cancers

MDPI

Suplementary Material

Regulation of Tissue Factor by CD44 Supports Coagulant Activity in Breast Tumor Cells

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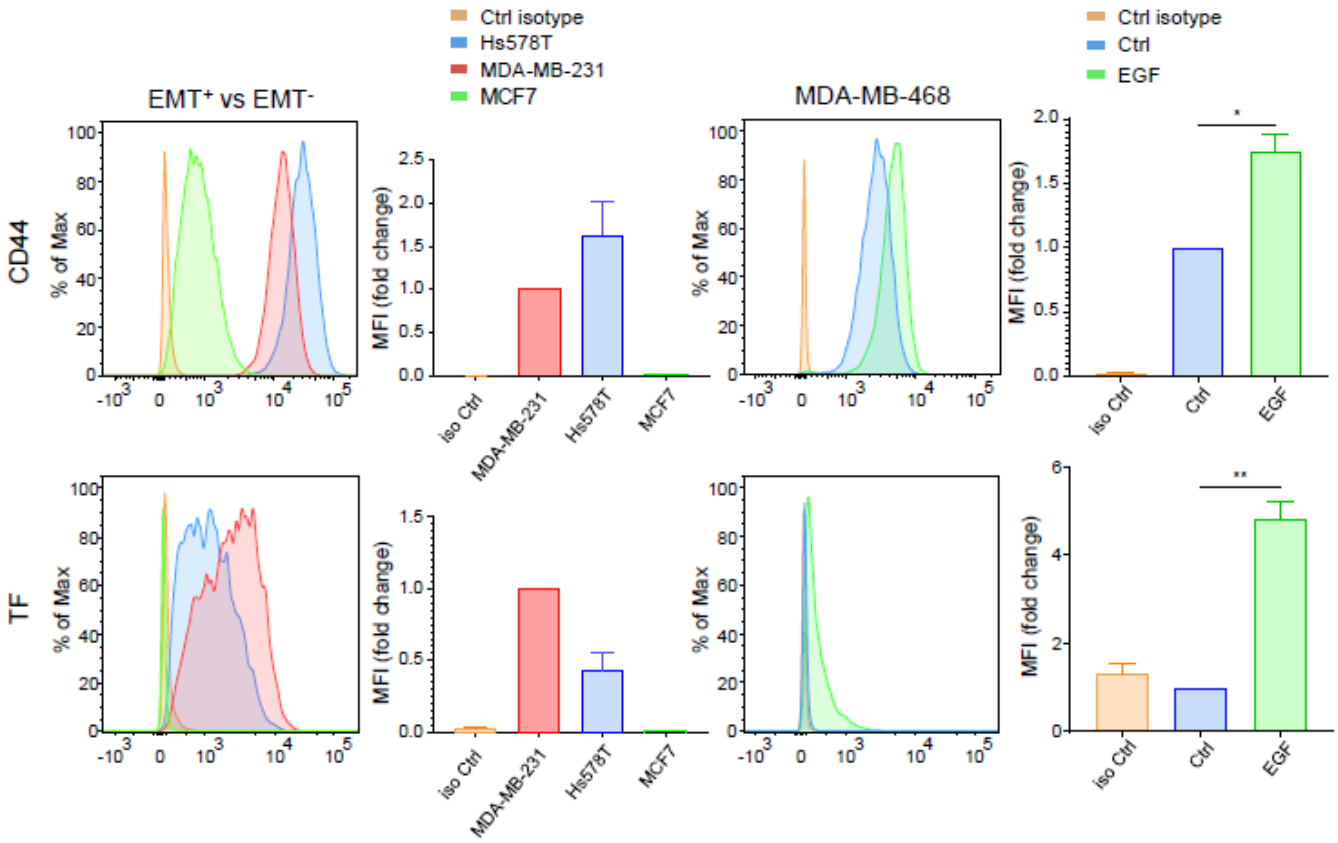


Figure S1. TF and CD44 coexpress in EMT cellular contexts. FACS analyses and associated Mean Fluorescence Intensity (MFI) quantifications of CD44 and TF in EMT$^+$ (MDA−MB−231 and Hs578T) vs EMT$^-$ (MCF7) and in EMT−inducible (MDA−MB−468 treated or not (Ctrl) with EGF to induced EMT) cell lines. *, $p < 0.05$; **, $p < 0.01$.

