Supplemental Information

Supplemental Figures

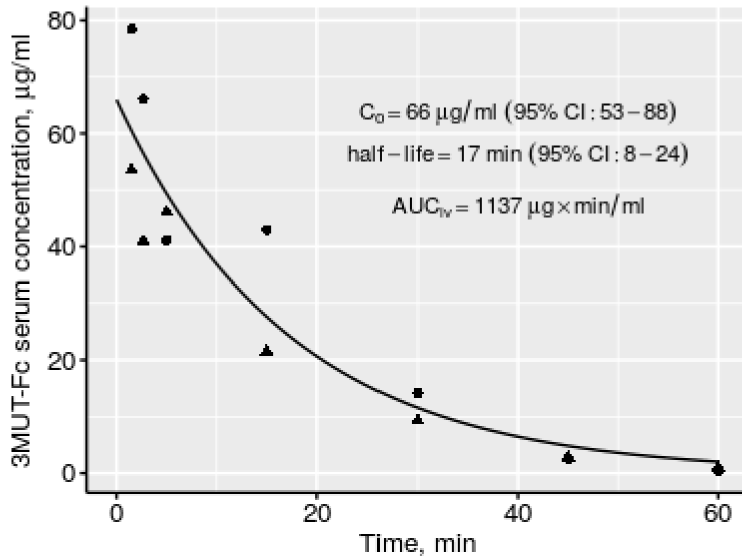
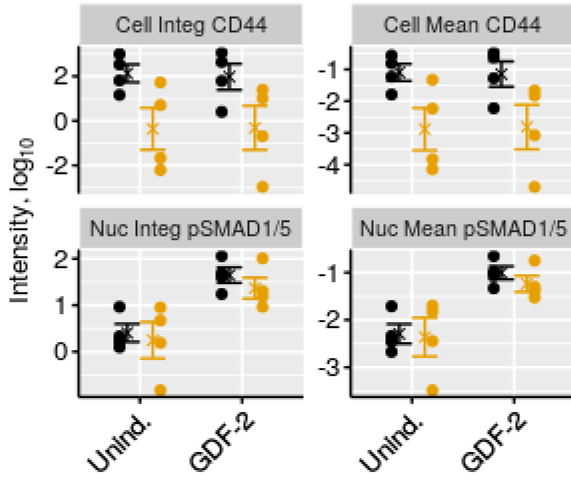
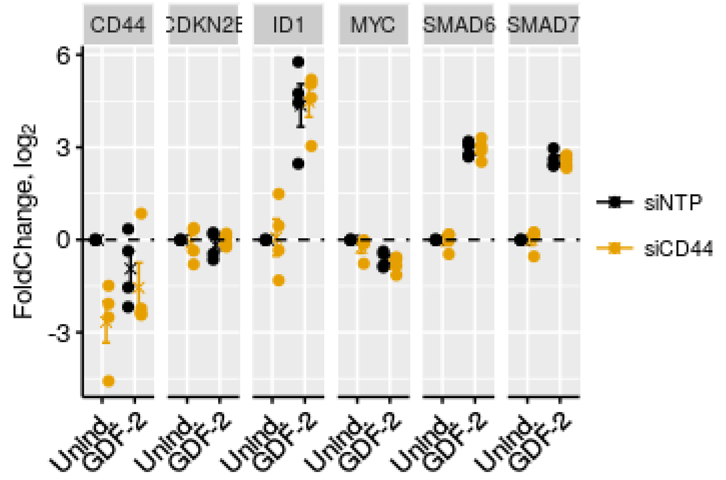


Figure S1. **CD44-3MUT-Fc serum half-life**. Related to Figure 2. CD44-3MUT-Fc showed 158 mL/h clearance and 17 min serum half-life in rats after intravenous administration. Volume of distribution was 45 ml or 18 %TBW. Dots or triangles represent CD44-3MUT-Fc concentrations predicted from 100- or 50-fold sample dilutions respectively.

