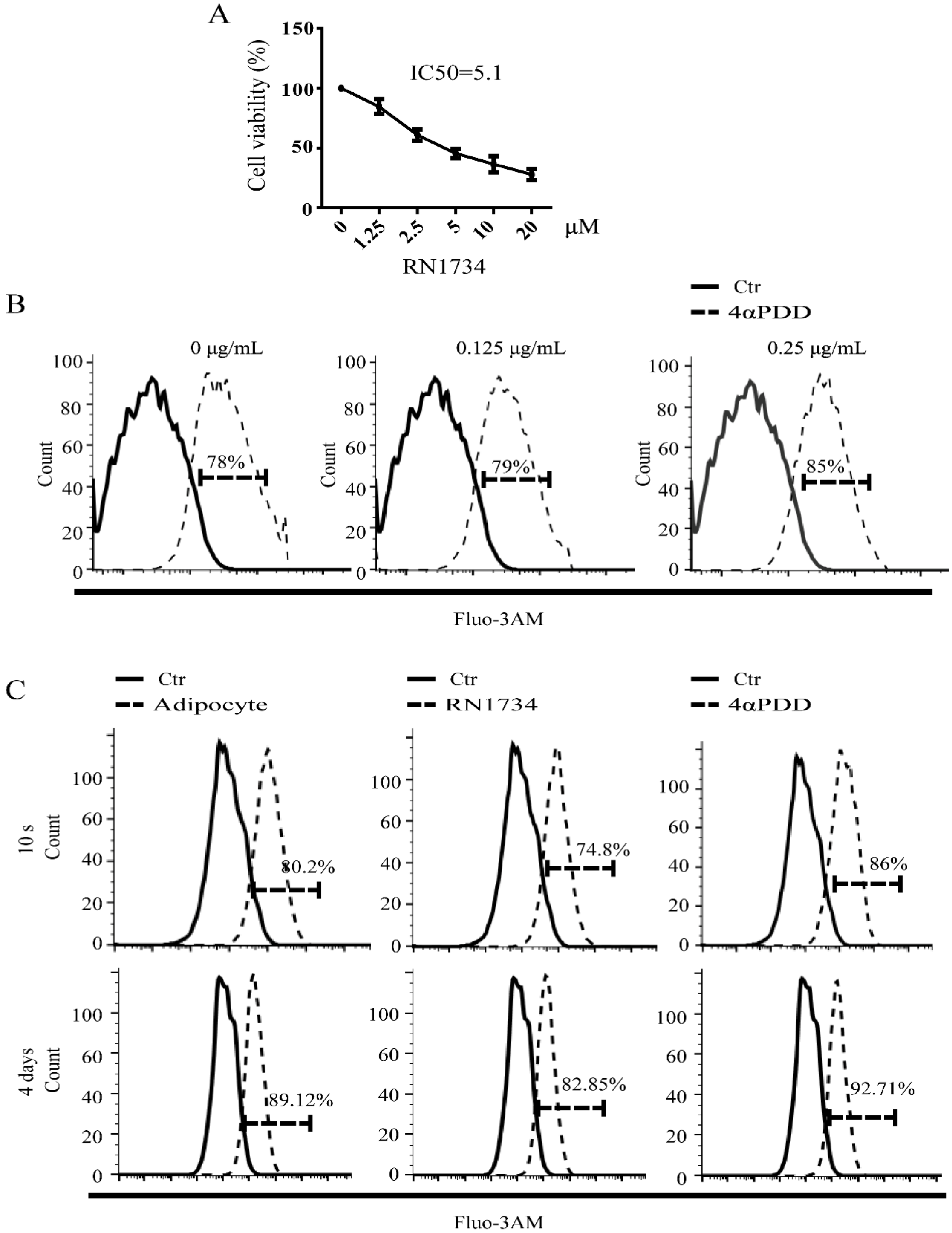
BM adipocytes were treated with shTRPV4 and shTGFβRII for 6 days, and fixed with 4% paraformaldehyde. Alexa Fluor 493/503-conjugated Bodipy (Sigma, USA) was added at a concentration of 2 μg/mL, and stained for 30 min at room temperature. BM adipocytes treated with FBL-3 cells and 4αPDD (0.25 μg/mL) were also stained as described above. BM sections of normal obese mice, AML mice and AML mice treated with 4αPDD were stained with CD117 (rabbit anti-CD117 multiclonal antibody, SAB, USA). DAPI was then dyed for 15 min at room temperature. Fluorescent images were captured using a fluorescence microscope system (Olympus, Japan).

