Supplementary Information

Adipose-derived stem cells enhance human breast cancer growth and cancer stem cell-like properties through adipsin

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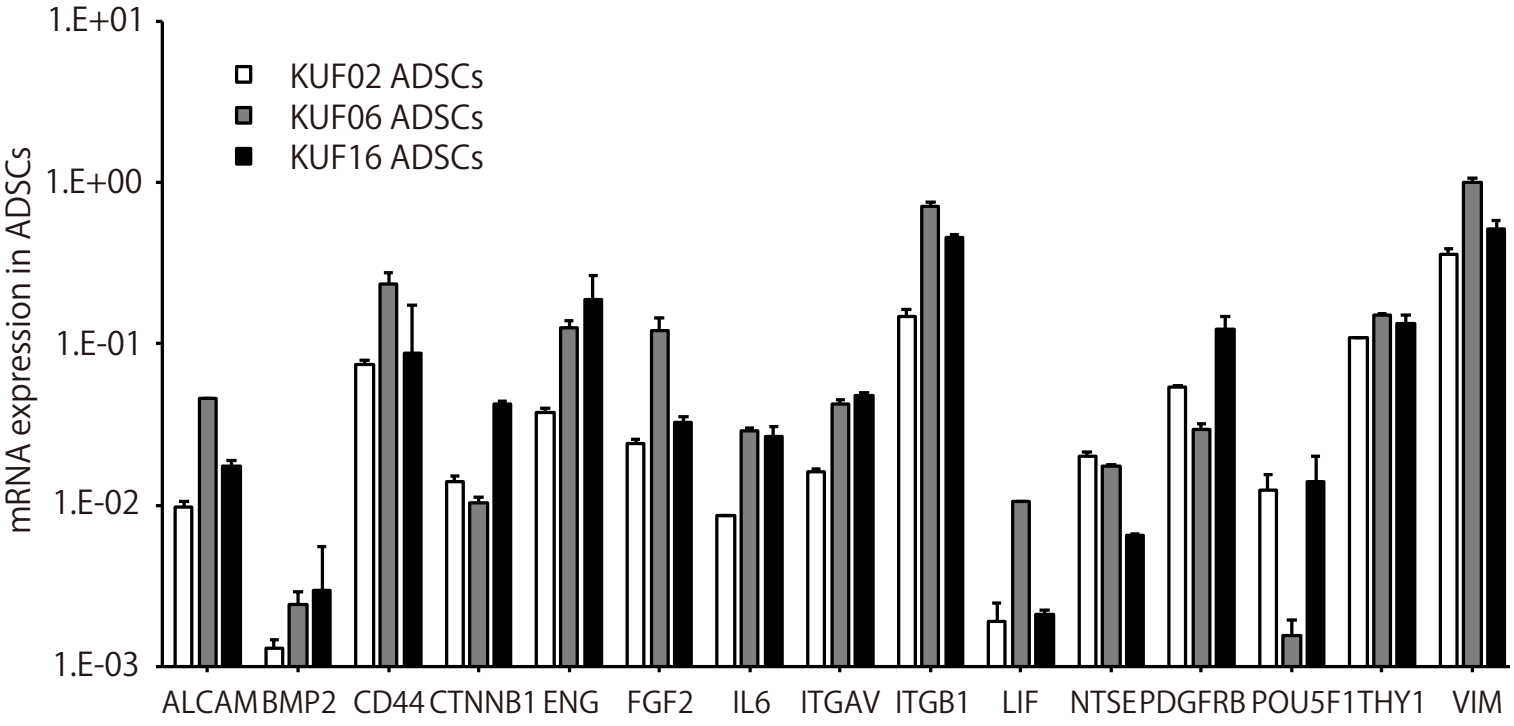

Figure S1. Expression profile of mesenchymal stem cell-related genes in human mammary ADSCs. Gene expression profile of three mammary ADSCs were analyzed using the Human Mesenchymal Stem Cell RT^2 Profiler PCR Array (PAHS-082ZA, QIAGEN). Expression level of the representative mesenchymal stem cell genes were presented. Relative gene expression was calculated by using the $\Delta\Delta$CT method with *GAPDH* as an internal control.