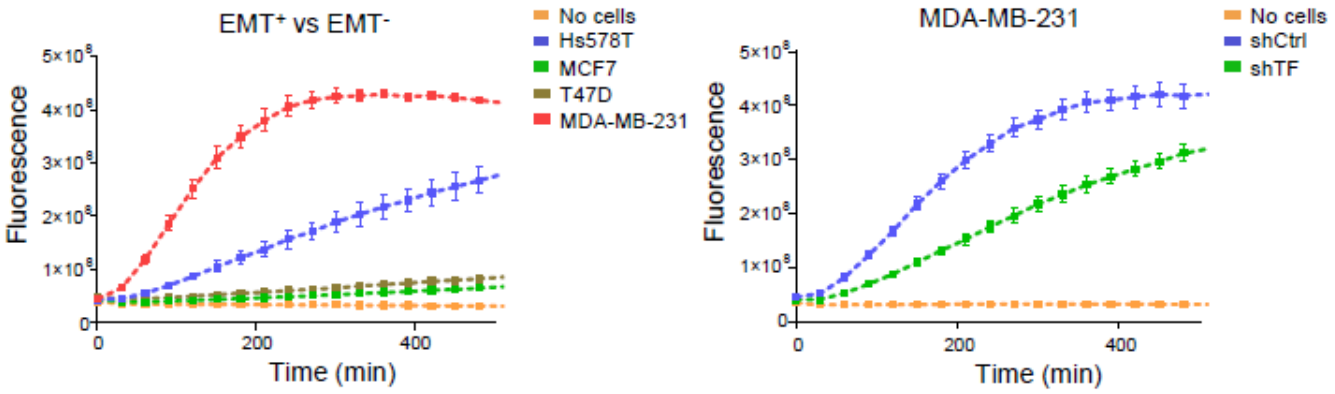
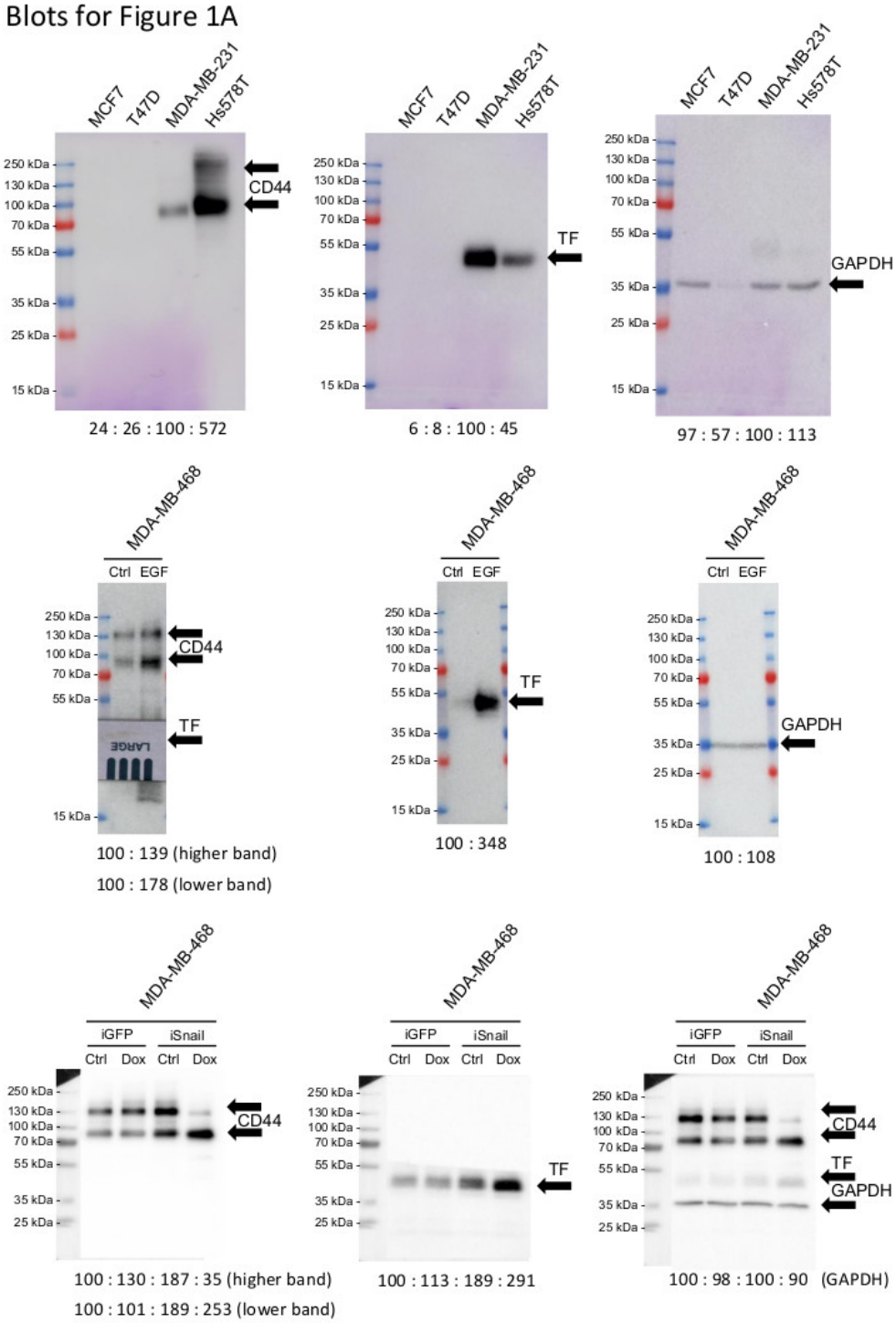
