Supplemental Data

Receptor and molecular mechanism of AGGF1 signaling in endothelial cell functions and angiogenesis

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Running title: Integrin α5/β1 as a receptor for AGGF1 in ECs

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Supplemental Figures

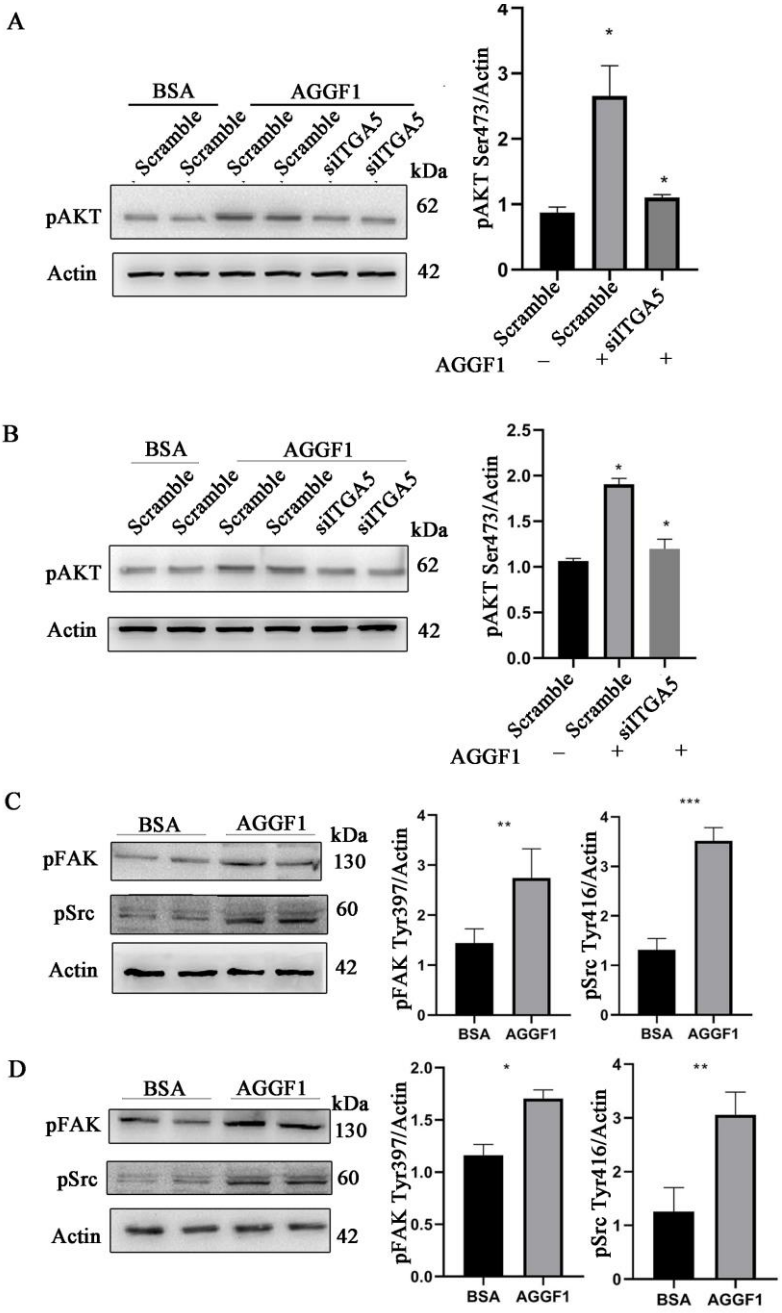

Figure I. AGGF1 can activate the AKT, FAK and Src signaling pathways through integrin α5 (encoded by the *ITGA5* gene) in both mouse aortic endothelial cells (MAECs) and human brain microvascular endothelial cells (HBMECs).

A and **B**, Western blot analysis showing the effects of siRNAs for *ITGA5* on the increased phosphorylation level of AKT in MAECs (**A**) and HBMECs (**B**) upon stimulation with purified AGGF1. **C** and **D**, Western blot analysis showing an increased phosphorylation level of FAK and Src in MAECs (**C**) or HBMECs (**D**) upon stimulation with purified AGGF1. Western blot data were quantified and analyzed using a Student's *t* test (**P*<0.05, ***P*<0.01, ****P*<0.001, n=3/group).