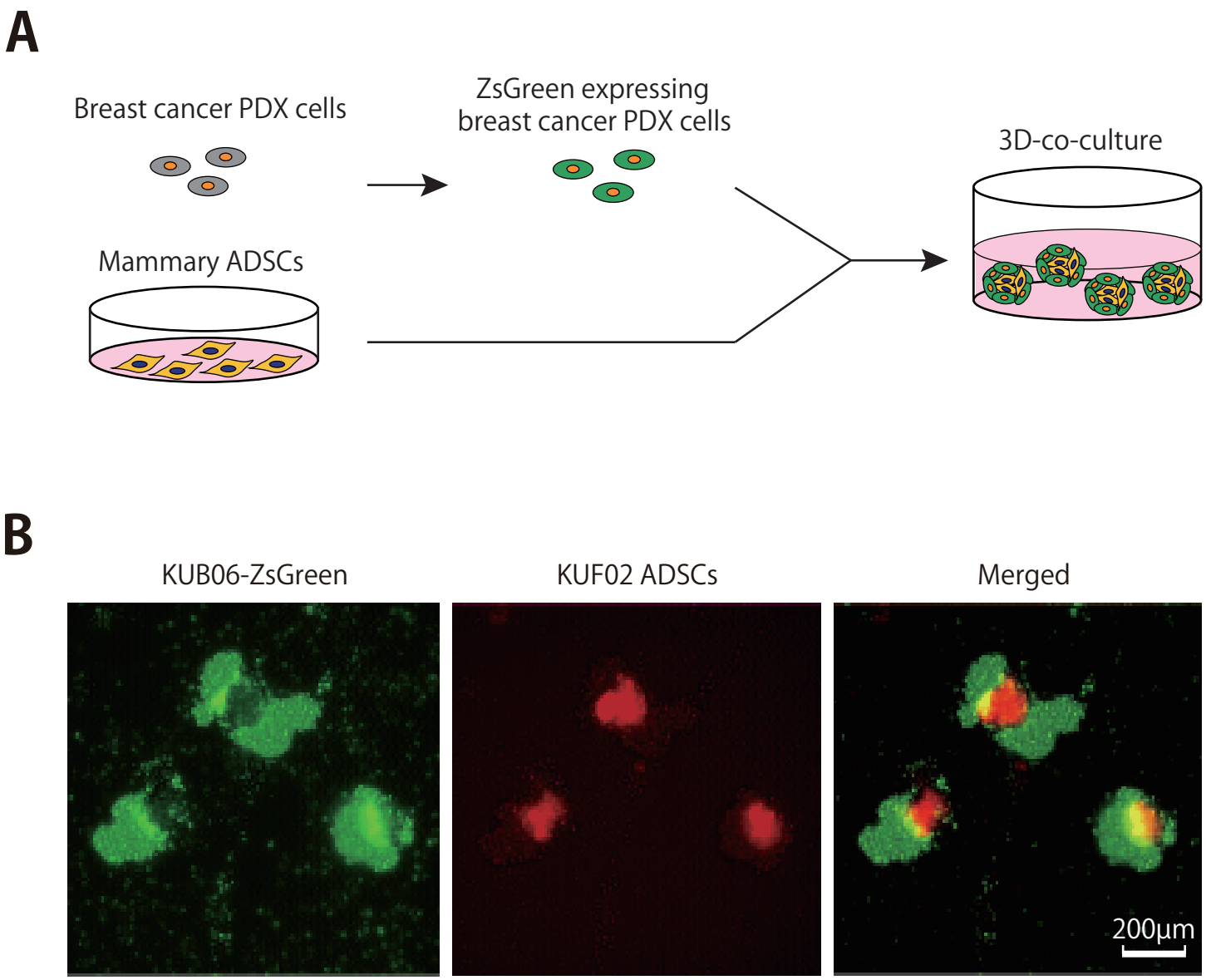

Figure S2. Close contact between breast cancer PDX cells and mammary ADSCs formed spheres in the three dimensional (3D) co-culture system. **(A)** Schematic illustration. **(B)** Close contact between breast cancer PDX cells and mammary ADSCs in the spheres. Fluorescent microscopic images of the spheres formed by ZsGreen expressing breast cancer PDX cells (KUB06-ZsGreen) and ADSCs (KUF02) co-cultured using a 3D-culture system. Green: ZsGreen-expressing KUB06 PDX cells, Red: ADSCs (weakly auto-fluorescent).

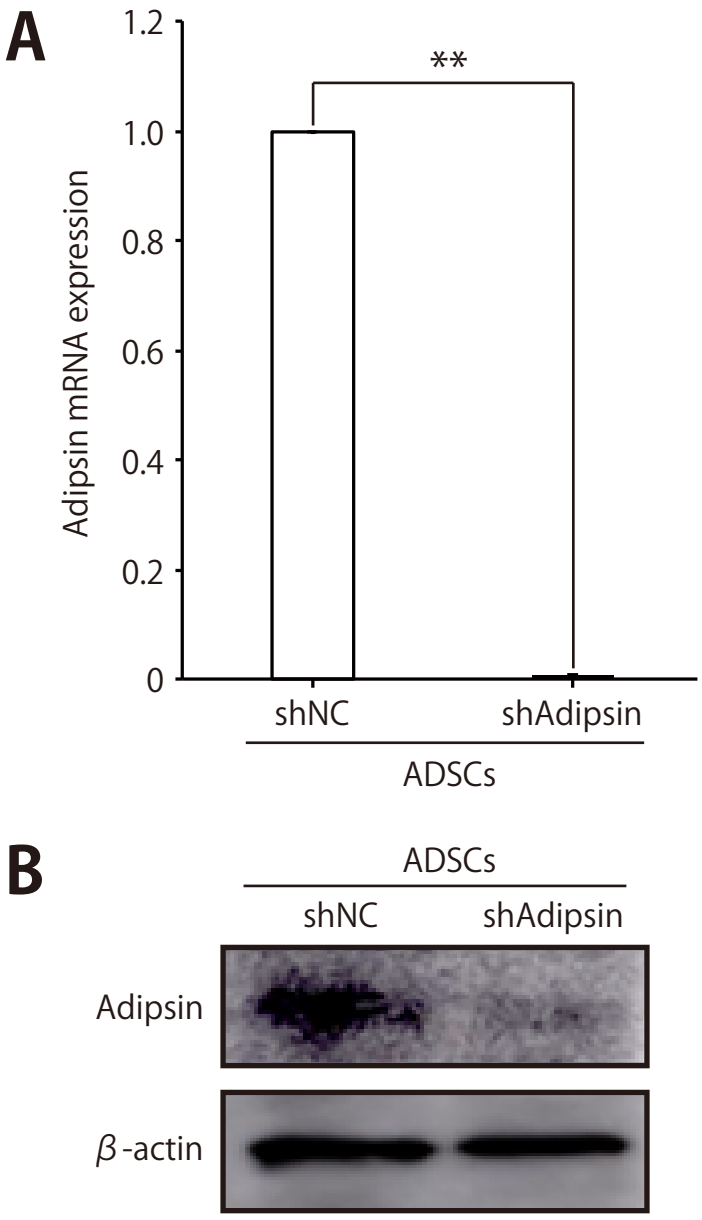

Figure S3. Effective knockdown of *adipsin* by shRNA in mammary ADSCs. **(A)** semi-quantitative PCR. *GAPDH* was used as a control. ** $p < 0.01$. **(B)** Western blot. β-actin was used as a control.