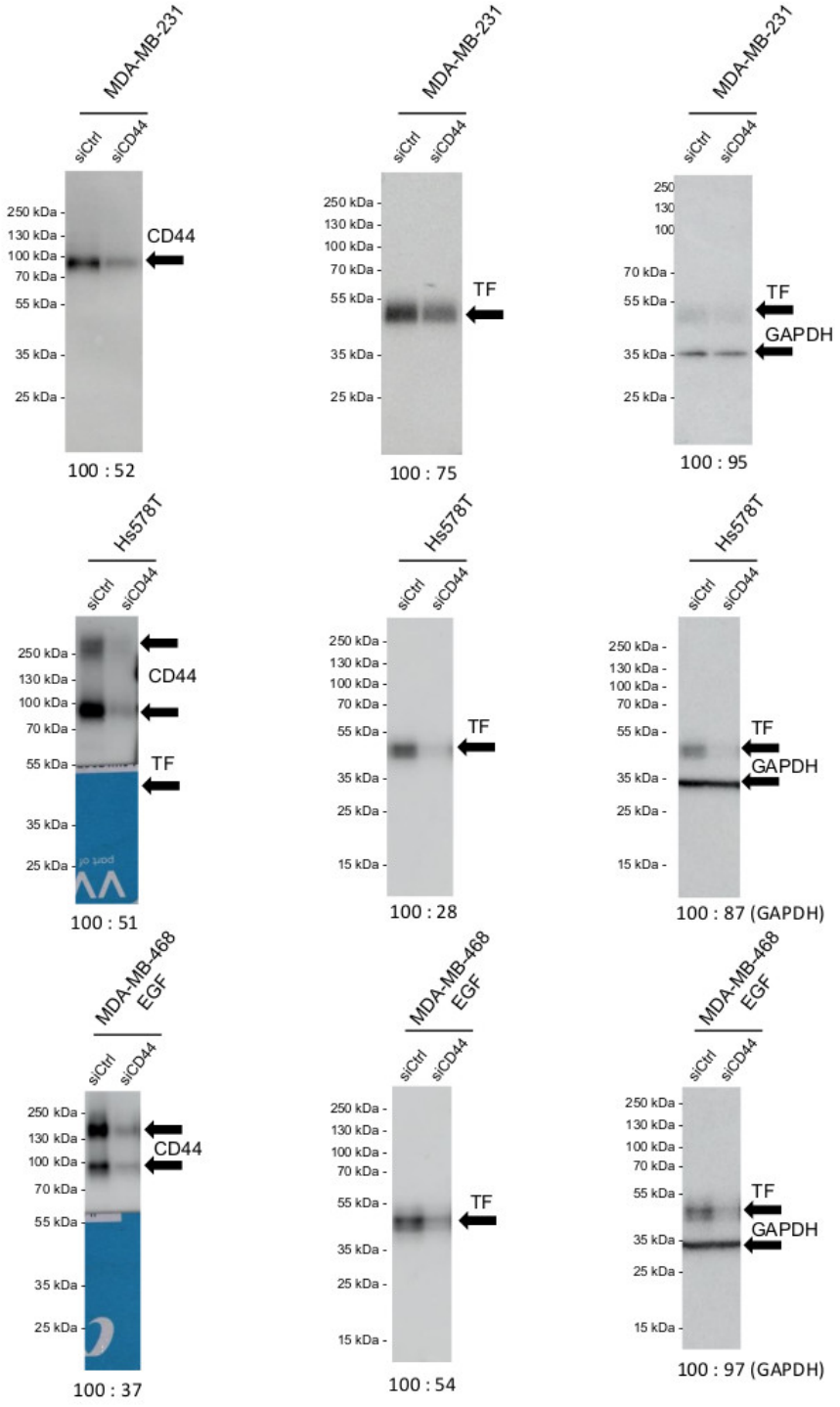