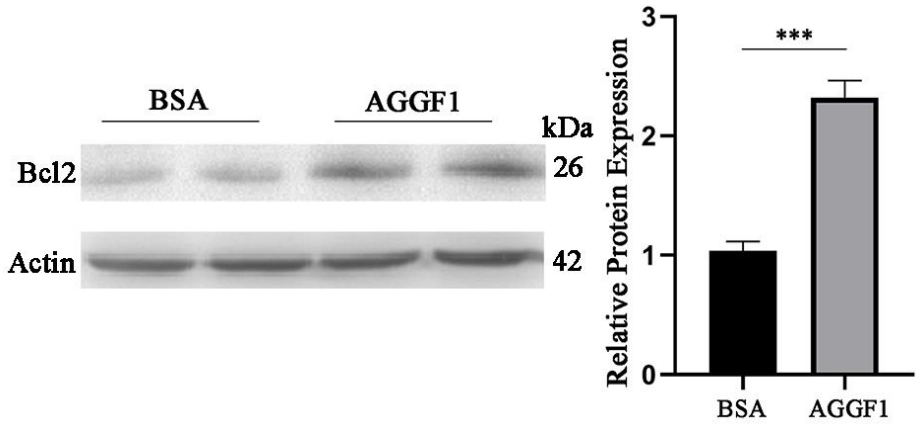

Figure II. AGGF1 treatment increases the expression level of Bcl2 involved in cell survival.

Representative Western blotting images showing that AGGF1 can increase the protein expression level of Bcl2 in HUVECs. HUVECs were starved overnight, treated with recombinant AGGF1 protein or BSA as control for 6 h, and then used for measuring the protein level of Bcl2. Western blot data were quantified and analyzed using a Student's *t* test (***P<0.001, n=3/group).

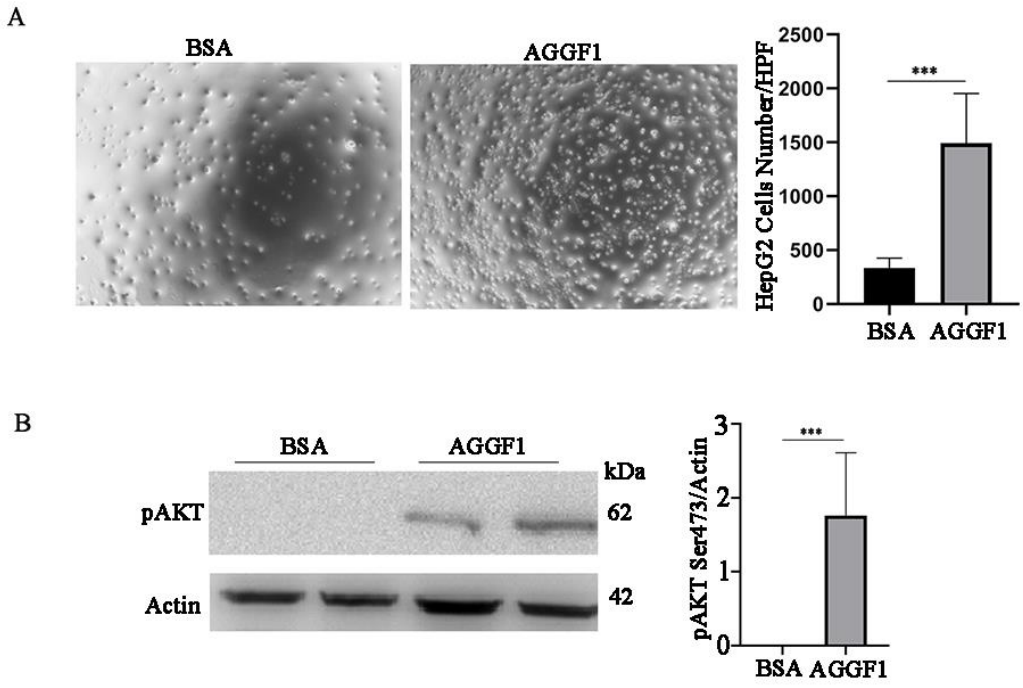

Figure III. AGGF1 can increase adhesion and activate AKT signaling in HepG2 cells.