Adipocyte measurement

The number and area of adipocyte were analyzed by Image-Pro-Plus 5.1 software. The resolution of the analysis software is 2048×1536 pixels (408×308 μm). 33×25 meshes could be automatically generated on the images. Each crossing between vertical and horizontal lines was recorded as a point. The software automatically stored the number of positive adipocytes and calculated the corresponding results.

Supplementary Figure

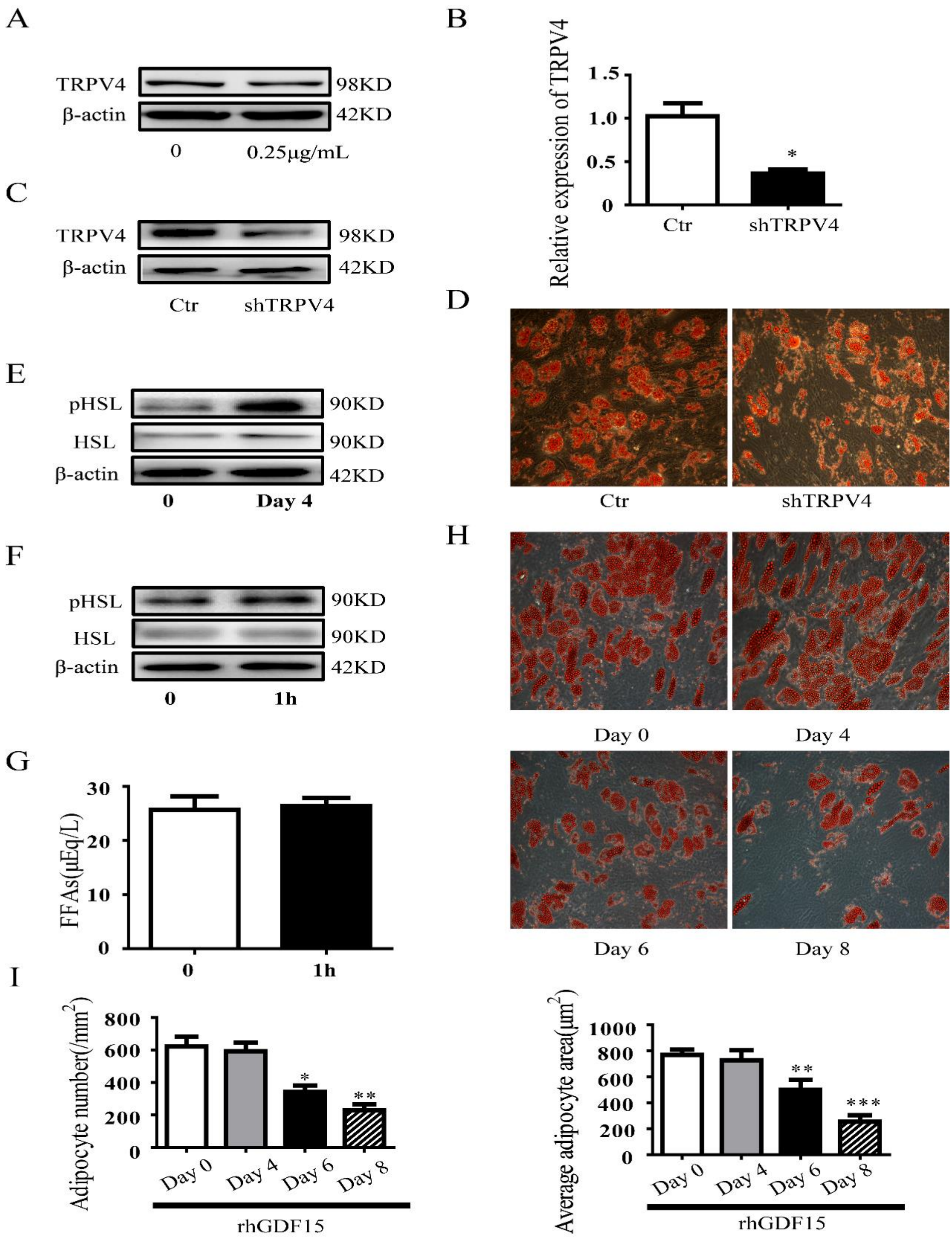
Supplementary Figure S1



Supplementary Figure S1. TRPV4 regulates intracellular calcium. (A) Dose response for the cellular viability of bone marrow (BM) adipocytes treated with RN1734. The IC50 values were calculated using a Nonlinear regression analysis. (B)

Histogram showed the Fluo3-AM fluorescence in BM adipocytes as a measure of the cytosolic free Ca^{2+} concentration. BM adipocytes treated with different concentrations of 4α-phorbol 12,13-didecanoate (4αPDD). The Fluo 3-AM level of calcium was measured by flow cytometry. (C) Histogram showed the Fluo3-AM fluorescence in BM adipocytes as a measure of the cytosolic free Ca^{2+} concentration. BM adipocytes treated with RN1734 and 4αPDD for 10 seconds (s) and 4 days. The Fluo 3-AM level of calcium was measured by flow cytometry. Three independent experiments were performed.

