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Supplemental Information

ZNF281-miR-543 Feedback Loop Regulates Transforming Growth Factor-β-Induced Breast Cancer Metastasis

Wei Ji, Qiang Mu, Xiang-Yu Liu, Xu-Chen Cao, and Yue Yu

Supplemental Material and Methods

siRNA, miRNA and antibodies

The miR-543 mimic, miR-543 inhibitor, or the appropriate scrambled controls were purchased from RiboBio (Shanghai, China). The Snail, ZEB1 and ZNF281 gene-specific short interfering (siRNA), and non-specific control siRNA were also purchased from RiboBio. Recombinant human TGFβ1 and EGF were purchased from R&D Systems (Redmond, WA, USA). Antibodies E-cadherin, N-cadherin, Vimentin, ZO-1, p-SMAD2 and β-actin were purchase from Cell Signaling Technology (Beverly, MA, USA). Antibodies Snail, ZEB1 and ZNF281 were obtained from Abcam (Cambridge, MA, USA).

Plasmids construction

The 3′-UTR of ZNF281 containing miR-543 binding site was amplified from genomic DNA and cloned into psiCHECK2 vector (Promega, Madison, WI, USA) to generate ZNF281-wt. Site-directed mutagenesis was performed using the Site-Directed Mutagenesis Kit (TransGene, Beijing, China) to generate the ZNF281 3′-UTRmut reporter vector (ZNF281-mut). The ORF of human ZNF281 gene was amplified by PCR in 293FT cell line, the amplified fragments were subcloned into the pcDNA3.0/HA vector. The miR-543 promoter region (-1000 to +1) and the E-boxs mutated fragments were cloned into pGL3-Basic vector (Promega; P-wt, P-M1, P-M2, P-M1+2). The human Snail (-1794 to +83) and ZEB1 (-1932 to +101) promoter was amplified from genomic DNA using specific primers and cloned into pGL3-Basic vector. All constructs were confirmed by sequencing.

Western blotting

Total protein was extracted by using RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% mM EDTA, 0.5% NP40) containing protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Indianapolis, IN, USA) and centrifuged for 20 min at 12, 000 rpm. 50 μg of total protein was loaded and separated on the 10% sodium dodecyl sulfate -polyacrylamide gradient gel. The proteins were then transferred onto PVDF membranes (Millipore, Bedford, MA, USA) and blocked with 5% non-fat milk at room temperature for 1 hour. The membranes were then incubated at 4 °C overnight with primary antibodies. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and proteins were then detected using the ECL reagent (Millipore).

Immunofluorescence

Cells were seeded onto glass coverslips in 24-well plates, washed with PBS, fixed in 4% formaldehyde solution for 30 min and then permeabilized with 0.2% Triton X-100/PBS for 15 min. Cells were blocked with 2% BSA in PBS for 30 min. Coverslips were incubated with primary antibodies overnight at 4°C, followed by incubation with FITC-/TRITC-conjugated secondary

antibodies for 1 h at room temperature, and then stained with DAPI. Finally, coverslips were observed under a fluorescence microscope.

Table S1. Oligonucleotides used for RT-qPCR

Name	Sequence (5' to 3')
VIM up	ACGTTCGTCAGCAGTATGAAA
VIM low	GTTAGCAGCCTCAGAGAGGTC
CDH1 up	CAGCCACAGACGCGGACGAT
CDH1 low	CTCTCGGTCCAGCCCAGTGGT
CDH2 up	TCGCCATCCAGACCGACCCA
CDH2 low	GCAGTTGACTGAGGCGGGTGC
TJP1 up	CAGGAAATCTATTTCAAGGTCTGC
TJP1 low	CATCACCAAAGGACTCAGCA
ZNF281 up	GAGCAGCAGGTGCCAAGGTG
ZNF281 low	CTCCCCGTCCCGTGTCAATT
SLUG up	TTCGGACCCACACATTACCT
SLUG low	TTGGAGCAGTTTTTGCACTG
SNAIL up	GTCCGTCTGCCGCACCTGAG
SNAIL low	ACACGGCGGTCCCTACAGC
TWIST1 up	GCAAGAAGTCGAGCGAAGAT
TWIST1 low	GCTCTGCAGCTCCTCGAA
ZEB1 up	TCAAAAGGAAGTCAATGGACAA
ZEB1 low	GTGCAGGAGGGACCTCTTTA
ACTB up	AGGCCAACCGCGAGAAGATGACC
ACTB low	GAAGTCCAGGGCGACGTAGCAC

Table S2. Oligonucleotides used for ChIP

Name	Sequence (5' to 3')
miR-543 E-box-1 up	ACGGCCGAACCCAAGGCAAG
miR-543 E-box-1 low	GTCACCACTGTCGATCAGCC
miR-543 E-box-2 up	GCTCAGAGGCTCCATCAGGG
miR-543 E-box-2 low	CTGGGAGACATCTAACAGGT
ZEB1 Z1 up	GACTCGAGCATTTAGACACA
ZEB1 Z1 low	GCAGAGAGCACTACTTTCTA
ZEB1 Z2 up	GAGCGGCTGTTGCTTCTTTC

ZEB1 Z2 low	CGCGCACAAACCCCACGCAA
ZEB1 Z3 up	GGAACAAGGCAGGAAAGGTA
ZEB1 Z3 low	ACTGCAGCTGCGGTGCAAGT
ZEB1 Z4 up	TAATGGGCGGCAACGGCCCT
ZEB1 Z4 low	CCTAAACACGTATTTCCTCG
Snail S1 up	GAGCAGCAGGTGCCAAGGTG
Snail S1 low	CTCCCCGTCCCGTGTCAATT
Snail S2 up	GACAGTAGTTCTGCCCTTCA
Snail S2 low	GGGACCTGGTTAGAGTTTCG
Snail S3 up	AGTCCAAACTCCTACGAGGC
Snail S3 low	AGGGAAGTGTGCTTTGGTGG
Snail S4 up	TGATGTGCGTTTCCCTCGTC
Snail S4 low	AAGCGAGGCCTCTGCGAGGT