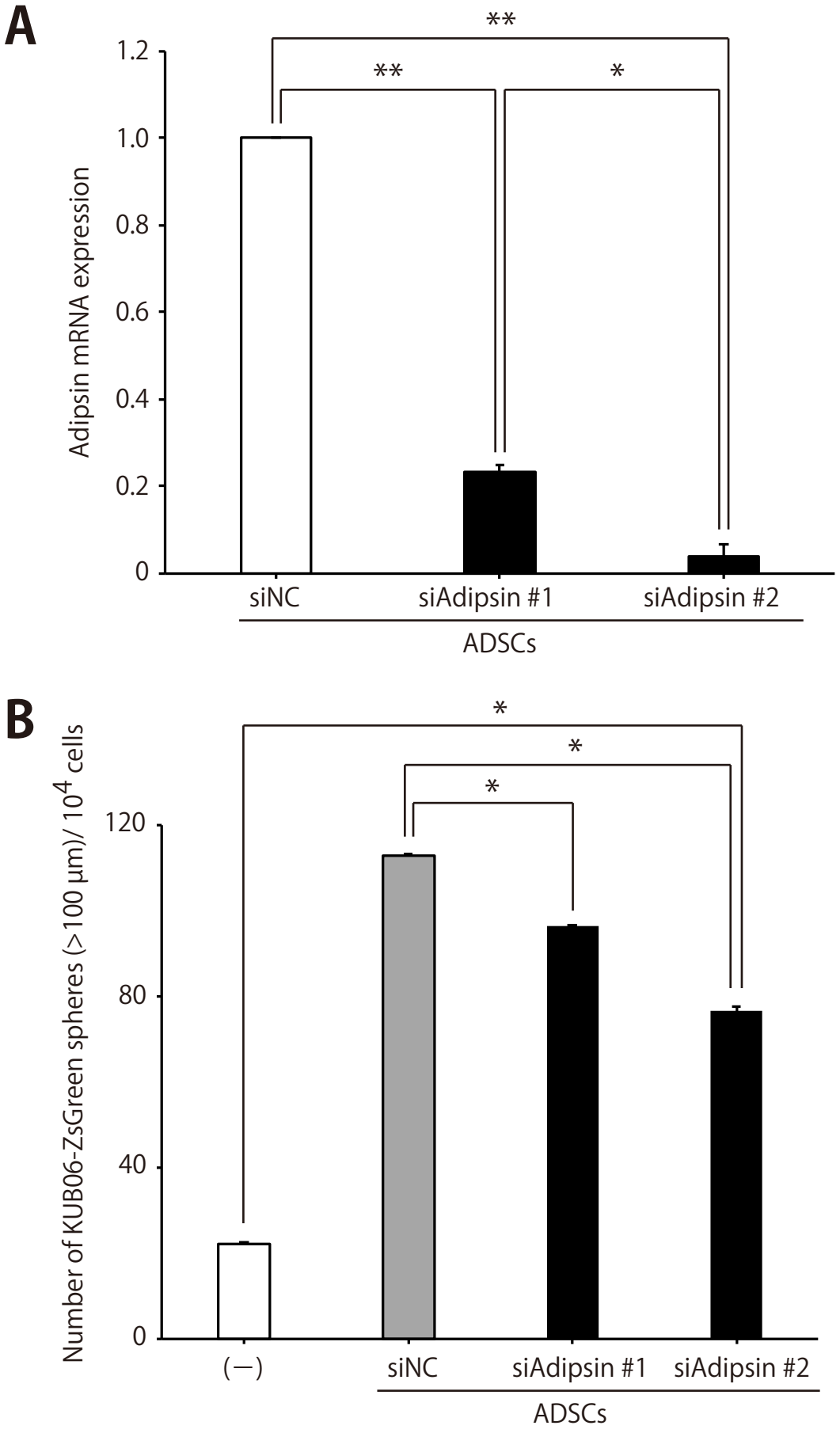

Figure S4. Suppression of the sphere-forming ability of breast cancer PDX cells by siRNA-mediated knockdown of *adipsin* in mammary ADSCs. **(A)** Reduction of *adipsin* mRNA levels in the KUF06 ADSCs transfected with siRNAs against *adipsin* (si*Adipsin*#1 and #2). *GAPDH* was used as a control. *$p < 0.05$, **$p < 0.01$. **(B)** Number of the spheres formed by breast cancer PDX (KUB06-ZsGreen) cells co-cultured with ADSCs transfected with siRNAs against *adipsin* (si*Adipsin*#1 or #2), or negative control (si*NC*) (PDX to KUF ratio 5:1) for 7 days. *$p < 0.05$.