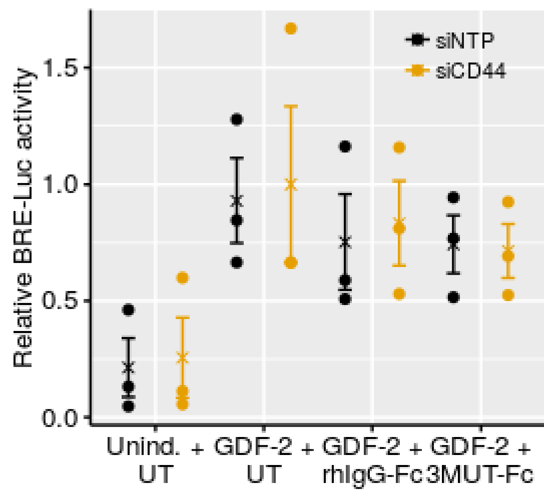
A



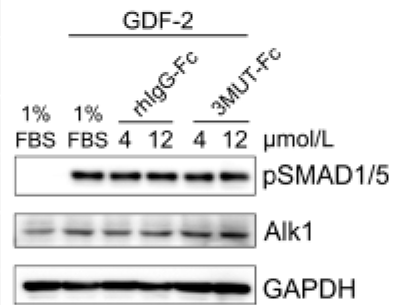
B



C



D



E

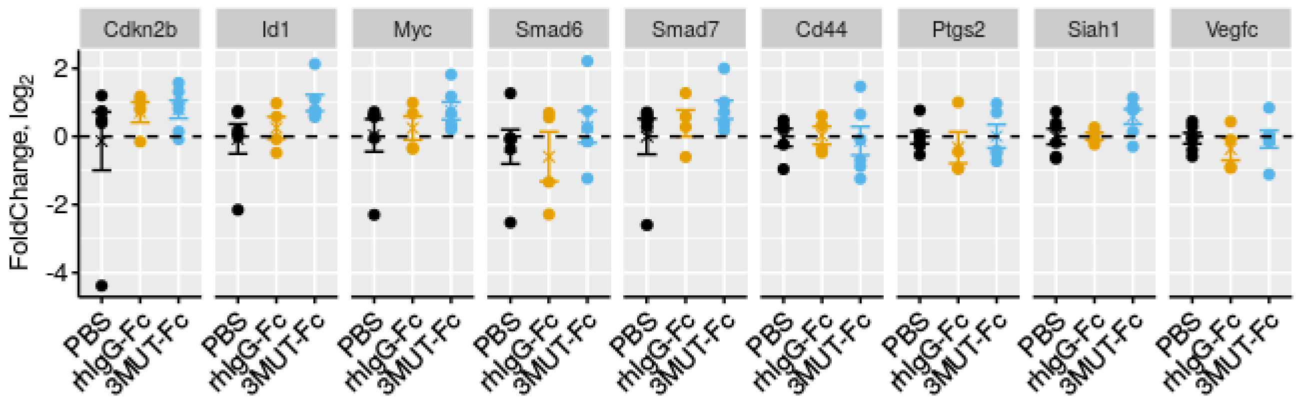


Figure S2. **CD44 is not involved in GDF-2/ALK1-dependent SMAD signaling**. Related to Figure 4. **(A,B)** siRNA transfected HUVECs were starved in 1% FBS media for overnight. After starving, cells were stimulated 2 h with 2.5 ng/ml GDF-2. **(A)** Fluorescent confocal microscopy analysis of p-SMAD1/5 nuclear location in CD44-silenced HUVEC in response to GDF-2 stimulation. Integrated and mean intensities per cell or in nuclei of anti-CD44 or anti-p-SMAD1/5 stainings are shown, respectively. **(B)** Transcription of SMAD target genes in CD44-silenced HUVEC in response to GDF-2 stimulation. Gene expression is shown relative to uninduced siNTP-transfected cells. Points are means from independent experiments (**A,B**). N = 4 independent experiments. **(C)** BMP-responsive element reporter activity of 10 ng/ml GDF-2 stimulated HUVECs transfected with CD44 siRNA and treated with CD44-3MUT-Fc. **(D)** Western blot analysis of HUVEC treated with CD44-3MUT-Fc in 1% FBS starvation media for 6 h and subsequently stimulated with 10 ng/ml GDF-2 for 30 min. **(E)** In vivo expression of SMAD target genes in mice treated with CD44-3MUT-Fc. RNA was isolated from lungs of mice treated with 15 mg/kg CD44-3MUT-Fc or hIgG-Fc in angiogenesis experiments described in Fig 1. Dots are individual mice, cross is mean and errorbar shows ± SEM. N = 6 mice from two independent experiments. siNTP - non-targeting siRNA pool, siCD44 - CD44-targeting pool. See also Supplemental Experimental Procedures.