A, Cell adhesion assays showing adhesion of HepG2 cells to wells coated with 5 μg/ml of AGGF1. BSA was used as a negative control. **B**, Western blotting showing that AGGF1 increase the level of phosphorylated AKT in HepG2 cells. Data were analyzed using a Student's *t* test (***P<0.001, n=6/group).

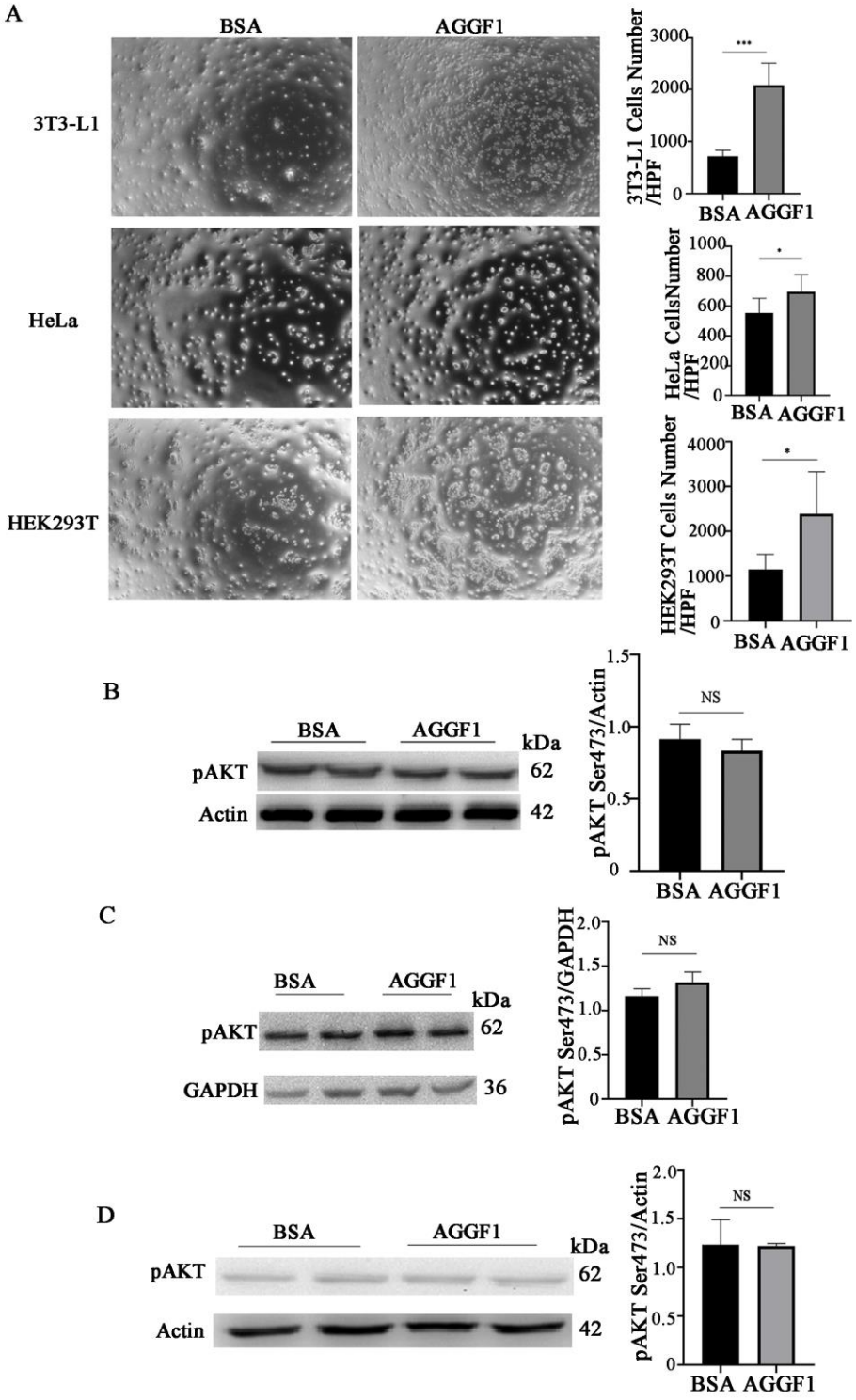

Figure IV. AGGF1 can physically bind to 3T3-L1 Cells, HeLa Cells and HEK293T Cells, but fails to activate AKT.