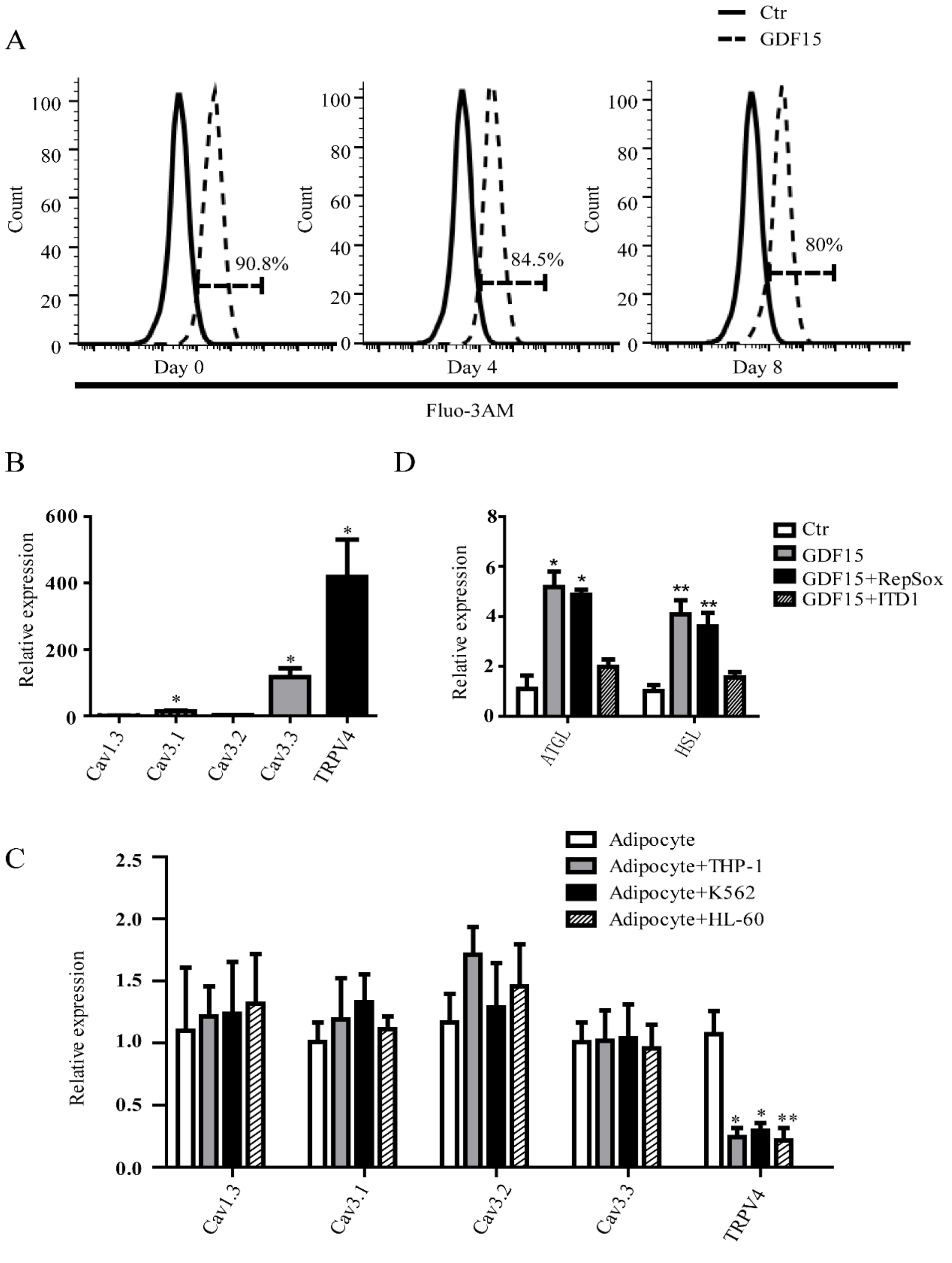
Supplementary Figure S2



Supplementary Figure S2. GDF15 promotes remodeling of bone marrow adipocytes. (A) Western blot was used to verify the expression of TRPV4 in BM adipocytes treated with 4αPDD. (B) RT-qPCR analysis of the expression of TRPV4 mRNA in BM adipocytes infected with shTRPV4 lentivirus for 48 hours (h). (C)