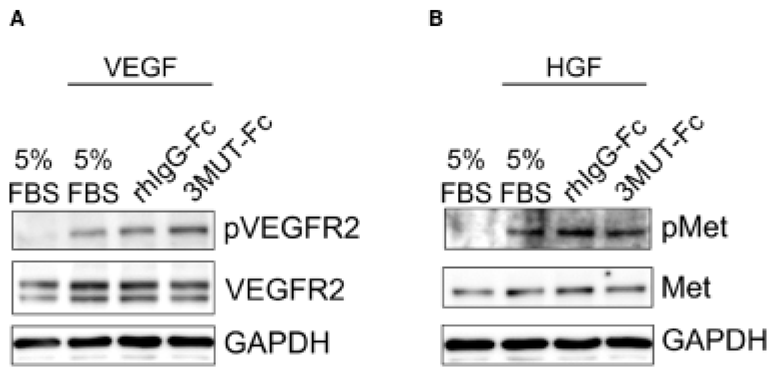
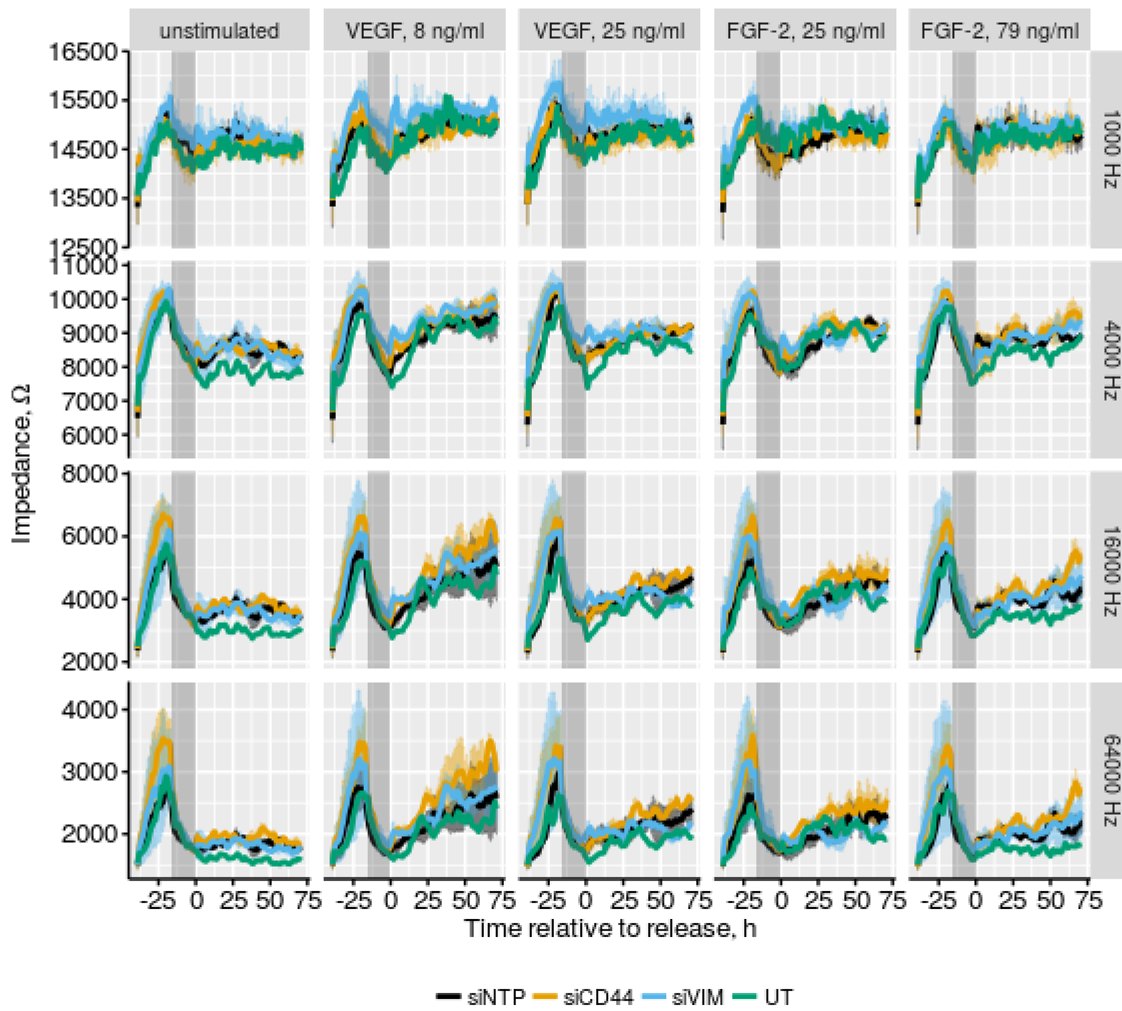