Figure S2. Validation of the enzymatic fluorescent coagulation assay. Enzymatic fluorescent coagulation assays performed in EMT^+ (MDA−MB−231 and Hs578T) vs EMT^- (MCF7 and T47D), and in EMT^+ (MDA−MB−231) cell lines transduced with a shRNA directed against TF (shTF) or a non−targeting shRNA (shCtrl). Fluorescence intensity was followed during 8 hrs.

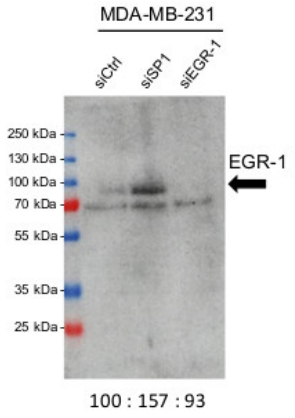
Blots for Figure 1A



Blots for Figure 2A



Blots for Figure 5A



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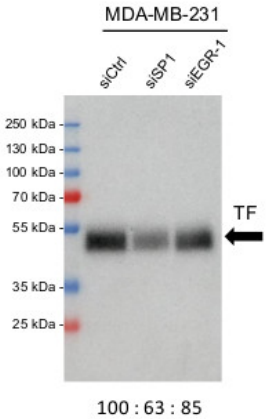

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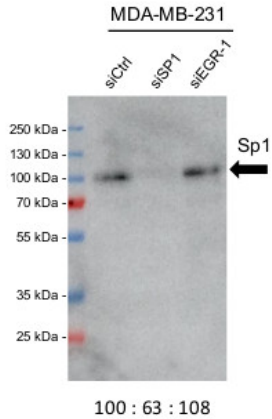