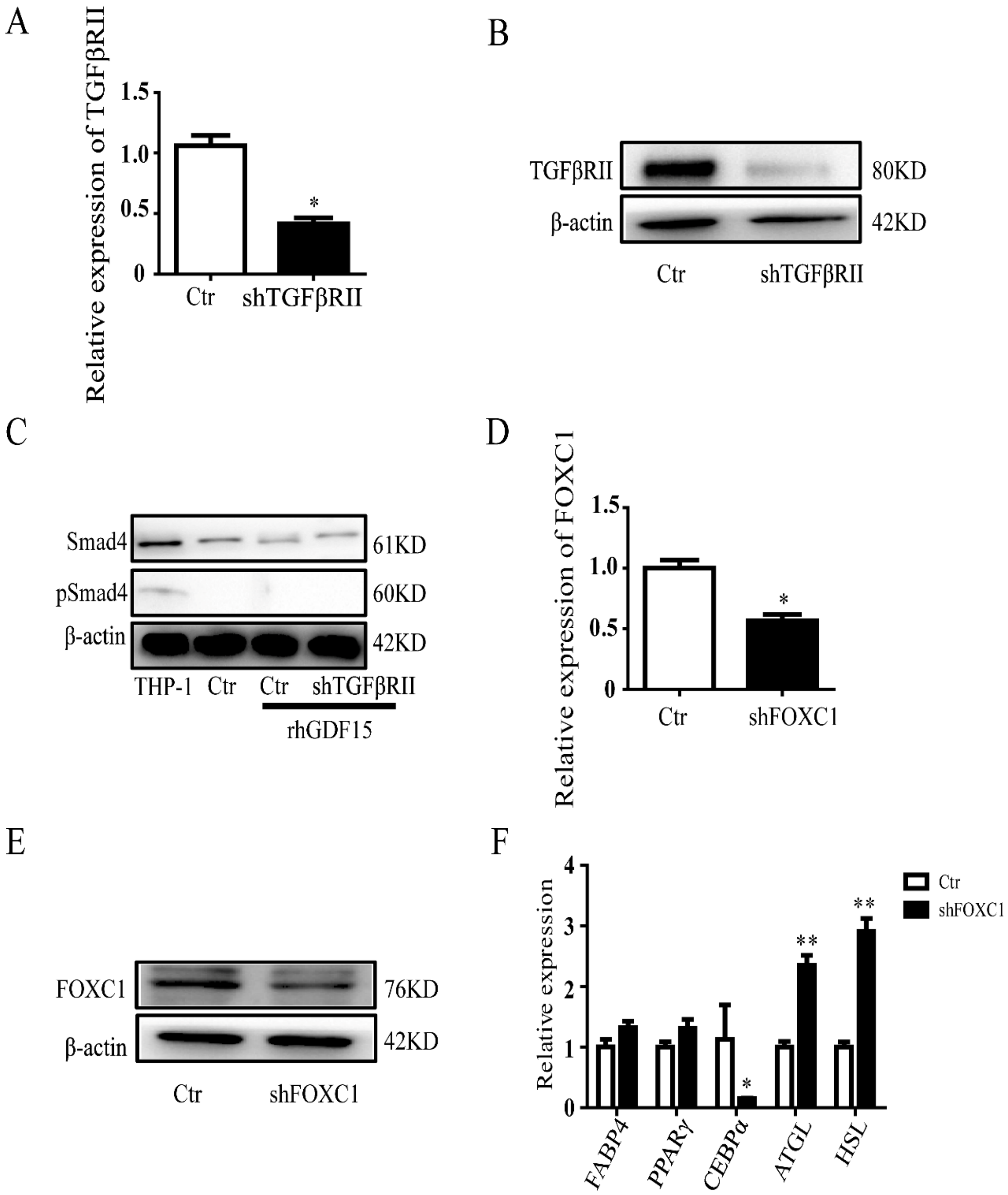
Western blot analysis of the knockdown efficiency of TRPV4 protein in BM adipocytes. (D) BM adipocytes were infected with shTRPV4 lentivirus for 6 days. Adipocytes were stained by Oil red O (ORO) staining. (E, F) showed the expression of HSL and pHSL in BM adipocytes treated with recombinant human GDF15 (rhGDF15) for 4 days and 1 h by Western blot analysis. (G) The content of FFAs in the supernatant of BM adipocytes treated with rhGDF15 for 1 h was detected using the colorimetric method. (H) BM adipocytes treated with rhGDF15 (200 ng/mL) for 0, 4, 6, 8 days. Adipocytes were stained by Oil red O (ORO) staining. All images were at a magnification of 200×. (I) The number and average area of adipocytes from the indicated groups were measured by using Image-Pro-Plus 5.1. Three independent experiments were performed. ****P* < 0.001, ***P* < 0.01, **P* < 0.05.