Figure S3. **CD44-3MUT-Fc effect on angiogenic growth factor receptor activation**. Related to Figure 4. Western blot analysis of synchronized HUVEC pretreated with CD44-3MUT-Fc in 5% FBS media for 1 h and subsequently released from starvation by stimulation with 25 ng/ml VEGF **(A)** or 63 ng/ml HGF **(B)**. After growth factor stimulation, cells were further grown for 72 h before western blot analysis was performed.

A



B

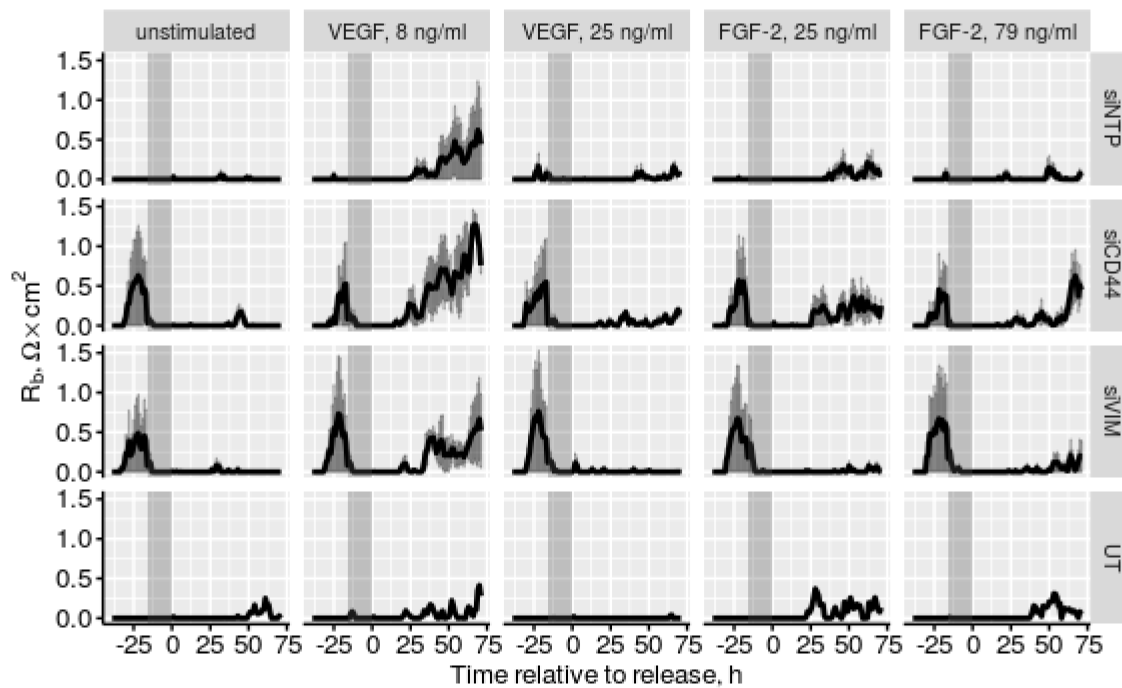


Figure S4. **CD44 silencing augments EC growth, barrier formation is functional**. siRNA transfected HUVECs were plated onto 96-well electrode arrays. After 24 h, cells were starved in 1% FBS media for overnight (gray area). After starving, cells were released from cell cycle block by addition of different concentrations of FGF-2 or VEGF. Following stimulation, HUVEC growth was monitored by measuring electrode impedance at different AC frequencies (**A**). Measurements recorded at 16000 Hz are shown in Figure 5C. (**B**) After experiment, barrier function (Rb) of cell layer was modeled using ECIS software (Giaever and Keese, 1991). Data are represented as mean ± SEM. Related to Figure 5.