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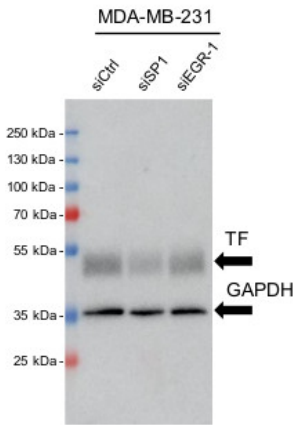

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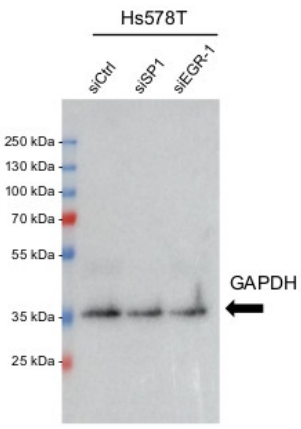

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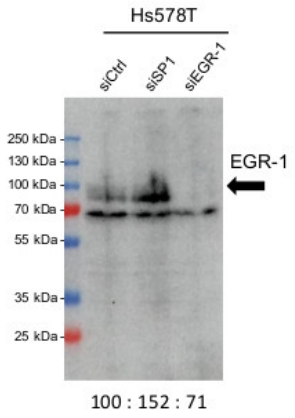

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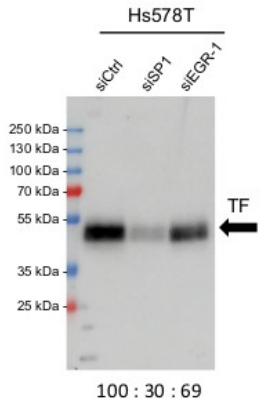

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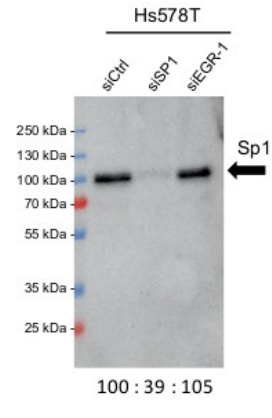

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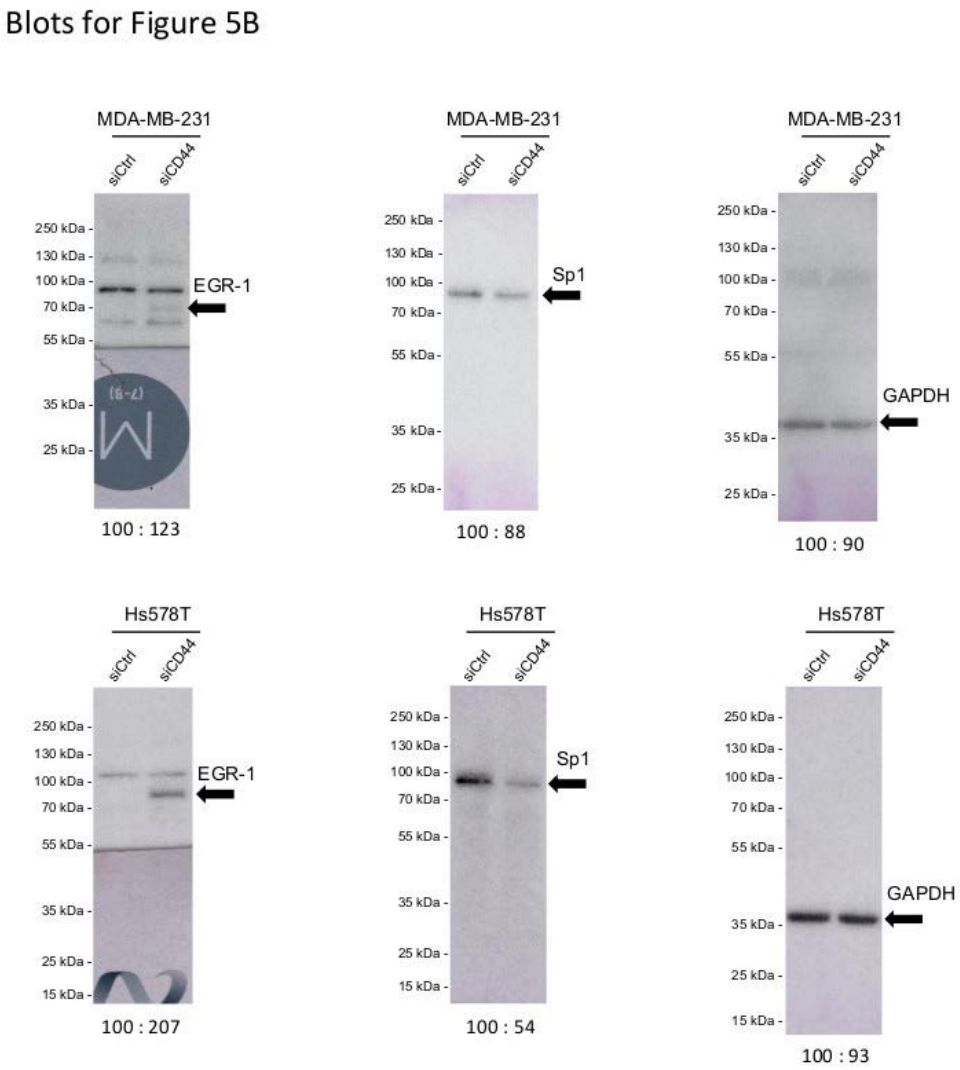