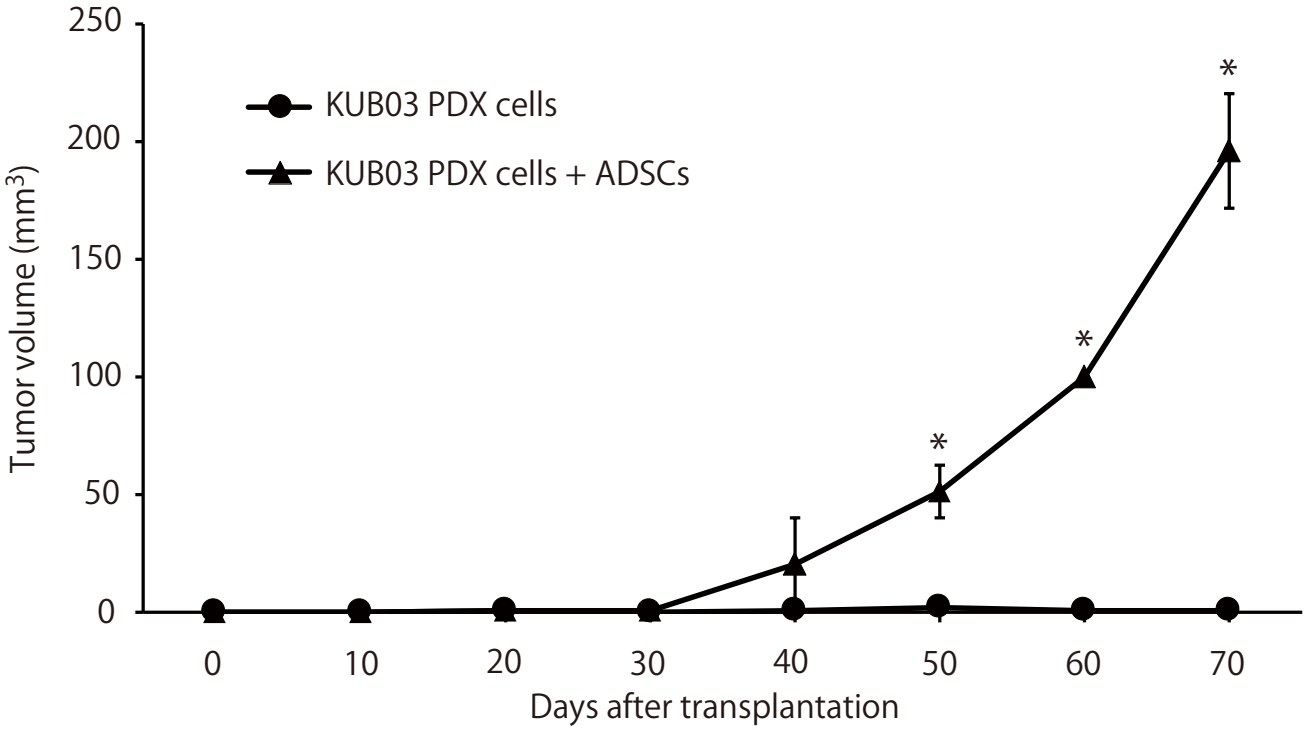

Figure. S5. Enhancement of the hormone-receptor-negative breast cancer PDX growth by co-transplantation with mammary ADSCs. One hundred thousand hormone-receptor-negative breast cancer PDX cells (KUB03 PDX cells) were transplanted with or without 1 x 10^5 KUF02 ADSCs into mammary fat pad regions of NOD/SCID mice. Tumor volume was measured twice a week. *$p < 0.05$.