Supplemental Experimental Procedures

CD44-3MUT-Fc serum half-life

F344/NCrHsd male rats were from Harlan, Netherlands. Rats carried polyurethane round tipped jugular vein catheter for blood sampling (Harlan Laboratories Surgical Services). After pre-serum blood sample was taken, rats were injected intravenously via tail vein with 3 mg of CD44-3MUT-Fc in 1ml volume. Blood samples were collected using jugular vein catheter at different time points. Blood samples were held at +37°C for 30 min to allow clot formation and then centrifuged at 1300·g for 10 min at RT. The supernatants were collected and stored at -20°C until assayed. For sandwich ELISA microwell plates were coated with mouse anti-human IgG1 antibody clone G17-1 (BD Biosciences). Blocking was performed with 1.5% BSA/PBS. Standards were step-diluted (40 µg/ml – 0 µg/ml) in 0.5% BSA/PBS supplemented with 5%, 2% or 1% rat serum. Samples taken at different time points: pre-serum, asap to 24 hours were diluted 1:50 or 1:100 in 0.5% BSA/PBS solution and applied to wells. Biotin mouse anti-human IgG antibody clone G18-145 (BD Biosciences) and streptavidin-HRP was used for detection. Tetramethylbenzidine was used for color development. Concentration at time zero and half-life was estimated from two-parameter exponential decay model with function $f(x)=d(exp(-x/e))$, where d is upper limit at $x = 0$, and e is decay constant.

Bre-Luc Reporter Assay

For reporter assay siRNA transfected HUVECs were seeded in 24-well plates 25000 cells/well. The next day cells were co-transfected with Id1-promoter derived reporter construct BRE-Luc and pLacZ. BRE-Luc reporter plasmid was a gift from Martine Roussel & Peter ten Dijke (Korchynskyi and Dijke, 2002)(Addgene plasmid #45126). pLacZ was used for normalization of internal transfection efficiency. Transfection was carried out using jetPEI-HUVEC transfection reagent according to manufacturer's protocol. For transfection 900 ng BRE-Luc, 100 ng pLacZ and 2 µl jetPEI-HUVEC per well was used. Cells were incubated with transfection complex in 2% FBS-DMEM (high gucose) for 3 h. Then, transfection media was changed to 0.1% FBS containing starvation media and cells were further incubated with 4 µM hIgG-Fc or CD44-3MUT-Fc in the presence of 10 ng/ml GDF-2 for ON at 37°C. Cells were lysed and luciferase and β-galactosidase activity was determined using TECAN microtiter plate reader.

Fluorescence Confocal Microscopy

siRNA transfected HUVECs were seeded into 0.1% gelatin-coated 8-well microscopy slides 24000 cells/well. After 24 h cells were switched to 0.1% FBS containing starvation media (M199, 0.1% FBS, 4 mM L-glutamine, 25 mM Hepes pH 7.4) for 6 h and then induced with 2.5 ng/ml GDF-2 for 2 h at 37°C. For immunofluorescence staining cells were fixed in freshly made 4% formaldehyde in PBS 10 min on ice followed by 10 min at RT. Primary Ab (mouse anti-human CD44 antibody MEM-263 from Exbio; anti phospho-SMAD1/5/8 rabbit mAb from Cell Signaling Technology) and fluorescently labelled secondary Ab (Alexa Fluor 488 or -568 conjugated secondary antibodies from Molecular Probes) stainings were performed sequentially in PBS/0.1% BSA buffer containing 0.1% saponin for 1 h at RT or ON at 4°C. Nuclei were stained with Hoechst 33285 (Sigma). Slides were mounted using Mowiol 4-88 (Sigma). Images were acquired using a Zeiss LSM 510 microscope with Plan-Apochromat 20x/0.8 M27 objective (Carl Zeiss). For each independent experiment, three random fields per well were acquired from two wells per condition. Confocal image stacks were converted to maximum intensity projections using Fiji software (Schindelin et al., 2012). Maximum intensity projections of image channels were segmented and quantitated using CellProfiler software (Kamentsky et al., 2011).

Quantitative RT-PCR

For quantitative RT-PCR cells were seeded in 6-cm cell culture dish at density 320000 cells/plate. Treatments were performed as in immunofluorescence experiments (see the Fluorescence confocal microscopy section). Total RNA was isolated from cells or snap-frozen tissues using RNeasy Plus Mini kit (Qiagen). cDNA was synthesised using SuperScript VILO Kit (Invitrogen). Quantitative RT-PCR was performed using Hot Firepol Evagreen qPCR Mix Plus reagent (no ROX; Solis Biodyne) in LightCycler 480 II instrument (Roche). Relative gene expression was determined by ddCt method using GAPDH and ACTB as reference genes.

List of primers used for real-time qPCR experiments.