Supplementary Figure S3



Supplementary Figure S3. The effect of GDF15 on calcium channels in bone marrow adipocytes. (A) Histogram showing the Fluo3-AM fluorescence in BM adipocytes as a measure of the cytosolic free Ca^{2+} concentration. BM adipocytes treated with rhGDF15 for different time (4 days and 8 days). The Fluo 3-AM level of calcium was measured by flow cytometry. (B) RT-qPCR was used to analyze the relative expression of *Cav1.3*, *Cav3.1*, *Cav3.2*, *Cav3.3* and *TRPV4* in BM adipocytes. (C) RT-qPCR were used to analyze the expression of *Cav1.3*, *Cav3.1*, *Cav3.2*, *Cav3.3* and *TRPV4* in BM adipocytes co-cultured with leukemia cell lines (THP-1, K562, HL-60). (D) RT-qPCR was used to analyze *HSL* and *ATGL* mRNA in BM adipocytes induced by rhGDF15 after treatment with RepSox or ITD1 for 4 days. Three independent experiments were performed. $^{**}P < 0.01$, $^{*}P < 0.05$.

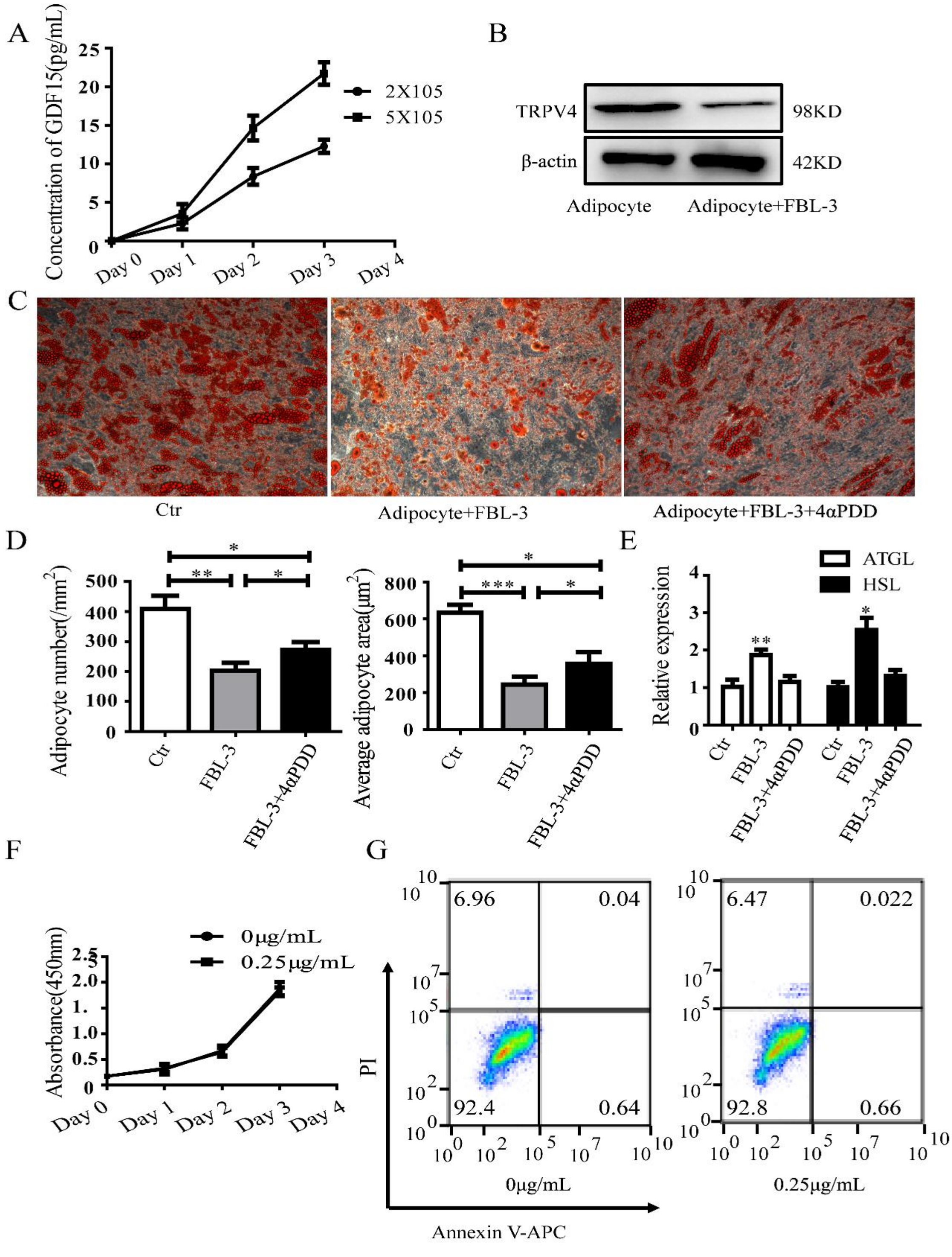
Supplementary Figure S4



Supplementary Figure S4. Validation of knockout efficiency of TGFβRII and FOXC1 genes. (A) RT-qPCR analysis of the expression of *TGFβRII* mRNA in BM adipocytes infected with shTGFβRII lentivirus for 48 h. (B) Western blot analysis of the knockdown efficiency of TGFβRII protein in BM adipocytes. (C) BM adipocytes were infected with shTGFβRII lentivirus for 48 h and then cultured with rhGDF15 for

4 days. The protein of Smad4 and pSmad4 was detected using Western blot analysis. THP-1 cells were used as positive control. (D) RT-qPCR analysis of the expression of *FOXC1* in BM adipocytes infected with shFOXC1 lentivirus for 48 h. (E) Western blot analysis of the efficiency of FOXC1 knockdown in BM adipocytes infected with shFOXC1 lentivirus. (F) RT-qPCR was used to analyze *FABP4*, *PPARγ*, *CEBPα*, *ATGL* and *HSL* mRNA levels after treatment with shFOXC1 lentivirus for 48 h. Three independent experiments were performed. ** $P < 0.01$, * $P < 0.05$.