A, Adhesion assays of 3T3-L1 cells, HeLa cells and HEK293T Cells to wells coated with 5 μg/ml of WT AGGF1. BSA was used as a negative control. **B**, Western blotting showing that AGGF1 cannot increase the level of phosphorylated AKT in 3T3-L1 cells. **C**, Western blotting showing that AGGF1 cannot increase the level of phosphorylated AKT in HeLa cells. **D**, Western blotting showing that AGGF1 cannot increase the level of phosphorylated AKT in HEK293T cells (Student t test; **P*<0.05, ***, *P*<0.001, NS, not significant, n=6/group).

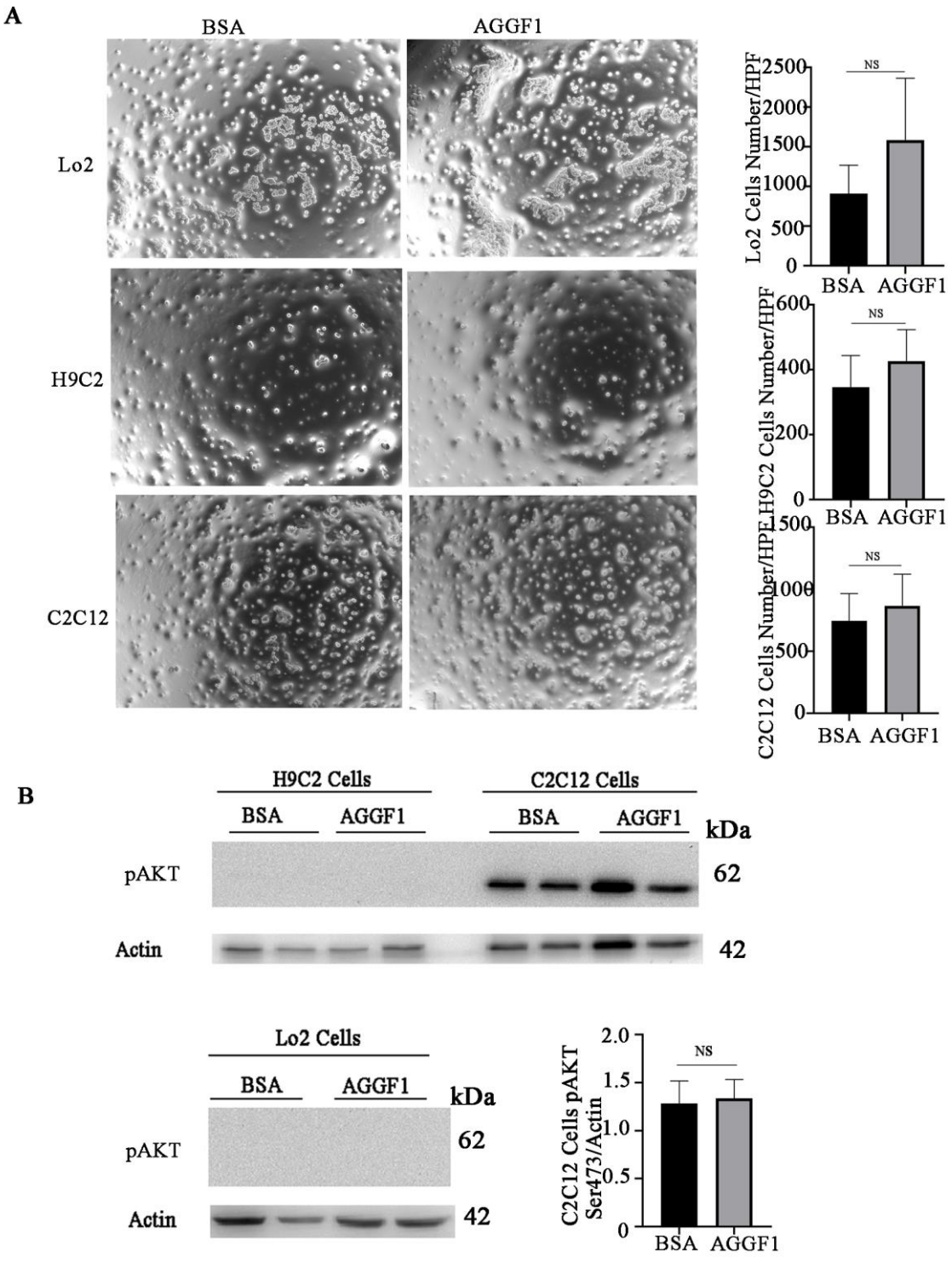

Figure V. AGGF1 cannot increase adhesion to cells in Lo2 Cells, H9C2 Cells and C2C12 Cells and fails to activate AKT.