Figure S3. Uncropped Western blot figures

Table S1. Overview of Study Design.

Analyses	Methods
CD44 and TF expression in EMT^{+} breast tumor cell lines	FACS, Western blotting for TF and CD44 on MDA−MB−231, Hs578T, MDA−MB−468 (induced or not to EMT).
Impact of CD44 silencing on TF expression	FACS, Western blotting for TF and CD44 after CD44 siRNA transfection in MDA−MB−231, Hs578T, MDA−MB−468 (induced or not to EMT).
Impact of CD44 silencing on coagulant properties	Enzymatic fluorescent coagulation assays and visual clot assays on MDA−MB−231, Hs578T, MDA−MB−468 (induced or not to EMT) transfected with CD44 siRNA.
Impact of CD44 silencing on metastatic seeding	Experimental metastasis assays on MDA−MB−231, Hs578T, MDA−MB−468 (induced or not to EMT) transfected with CD44 siRNA
Analysis of a TF transcriptional regulation by CD44	TF−promoter reporter assays Western blotting and RT−qPCR (for TF or Sp1 or EGR−1) after CD44 silencing, Sp1 silencing or mithramycin treatments in MDA−MB−231 cells and Hs578T cells.

Table S2. list of siRNA.

siRNA	Sequences
siCtrl 1	5'−CAGAACUAGAUUGCGAAUA−3'
siCtrl 2	5'−GCCCAAAUAAUGGUCAGAA−3'
siCtrl 3	5'−GAUACUAUCUAGUCUAGAC−3'

siCD44 1 [1]	5'−GCAGAUCGAUUUGAAUAUA−3'
siCD44 2 [1]	5'−GUAUGACACAUAUUGCUUC−3'
siCD44 3 [2]	5'−AAAUGGUCGCUACAGCAUC−3'
siSP1 1 [3]	5'− GCAACAUGGGAAUUAUGAA−3'
siSP1 2 [3]	5'−GGCAGACCUUUACAACUCA −3'
siSP1 3 [3]	5'−CCACAAGCCCAAACAAUCA−3'
siEGR1 1*	5'− CGACAGCAGUCCCAUUUAC −3'
siEGR1 2*	5'− GGACAUGACAGCAACCUUU −3'
siEGR1 3*	5'− GACCUGAAGGCCCUCAAUA −3'

* from Dharmacon™**.**

Table S3. list of primer sequences used for qPCR analyses.