Supplementary Figure S5

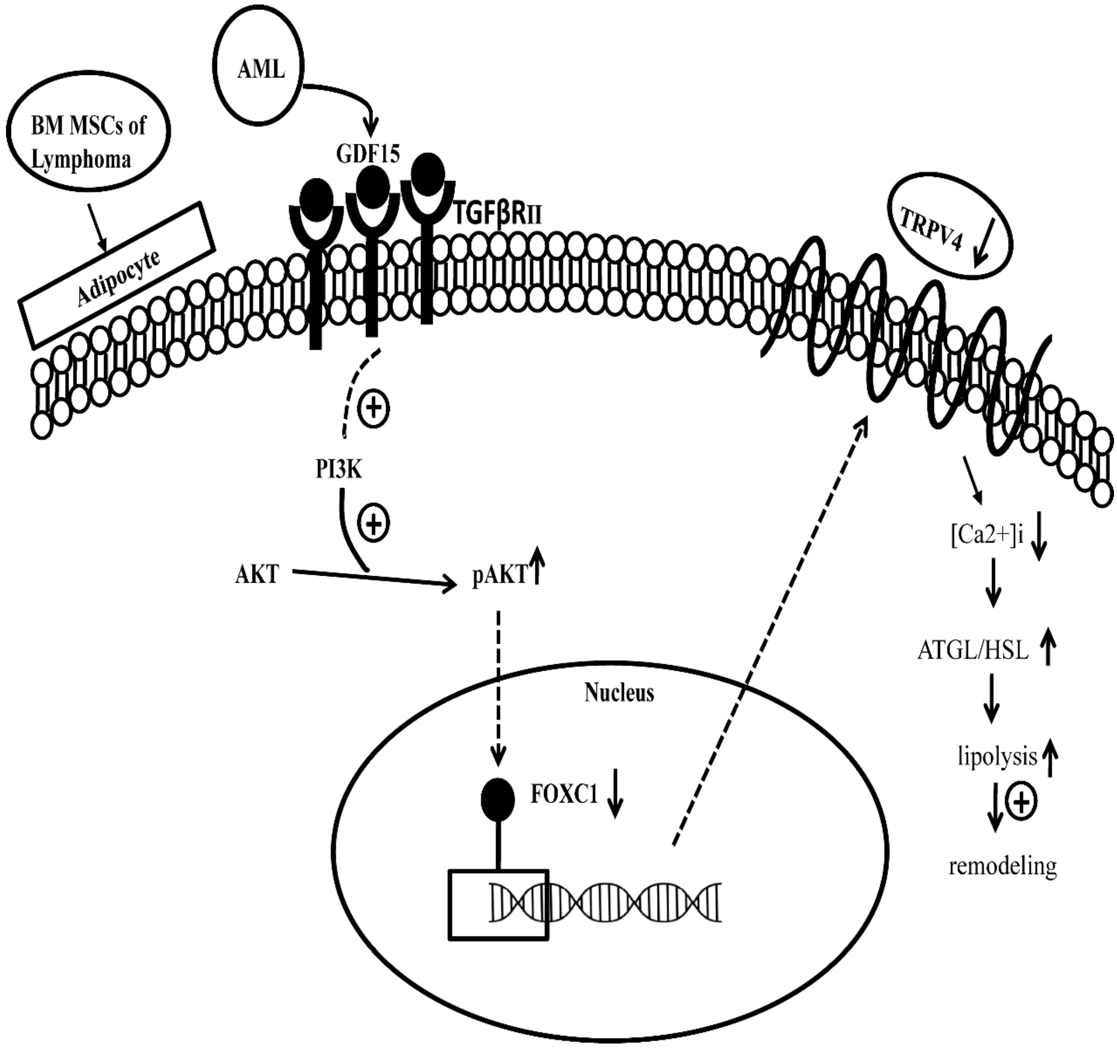


Supplementary Figure S5. FBL-3 cells remodel bone marrow adipocytes *in vitro*.

(A) Concentration of GDF15 in supernatant of FBL-3 cells (2×10^5/mL and 5×10^5/mL) by ELISA at different time (1, 2, 3 days). (B) Western blot analysis of the expression of TRPV4 protein after co-cultured of FBL-3 cells with BM adipocytes for 4 days. (C, D)

BM adipocytes co-cultured with dimethyl sulfoxide (Ctr), FBL-3 (2×10^5/mL) or FBL-3 (2×10^5/mL) and 4αPDD (0.25μg/mL) for 6 days. Adipocytes were stained by ORO staining. All images were at a magnification of 200×. The number and average area of adipocytes from the indicated groups were measured by using Image-Pro-Plus 5.1. (E) BM adipocytes co-cultured with dimethyl sulfoxide (Ctr), FBL-3 (2×10^5/mL) or FBL-3 (2×10^5/mL) and 4αPDD (0.25μg/mL) for 4 days. RT-qPCR was used to analyze *ATGL* and *HSL* mRNA. (F) CCK8 assay was used to analyze the effect of 4αPDD (0μg/mL and 0.25μg/mL) on FBL-3 cell proliferation. (G) Flow cytometry analysis of the apoptosis of FBL-3 cells treated with 4αPDD (0.25μg/mL). Three independent experiments were performed. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

Supplementary Figure S6



Supplementary Figure S6. Patterns of TRPV4 in bone marrow adipocytes regulated by GDF15 derived from leukemia cells. MSCs were derived from lymphoma patients without BM invasion and were induced into mature adipocytes *in vitro*. GDF15 secreted by leukemia cells binds to TGFβRII on BM adipocytes. Subsequently, the PI3K/AKT pathway in the adipocytes is activated, thereby inhibiting the expression of the transcription factor FOXC1. This series of processes leads to a decrease in the expression of TRPV4 in BM adipocytes and promotes BM adipocyte remodeling. Thus, Ca^{2+} influx ($[Ca^{2+}]_i$) is reduced, which promotes the expression of

ATGL and HSL in BM adipocytes. Eventually, BM adipocytes undergo lipolysis and remodeling.