A, Adhesion assays of Lo2 cells, H9C2 cells and C2C12 cells to wells coated with 5 μg/ml of WT AGGF1. BSA was used as a negative control. **B**, Western blotting showing that AGGF1 cannot increase the level of phosphorylated AKT in H9C2 cells, C2C12 cells and Lo2 cells (Student t test; NS, not significant, n=6/group).

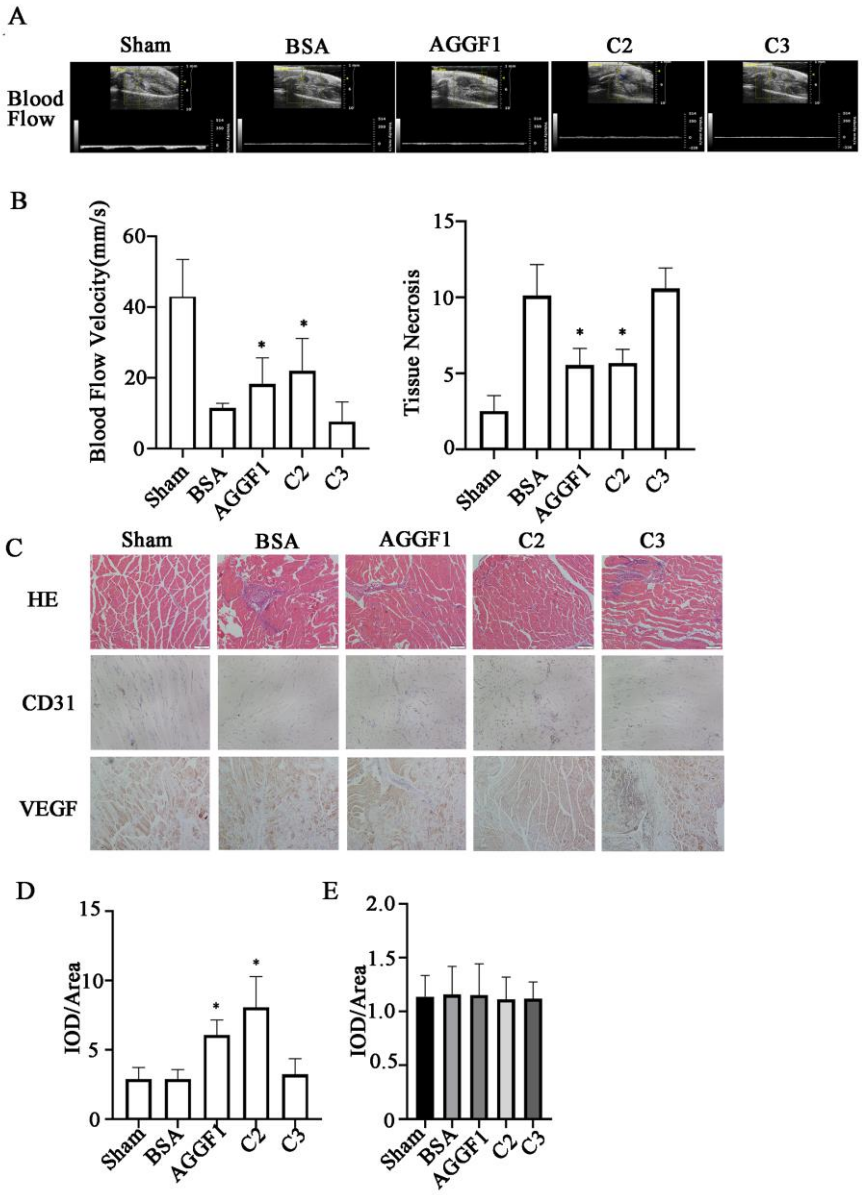

Figure VI. Analysis of WT AGGF1 and mutant C2 with the functional angiogenic domain, and mutant C3 without the domain in female mice.