Genes	Primers	Sequences
hFT	Forward	5'−CAGACAGCCCGGTAGAGTGT−3'
	Reverse	5'−CCACAGCTCCAATGATGTAGAA−3'
hGAPDH	Forward	5'−TGCCGTCTAGAAAAACCTGCCAAA−3'
	Reverse	5'−CTCTCTTCCTCTTGTGCTCTTGCT−3'
hGAPDH (nested−qPCR)	Forward	5'−ACCCTACTGATGATGACGTGAGCA−3'
	Reverse	5'−TGGAATGTGTCTTGGTCTCTGGTA−3'
mGAPDH	Forward	5'−TGTCCGTCGTGGATCTGAC−3'
	Reverse	5'−GAGTTGCTGTTGAAGTCGCA−3'

Table S4. list of antibodies used for western blotting analyses.

Anticorps	Clone	Source	References
Mouse anti−human tissue factor	VD8	Sekisui Diagnostics	ADG4508
Mouse anti−human CD44	2C5	R&D Systems	BBA10
Rabbit anti−human Sp1	Polyclonal	Cell Signaling	5931
Rabbit anti−human EGR−1	15F7	Cell Signaling	4153
Mouse anti−human GAPDH	6C5	Merck Millipore	MAB374
Goat anti−rabbit/HRP	Polyclonal	Cell Signaling	7074
Horse anti−mouse/HRP	Polyclonal	Cell Signalling	7076

Table S5. list of antibodies used for FACS analyses.

Anticorps	Clone	Source	References
Alexa fluor® 488 anti−mouse/human CD44	Monoclonal Rat IgG2b, κ Clone IM7	Biolegend	103015
Alexa fluor® 594 anti−human TF	Monoclonal Mouse IgG1 Clone −#323519	R&D Systems	FAB23391T
Alexa Fluor® 488 Isotype Ctrl Antibody	Monoclonal Rat IgG2b, κ	Biolegend	400625
Alexa Fluor® 594 Isotype Ctrl Antibody	Monoclonal Mouse IgG1	R&D Systems	FAB110T

Table S6. list of primer sequences used for sequencing.

Genes	Primers	Sequences
hCD44	Forward	5'− GCAGCACTTCAGGAGGTTACATCT−3'
	Reverse	5'− TGTGGACATGAAGATTGGGGTGTAA−3'

Table S7. list of plasmids used for dual−luciferase reporter assays.

Plasmids	Regions	Source	References
− 2106	−2106 nt to +121 nt	Addgene	15440 [4]
−227	−227 nt to +121 nt	Addgene	15442 [4]
−192	−192 nt to +121 nt	Addgene	15446 [4]
−111	−111 nt to +121 nt	Addgene	15447 [4]

Reference

1. Li, C.Z., Liu, B., Wen, Z-Q., Li, HY., Inhibition of CD44 expression by small interfering RNA to suppress the growth and metastasis of ovarian cancer cells in vitro and in vivo. Folia Biol (Praha), 2008. **54**(6): p. 180-6.
2. Singleton, P.A., Salgia, R., Moreno-Vinasco, L., Moitra, J., Sammani, S., Mirzapoiazova, T., Garcia, J.G.N., *CD44 regulates hepatocyte growth factor-mediated vascular integrity. Role of c-Met, Tiam1/Rac1, dynamin 2, and cortactin.* J Biol Chem, 2007. **282**(42): p. 30643-57.
3. J Bin, L., Kim, B.E., Hall, C.F., Leach, S.M., Leung, D.Y.M., Inhibition of transcription factor specificity protein 1 alters the gene expression profile of keratinocytes leading to upregulation of kallikrein-related peptidases and thymic stromal lymphopoietin. J Invest Dermatol, 2011. **131**(11): p. 2213-22.
4. Mackman, N., *Regulation of the tissue factor gene.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 1995. **9**(10): p. 883-9.