Supplementary Table S1. Expression levels of the 84 genes related to mesenchymal stem cells and their differentiation in the human mammary ADSCs (KUF).

Gene symbol	Expression level (relative to *GAPDH* mRNA)		
	KUF02	KUF06	KUF16
ABCB1	1.31E-04	1.31E-04	4.68E-04
ACTA2	2.44E-02	1.21E-01	6.58E-01
ALCAM	9.79E-03	4.56E-02	1.75E-02
ANPEP	3.59E-02	4.73E-02	1.32E-02
ANXA5	6.61E-02	1.84E-01	1.30E-01
BDNF	7.03E-04	1.62E-02	1.25E-03
BGLAP	3.24E-03	3.07E-04	6.06E-03
BMP2	1.29E-03	2.43E-03	2.98E-03
BMP4	6.56E-03	2.70E-03	7.88E-04
BMP6	5.16E-03	8.30E-03	6.29E-03
BMP7	2.78E-02	1.86E-04	1.18E-03
CASP3	5.92E-03	3.95E-03	8.61E-03
CD44	7.40E-02	2.35E-01	8.76E-02
COL1A1	1.65E+00	6.29E+00	6.95E+00
CSF2	5.24E-03	1.35E-04	4.40E-04
CSF3	3.43E-03	2.27E-04	1.80E-03
CTNNB1	1.41E-02	1.04E-02	4.26E-02
EGF	1.13E-02	3.70E-04	8.43E-03
ENG	3.73E-02	1.26E-01	1.87E-01
ERBB2	1.80E-02	8.89E-03	1.04E-02
FGF10	6.76E-02	3.08E-03	6.03E-02
FGF2	2.41E-02	1.20E-01	3.25E-02
FUT1	8.37E-03	3.29E-04	9.75E-03
FUT4	2.28E-03	6.74E-04	2.36E-03
FZD9	2.05E-01	1.64E+01	5.01E+02
GDF15	1.91E-02	2.32E-02	4.97E-02
GDF5	1.20E-03	2.56E-04	6.41E-04
GDF6	3.38E-03	1.41E-03	7.28E-03
GDF7	7.96E-04	8.83E-05	2.55E-04
GTF3A	3.75E-03	2.35E-03	4.36E-03
HAT1	2.23E-03	8.66E-04	2.36E-03
HDAC1	4.23E-03	4.60E-03	5.01E-03
HGF	3.38E-03	1.52E-04	7.06E-04
HNF1A	1.49E-03	1.70E-04	1.06E-03

ICAM1	5.13E-03	5.88E-02	9.56E-03
IFNG	3.17E-01	2.91E-05	6.54E-03
IGF1	5.49E-03	3.28E-04	2.00E-03
IL10	5.77E-04	7.74E-05	4.59E-04
IL1B	2.08E-03	1.85E-02	7.51E-04
IL6	8.64E-03	2.88E-02	2.69E-02
INS	9.03E-03	1.52E-04	1.32E-03
ITGA6	2.11E-03	3.31E-03	1.42E-03
ITGAV	1.61E-02	4.26E-02	4.81E-02
ITGAX	1.11E-03	9.29E-05	8.02E-04
ITGB1	1.49E-01	7.07E-01	4.54E-01
JAG1	3.86E-03	7.39E-04	1.51E-03
KAT2B	2.57E-03	3.38E-03	3.36E-03
KDR	1.27E-02	6.14E-04	7.26E-03
KITLG	2.15E-01	4.34E-03	4.26E-03
LIF	1.92E-03	1.05E-02	2.11E-03
MCAM	1.67E-03	7.57E-04	9.96E-03
MMP2	3.14E-01	3.70E-01	3.54E-01
NES	2.30E-03	1.80E-03	1.51E-03
NGFR	7.00E-04	1.46E-04	1.26E-03
NOTCH1	3.47E-03	1.87E-03	2.61E-03
NT5E	2.02E-02	1.75E-02	6.55E-03
NUDT6	6.88E-03	2.65E-04	2.68E-03
PDGFRB	5.37E-02	2.97E-02	1.24E-01
PIGS	6.64E-03	2.72E-03	7.55E-03
POU5F1	1.26E-02	1.56E-03	1.42E-02
PPARG	1.56E-03	2.59E-04	1.69E-03
PROM1	1.51E-03	7.40E-05	1.10E-03
PTK2	5.96E-03	9.62E-03	9.83E-03
PTPRC	7.34E-04	9.52E-05	3.84E-04
RHOA	3.80E-02	2.26E-02	6.01E-02
RUNX2	2.31E-03	3.14E-03	3.15E-03
SLC17A5	4.95E-03	6.74E-03	3.61E-03
SMAD4	7.30E-03	7.48E-03	1.09E-02
SMURF1	2.23E-02	4.85E-03	6.99E-03
SMURF2	2.85E-02	4.89E-02	1.84E-02
SOX2	1.82E-03	1.95E-04	1.82E-03
SOX9	2.20E-03	1.13E-02	1.06E-02
TBX5	6.69E-03	1.32E-02	7.80E-02