A, The treatment effect of WT and mutant AGGF1 proteins for PAD in a hindlimb ischemia mouse model. Representative Doppler ultrasound images are shown for female mice treated with WT AGGF1, mutant C2, and mutant C3. Sham mice and BSA were used as negative controls. **B**, Female mice were treated with AGGF1 or truncated proteins in the hind limb ischemia model. After 28 days, the blood flow was measured by Doppler ultrasound. The blood flow rate is shown on the left, and the degree of tissue necrosis is shown on the right. **C**, Representative images for H&E staining, CD31 immunostaining, and VEGF immunostaining for sections of ischemic hindlimb muscles. **D** and **E**, Data as in (**C**) were quantified and plotted for CD31 (**D**) and VEGF (**E**). IOD, Integrated Optical Density. Data were shown as mean±SEM and analyzed using one-way ANOVA with Dunnett multiple comparison tests (*P<0.05, n=6/group).

Supplemental Tables

Table I. Primers used for construction of serial N-terminal *AGGF1* deletion mutatis.

Primers	Sequences (5' to 3')	Deleted Amino Acids
AGGF1 N1□F	ATTG GAT CCC GAG CCT GAG CTG GCC CAG	□□1-18
AGGF1 N2□F	ATTG GAT CCC AAT AAA AAG TCT GAT GTA GAA	□□1-85
AGGF1 N3 F	ATTG GAT CCC GAC CAT TTT GCC TCA AAT TCA	□□1-164
AGGF1 N4□F	ATTG GAT CCC CAC AGC ACT GGT TTC TAT TAT	□□1-218
AGGF1 N5□F	ATTG GAT CCC TCT GCA ACA AAT GAG GAA AAG	□□1-283
AGGF1 N6 F	ATTG GAT CCC AAC ATC TCT AAT TCA ACA TCA	□□1-348
AGGF1 N7□F	ATTG GAT CCC ATT GTC ATT AGA TCA CCT GTG	□□1-412
AGGF1 N8□F	ATTG GAT CCC GTG GAT CAA GGC AGT CAA AAT	□□1-471
AGGF1 N9□F	ATTG GAT CCC ACC TGT GAT GGA TGT GAA	□□1-521
AGGF1 N10□F	ATTG GAT CCC ACA GAA TAC GAA GAT GAA AAG	□□1-573
AGGF1 N101 F	ATTG GAT CCC AAT CCA AAA TAT AAA GAT AGA G	□□1-583
AGGF1 N102 F	ATTG GAT CCC CGT AGG GAG CAG GTT GGA AGT	□□1-593
AGGF1 N103 F	ATTG GAT CCC TTC CAA AGA GAT GAT GCT	□□1-603
AGGF1 N104 F	ATTG GAT CCC CAT TCT GAA ATT ACT GAT AGC	□□1-613
AGGF1 N11□F	ATTG GAT CCC CGG AAG ATG TTG GAG AAG ATG	□□1-623
AGGF1 N12□F	ATTG GAT CCC CTC CAA AAC AAG AAC AAA AAA	□□1-673
AGGF1 N R	TAA AGC GGC CGC TCA CTC TAA AGT CCC TTT	

Table II. Primers used for construction of serial C-terminal *AGGF1* deletion mutants.

Primers	Sequences (5' to 3')	Deleted Amino Acids
AGGF1 C1 F	TATA GC GGC CGC TCA TTT GCC TGT CCC CAA	□□665-714
AGGF1 C2□F	TATA GC GGC CGC TCA ATG AAC AGA TGC AGG	□□615-714
AGGF1 C3□F	TATA GC GGC CGC TCA TTT CTT TAA TTC TTT	□□565-714
AGGF1 C4□F	TATA GC GGC CGC TCA AAA GGA TAA GAC AGT	□□514-714
AGGF1 C5□F	TATA GC GGC CGC TCA ATG GTC AAA ATA AAT	□□465-714
AGGF1 C6□F	TATA GC GGC CGC TCA GAC AAT TAC TCT AAT	□□415-714
AGGF1 C7□F	TATA GC GGC CGC TCA AGT CTC CAT GAT TTT	□□365-714
AGGF1 C8□F	TATA GC GGC CGC TCA TGC GAA ATT TTC TTC	□□315-714
AGGF1 C9□F	TATA GC GGC CGC TCA AGA AGT CGG ATA AGG	□□265-714

AGGF1	TATA	GC GGC CGC TCA TCC AGT ATT TTC ATC	□□215-714
AGGF1	TATA	GC GGC CGC TCA CAC TTG TCT ATA TTT	□□165-714
AGGF1	TATA	GC GGC CGC TCA GTC ATT GTA GTA CGT	□□115-714
AGGF1	TATA	GC GGC CGC TCA TTC ATT TCT CCC ACG	□□85-714
AGGF1 C R	AA TTG GAT CCG ATG GCC TCG GAG GCG CCG		

Table III. Sequences for siRNAs.