Gene	Forward primer (5’-3’)	Reverse primer (5’-3’)
Cdkn2b	CCCTGCCACCCTTACCAGA	CAGATACCTCGCAATGTCACG
Id1	ACCCTGAACGGCGAGATCA	TCGTCGGCTGGAACACATG
Myc	ATGCCCCTCAACGTGAACTTC	GTCGCAGATGAAATAGGGCTG
Smad6	TTCTCGGCTGTCTCCTCCTGA	GTGGCCTCGGTTTCAGTGTAAGA
Smad7	GGCCTATCCACAGGCTTCTGA	GTGACAGGCGGCAGTAAGACA
Gapdh	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
Ptgs2	AGATTGCTGGCCGGGTTGCTG	CAGGGAGAAGCGTTTGCGGT
Actb	GCCCTAGGCACCAGGGTGTG	GGGGCCACACGCAGCTCATT
Vegfc	GGGGGCGAGGTCAAGGCTTTT	GCCTTTCCGCAGCTGGCACT
Cd44	TGCCTCAGCCCCTCCTGAAGA	TGGAGCCGCTGCTGACATCG
Siah1a	AGGAATTCCAGAAAGGCAAGG T	AGAGACAAGAGCATCCTGCAC
CDKN2B	AAGCTGAGCCCAGGTCTCCTA	CCACCGTTGGCCGTAAACT
ID1	GGCTGTTACTCACGCCTCAAG	CCAACTGAAGGTCCCTGATGTAG
MYC	CGTCTCCACACATCAGCACAA	CACTGTCCAACTTGACCCTCTTG
SMAD6	TCTCCTCGCGACGAGTACAAG	GGAGCAGTGATGAGGGAGTTG
SMAD7	AGAGGCTGTGTTGCTGTGAATC	GCAGAGTCGGCTAAGGTGATG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Western Blot Analysis of CD44-3MUT-Fc Effect on Activation of Angiogenic Growth Factor Receptors

To evaluate whether CD44-3MUT-Fc affects VEGF, HGF or GDF-2 mediated receptor activation, HUVEC were seeded in 0.1% gelatin-coated 6-well plates 80000 cells/well. VEGF and HGF stimulated cells were grown and treated as described in Materials and methods (see HUVEC growth and treatments section). Briefly, serum-starved HUVEC were treated for 72 h with 4 µM rhIgG-Fc or CD44-3MUT-Fc in the presence of 25 ng/ml VEGF or 63 ng/ml HGF. After treatments cells were lysed in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1 mM Na3VO4, 10 mM NaF and protease inhibitor coctail. For analysing GDF-2 mediated SMAD1/5 activation in response to CD44-3MUT-Fc treatment, 24 h after seeding cells were treated with 4 and 12 µM rhIgG-Fc or CD44-3MUT-Fc in 1% FBS containing starvation media (M199, 1% FBS, 4 mM L-glutamine, 25 mM Hepes pH 7.4) for 6 h and then stimulated with 10 ng/ml GDF-2 for 30 min at 37°C. Subsequently cells were lysed in 70 µl of 1x Laemmli’s sample buffer. 10 µg of VEGF and HGF or 20 µl of GDF-2 stimulated samples were subjected to western blot analysis, which was carried out essentially as described in Materials and methods (see Western blot analysis section). Except, 5% BSA-TBST was used for primary Ab incubations and 5% or 2% skimmed milk-TBST respectively for blocking and secondary Ab incubations. Following Abs were used for western blot: anti-GAPDH mouse mAb 1/10000 from Millipore; anti phospho-SMAD1/5/8 rabbit mAb (D5B10) 1/1000, anti-phospho-VEGFR2(Tyr1175) rabbit mAb (D5B11) 1/1000, anti-phospho-Met(Tyr1234/1235) rabbit mAb (D26) 1/1000 and anti-VEGFR2 rabbit mAb (55B11) 1/1500 from Cell Signaling Technology; anti-Met rabbit pAb (C-28) 1/1000 and anti-Alk-1 goat pAb (D-20) 1/1000 from Santa Cruz Biotechnology.

List of siRNA target sequences.