TERT	1.67E-03	3.41E-04	2.35E-03
TGFB1	1.06E-02	3.26E-02	3.23E-02
TGFB3	5.68E-03	1.22E-03	1.25E-02
THY1	1.09E-01	1.52E-01	1.34E-01
TNF	9.20E-04	1.54E-04	5.08E-04
VCAM1	1.53E-03	1.86E-04	7.71E-04
VEGFA	4.88E-03	1.02E-02	1.21E-02
VIM	3.62E-01	1.01E+00	5.20E-01
VWF	1.60E-03	9.31E-05	1.07E-03
WNT3A	3.76E-01	9.28E-04	2.21E-01
ZFP42	4.72E-04	2.11E-04	1.07E-03
ACTB	2.35E-02	1.85E-02	1.15E+01
B2M	5.32E-02	1.16E-01	1.59E-01
GAPDH	1.35E+01	9.12E-01	7.81E+00
HPRT1	1.23E-03	1.24E-03	2.01E-01
RPLP0	1.84E-01	6.65E-02	3.19E-01
HGDC	7.93E-04	5.97E-05	1.76E-03

Supplementary Table S2. Characteristics of the patients whose expression level of *adipsin* mRNA in mammary adipose tissue was measured.

Characteristics (n = 20)		
Age - median (range)	68	(47 - 88)
Gender - No. (%)		
Female	20	(100)
Menopause - No. (%)		
Yes	17	(85)
No	3	(15)
Body Mass Index (kg/m^2) - median (range)	23	(17 - 44)
Comorbidity - No. (%)		
Hypertension	9	(45)
Diabetes mellitus	3	(15)
Tumor stage - No. (%)		
T1	7	(35)
T2	13	(65)
Lymph node stage - No. (%)		
N0	14	(70)
N1	2	(10)
N2	4	(20)
Metastasis stage - No. (%)		
M0	20	(100)
Estrogen receptor (ER) - No. (%)		
Positive	17	(85)
Negative	3	(15)
Progesterone receptor (PgR) - No. (%)		
Positive	15	(75)
Negative	5	(25)
Human epidermal growth factor receptor 2 (HER2) - No. (%)		
Positive	0	(0)
Negative	20	(100)
Triple negative breast cancer - No. (%)		
Yes	3	(15)
No	17	(85)
Ki-67 index (%) - median (range)	6	(1 - 60)