Gene	siRNA Sequences (5' to 3')
ITGA5-1	AAGAATCTCAACAACTCGCAAAGCGAC
ITGA5-2	CTCCACAGATAACTTCACCCGAA
ITGB1-1	GGAACAGCAGAGAAGCTCA
ITGB1-2	GCGCATATCTGGAAATTTG
PTK2-1	GGTCGAATGATAAGGTGTA
PTK2-2	GAAGAGCGATTATATGTTA
Src-1	GCCTCAACGTGAAGCACTA
Src-2	CAAGAGCAAGCCCAAGGAT

Major Resources Tables

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
MAECs	Procell	unknown	https://www.procell.com.cn/view/2614.html

Antibodies

Target antigen	Source	Catalog#	Working concentration	Persistent ID / URL
AGGF1	Proteintech	1189-1	0.7 ug/ml	https://www.ptgcn.com/products/AGGF1-Antibody-11889-1-AP.htm
Integrin α5	Proteintech	10569-1	0.5 ug/ml	https://www.ptgcn.com/products/ITGA5-Antibody-10569-1-AP.htm
pFAK Try397	Cell Signaling Technology	8556	1:1000	https://www.cellsignal.cn/products/primary-antibodies/phospho-fak-tyr397-d20b1-rabbit-mab/8556?site-search-type=Products&N=4294956287&Ntt=fak&fromPage=plp
GAPDH	Proteintech	60004-1	0.166 ug/ml	https://www.ptgcn.com/products/GAPDH-Antibody-60004-1-Ig.htm
pSRC Try416	Cell Signaling Technology	59548	1:1000	https://www.cellsignal.cn/products/primary-antibodies/phospho-src-family-tyr416-e6g4r-rabbit-mab/59548?site-search-type=Products&N=4294956287&Ntt=src&fromPage=plp
pAKT Ser473	Cell Signaling Technology	4060	1:1000	https://www.cellsignal.cn/products/primary-antibodies/phospho-akt-ser473-d9e-xp-rabbit-mab/4060?site-search-type=Products&N=4294956287&Ntt=akt&fromPage=plp
AKT	Cell Signaling Technology	4691	1:1000	https://www.cellsignal.cn/products/primary-antibodies/akt-pan-c67e7-rabbit-mab/4691?site-search-type=Products&N=4294956287&Ntt=akt&fromPage=plp
SRC	Cell Signaling Technology	2109	1:1000	https://www.cellsignal.cn/products/ primary-antibodies/src-36d10-rabbit-mab/2109?site-search-type=Products&N=4294956287&Ntt=src&fromPage=plp
Integrin b1	Proteintech	12594-1	0.9ug/ml	https://www.ptgcn.com/Products/ITGB1-Antibody-12594-1-AP.htm
BCL2	Proteintech	12789-1	0.5 ug/ml	https://www.ptgcn.com/products/BCL2-Antibody-12789-1-AP.htm

Other reagents

Reagent	Source	Catalog#	Persistent ID/URL
Hanks' Balanced Salt Solution	Thermo Scientific	88284	https://www.thermofisher.com/order/catalog/product/88284?SID=srch-srp-88284#/88284?SID=srch-srp-88284
Zeba™ Spin Desalting Columns	Thermo Scientific	89893	https://www.thermofisher.com/order/catalog/product/89893?SID=srch-hj-89893#/89893?SID=srch-hj-89893
Cell dissociation buffer	Thermo Scientific	13151014	https://www.thermofisher.com/order/catalog/product/13151014?SID=srch-srp-13151014#/13151014?SID=srch-srp-13151014
Protein A/G Plus Agarose	Thermo Scientific	20424	https://www.thermofisher.com/order/catalog/product/20424?SID=srch-srp-20424#/20424?SID=srch-srp-20424