Gene	Entrez gene id	Catalog #	Target sequence
CD44	960	J-009999-06	GAAUAUAACCUGCCGCUUU
CD44	960	J-009999-07	CAAGUGGACUCAACGGAGA
CD44	960	J-009999-08	CGAAGAAGGUGUGGGCAGA
CD44	960	J-009999-09	GAUCAACAGUGGCAAUGGA
VIM	7431	L-003551-06	UCACGAUGACCUUGAAUAA
VIM	7431	L-003551-07	GAGGGAAACUAAUCUGGAU
VIM	7431	L-003551-08	UUAAGACGGUUGAAACUAG
VIM	7431	L-003551-09	GGAAAUGGCUCGUCACCUU
NTP	NA	D-001810-10-05	UGGUUUACAUGUCGACUAA
NTP	NA	D-001810-10-05	UGGUUUACAUGUUGUGUGA
NTP	NA	D-001810-10-05	UGGUUUACAUGUUUUCUGA
NTP	NA	D-001810-10-05	UGGUUUACAUGUUUUCCUA

Reproducibility

This article and supplemental information is written in knitr (Xie, 2016), an R package for reproducible research. For reproducibility, all graphs, computations and statistics were computed at the same time that the text was typeset. The source code of the article and supplemental information is available upon request. The data files are available upon request.

List of loaded R packages.

Package	Version	Date	Source
boot	1.3-17	2015-06-29	CRAN (R 3.2.1)
bootES	1.2	2015-08-14	CRAN (R 3.2.3)
broom	0.4.0	2015-11-30	CRAN (R 3.2.2)
coda	0.18-1	2015-10-16	CRAN (R 3.2.2)
DBI	0.3.1	2014-09-24	CRAN (R 3.2.1)
dplyr	0.4.3	2015-09-01	CRAN (R 3.2.2)
drc	2.5-12	2015-04-14	CRAN (R 3.2.1)
foreach	1.4.3	2015-10-13	CRAN (R 3.2.2)
Formula	1.2-1	2015-04-07	CRAN (R 3.2.1)
geepack	1.2-0.1	2016-01-27	CRAN (R 3.2.3)
ggplot2	2.0.0.9001	2016-02-15	Github (hadley/ggplot2@067e096)
ggthemes	3.0.1	2016-01-10	CRAN (R 3.2.3)
glmnet	2.0-2	2015-04-12	CRAN (R 3.2.2)
gridExtra	2.0.0	2015-07-14	CRAN (R 3.2.1)

Package	Version	Date	Source
gtable	0.1.2	2012-12-05	CRAN (R 3.2.3)
Hmisc	3.17-1	2015-12-18	CRAN (R 3.2.3)
knitr	1.12.3	2016-01-22	CRAN (R 3.2.3)
lattice	0.20-33	2015-07-14	CRAN (R 3.2.1)
lubridate	1.5.0	2015-12-03	CRAN (R 3.2.3)
magrittr	1.5	2014-11-22	CRAN (R 3.2.1)
MASS	7.3-45	2015-11-10	CRAN (R 3.2.2)
Matrix	1.2-3	2015-11-28	CRAN (R 3.2.2)
MESS	0.3-2	2014-10-21	CRAN (R 3.2.2)
plyr	1.8.3	2015-06-12	CRAN (R 3.2.1)
png	0.1-7	2013-12-03	CRAN (R 3.2.1)
ProjectTemplate	0.6	2014-10-06	CRAN (R 3.2.1)
reshape2	1.4.1	2014-12-06	CRAN (R 3.2.1)
rjags	4-5	2016-01-06	CRAN (R 3.2.3)
RSQLite	1.0.0	2014-10-25	CRAN (R 3.2.1)
runjags	2.0.2-8	2015-09-14	CRAN (R 3.2.3)
scales	0.3.0.9000	2016-02-15	Github (hadley/scales@ad60fbe)
survival	2.38-3	2015-07-02	CRAN (R 3.2.1)
tidyr	0.4.1	2016-02-05	CRAN (R 3.2.3)

Supplemental References

Giaever, I., and Keese, C.R. (1991). Micromotion of mammalian cells measured electrically. Proc. Natl. Acad. Sci. U.S.A. *88*, 7896–7900.

Kamentsky, L., Jones, T.R., Fraser, A., Bray, M.A., Logan, D.J., Madden, K.L., Ljosa, V., Rueden, C., Eliceiri, K.W., and Carpenter, A.E. (2011). Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. Bioinformatics *27*, 1179–1180.

Korchynskyi, O., and Dijke, P. ten (2002). Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J. Biol. Chem. *277*, 4883–4891.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.

Xie, Y. (2016). Knitr: A general-purpose package for dynamic report generation in r.