Supplementary Table S3. Primer sequences for semi-quantitative real-time PCR

Gene name		Primer sequence (5'-3')
Adipsin	Forward	GGAGCAGTGGGTGCTGAG
	Reverse	AGCTGTAGCAGCAGGAGGTC
PPARG	Forward	GAGCCCAAGTTTGAGTTTGC
	Reverse	CTGTGAGGACTCAGGGTGGT
GAPDH	Forward	AGAAGGCTGGGGCTCATTTG
	Reverse	AGGGGCCATCCACAGTCTTC
CD44	Forward	AAAGGAGCAGCACTTCAGGA
	Reverse	TGTGTCTTGGTCTCTGGTAGC
CXCR4	Forward	AATCTTCCTGCCCACCATCTA
	Reverse	AGCCTGTACTTGTCCGTCAT
SNAI2	Forward	CCTTTTTCTTGCCCTCACTGC
	Reverse	GGCTTCGGAGTGAAGAAATGC
SNAI1	Forward	CGAGCTGCAGGACTCTAAT
	Reverse	CCACTGTCCTCATCTGACA
ZEB1	Forward	GCACCTGAAGAGGACCAGAG
	Reverse	TGCATCTGGTGTTCCATTTT
BMI1	Forward	GTCCAAGTTCACAAGACCAGACC
	Reverse	ACAGTCATTGCTGCTGGGCATCG

Supplementary Table S4. Sequences of siRNA and shRNA against *adipsin*

siRNA or shRNA	Product ID	Sequence (5'-3')
Adipsin siRNA #1	Hs_CFD_1747	CUGCUACAGCUGUCGGAGATT
siRNA #2	Hs_CFD_1750	CCUCCAAGCGCCUGUACGATT
Adipsin shRNA	TRCN0000057208	CCGGCGCGACTCCATCTCTACAAATCTCG-AGATTTGTAGAGATGGAGTCGCGTTTTTG

Supplementary Materials and Methods

Semi-quantitative RT-PCR analysis

Total RNA was extracted from three human ADSCs (KUF02, KUF06, and KUF16) using an RNeasy Mini Kit (QIAGEN, Hilden, Germany), and reverse transcribed using a PrimeScript RT reagent kit (Takara Bio, Kusatsu, Japan). A Human Mesenchymal Stem Cell RT^2 Profiler PCR Array (PAHS-082ZA, QIAGEN) was used to analyze 84 specific gene expressions related to human mesenchymal stem cells. Real-time PCR was performed using KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and the Thermal Cycler Dice (Takara Bio). Dissociation curves were analyzed after each reaction to assess quantification specificity. Thermal cycling conditions for PCR amplification were as follows: 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 35 sec at 55°C, and 30 sec at 72°C. Relative gene expression was calculated by using the ΔΔCT method with *GAPDH* as an internal control.

Cytokine array

KUF06 ADSCs were 3D-cultured for 3 days. The culture supernatants were collected by centrifugation at 300 g for 10 min at 4°C. Expression profile of the 102 human cytokines was analyzed using Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacture's instruction. Images of the membrane were taken using the Amersham Imager 600 (GE Healthcare, Little Chalfont, U.K.).

Western blotting

Protein extraction and Western blotting were performed as previously described [1]. Briefly, the cells were lysed with a lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP40) containing protease and phosphatase inhibitors (100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 2 mg/mL aprotinin, 5 mg/mL leupeptin) for 20 min on ice. Samples were separated on SDS-12% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane using Trans-Blot Turbo Mini PVDF Transfer system (Bio-Rad, Hercules, CA, USA). After blocking with 3% bovine serum albumin, filters were incubated with an anti-adipsin (1:400, AF1824, R&D Systems) or an anti-β-actin (1:3000, A5441, Sigma, St. Louis, MO, USA) antibody. HRP conjugated rabbit anti-goat immunoglobulin G (1:2000, P0160, DakoCytomation, Glostrup, Denmark) or goat anti-mouse IgG (1:3000, 1706516, Bio-Rad) antibody was then added and the bands were detected using the Chemi-Lumi One L (nacalai tesque, Kyoto, Japan) and the Amersham Imager 600 (GE Healthcare).

Reference

1. Imamura Y, Mukohara T, Shimono Y, Funakoshi Y, Chayahara N, Toyoda M, et al. Comparison of 2D- and 3D-culture models as drug-testing platforms in breast cancer. *Oncol Rep* 2015; **33**: 1837-1843.