# RHOJ Induces Epithelial-Mesenchymal Transition by IL-6/STAT3 to Promote Invasion and Metastasis in Gastric Cancer

## Supplementary materials and methods

### Tissue specimens

A total of 30 formalin-fixed, paraffin-embedded GC samples (8 EMT-subtype GC samples and 22 Non-EMT-subtype-GC samples) were randomly recruited from the pathology department of Nanjing Drum Tower Hospital. All of these samples were cut into 4 μm slices and used for IHC staining.

### Cell culture

Human GC cells line (MKN-45, MKN-28, MGC-803, BGC-823, SNU-1, HGC-27, SGC7901) and human umbilical vein endothelial cells (HUVEC) were purchased from iCell Bioscience Inc (Shanghai, China). Human GC cells line (SNU-719, SNU-216) and human embryonic kidney 293T (HEK-293T) cells were obtained from were derived from Cobioer Biosciences (Nanjing, China). Maintained at 37°C and 5% $CO_2$, all cells were cultured in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (Sigma, USA) and 1% penicillin/streptomycin (Sangon Biotech, Shanghai, China).

### Establishment of stable RHOJ knockdown/overexpression GC cells line

To generate the stable RHOJ knockdown/overexpression GC cell line, lentiviral constructs of RHOJ knockdown and RHOJ overexpression were used. pLKO.1-shScramble and pLKO.1-shRHOJ vectors were acquired from

23 Sigma-Aldrich. pLenti-CMV-GFP/puro and pLenti-CMV-RHOJ vectors were
24 purchased from Miaoling Biotechnology (Wuhan, China). pLKO.1-shRHOJ vector
25 sequences were as follows: pLKO.1-shRHOJ-1 (forward)
26 5'-GCCCGTTTGCTGTATATGAAA-3', pLKO.1-shRHOJ-3 (forward)
27 5'-CATCTGCTTCTCTGTCGTAAA-3'. Briefly, pLKO.1-shRHOJ plasmids
28 containing the RHOJ knockdown target sequences were transduced into SGC7901
29 and SNU-1 cells, while pLenti-CMV-RHOJ plasmids containing the RHOJ
30 overexpression target sequences were transduced into SNU-1 and MKN-45 cells.
31 After 48 h of infection with corresponding lentiviral constructs, puromycin-resistant
32 single clones were screened (screening concentration was 2 μg/mL). Approximately
33 after 4 weeks, the single clones proliferated and the RHOJ knockdown/overexpression
34 clones were identified by qPCR and western blotting.

35 **Quantitative real-time PCR (qPCR)**

36 Total RNA was extracted from GC cells using the Total RNA rapid extraction reagent
37 (Yfxbio, Nanjing, China). qPCR was performed using a 5×All-In-One RT MasterMix
38 (G592, ABMgood, USA) and Hieff® qPCR SYBR Green Master Mix (11201ES50,
39 Yeasen, Shanghai, China), according to the manufacturer's instruction. The following
40 primer sequences were selected: GAPDH, (forward)
41 5'-ACCCAGAAGACTGTGGATGG-3', (reverse)
42 5'-TTCAGCTCAGGGATGACCTT-3', RHOJ, (forward)
43 5'-CCTGAGTGACAGAGAAAGAACC-3', (reverse)
44 5'-GGAGTGTGTGCGTATGAAAGA-3', TNF-α, (forward)

45 5'-GAGGCCAAGCCCTGGTATG-3', (reverse) 5'-CGGGCCGATTGATCTCAGC-3',
46 IL-1β, (forward) 5'-TGAAATGATGGCTTATTACAGTGG-3', (reverse)
47 5'-GTAGTGGTGGTCGGAGATTCGTAG-3', IL-6, (forward)
48 5'-GGCACTGGCAGAAAACAACC-3', (reverse)
49 5'-GCAAGTCTCCTCATTGAATCC-3'.

50 **Western blotting**

51 Total proteins from GC cells were homogenized in Lysis Buffer (50 mM Tris, 150
52 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, pH 7.4) containing 1% protease
53 inhibitors and 1% phosphatase inhibitors. After centrifuging at 12,000 rpm for 10 min
54 at 4°C, the total protein content was measured by Pierce BCA Protein Assay Kit
55 (23227, Thermo) and denatured for 10 min at 100°C. Proteins were separated by 10%
56 SDS-PAGE and transferred to nitrocellulose membranes and blocked for 1.5 h with 5%
57 skim milk (PBS diluted). Then membranes were respectively incubated with the
58 following primary antibodies: RHOJ (1:500, Abnova, H00057381-M01), E-cadherin
59 (1:1000, Cell Signaling Technology, #3195), Vimentin (1:1000, Cell Signaling
60 Technology, #5741), N-cadherin (1:2000, Proteintech, 22018-1-AP), ZEB1 (1:1000,
61 Abclonal, A5600), SNAI2 (1:1000, Proteintech, 12129-1-AP), STAT3 (1:5000,
62 Thermo, MA1-13042), p-STAT3 (Tyr705) (1:2000, Cell Signaling Technology,
63 #9145S), p-STAT3-S727 (1:1000, Abclonal, AP0715), VEGFA (1:1100, Sangon,
64 D360788-0025), and GAPDH (1:100000, Proteintech, 60004-1-Ig), and then were
65 detected using specific secondary antibodies (1:10000, Bioworlde,
66 BS13278/BS12478). Protein bands were visualized by the ECL kit (P10200, NCM

67 Biotech, Suzhou, China). The relative levels of all individual proteins were based on

68 GAPDH.

69 **Immunofluorescence (IF)**

70 Seeded in co-focal dishes ($2\times10^5$ cells/well), cells were fixed with 4%

71 paraformaldehyde and then ruptured the cell membranes by 0.3% Triton (PBS diluted)

72 for 10 min. After three times of PBS washing, non-specific antigen-binding sites were

73 blocked for 1.5 h with 5% BSA (PBS diluted). Cells were then separately incubated

74 with E-cadherin (1:100, Abclonal, A11492) and Vimentin (1:100, Proteintech,

75 60330-1-Ig) overnight at 4°C. Next day, after three times of PBS washing, cells were

76 incubated with corresponding secondary antibodies attached with FITC (1:100,

77 Abclonal, AS001) or Cy3 (1:100, Abclonal, AS007) for 1.5 h. The nuclei were then

78 stained with DAPI for 5 min and terminal images were captured using a confocal

79 microscope from the Analysis and Testing Center at NMU.

80 **Immunohistochemistry (IHC)**

81 Human GC samples and mice lungs samples were fixed with 4% paraformaldehyde

82 and paraffin-embedded before cutting it into 4 μm thick sections. The tissue sections

83 were routinely dewaxed, rehydrated, and subjected to antigen retrieval by heating in

84 sodium citrate (pH 6.0) for 10 min. After blocking with 3% $H_2O_2$, sections were

85 incubated with the primary antibody (5% BSA diluted): RHOJ (1:150, ORIGENE,

86 TA505592), E-cadherin (1:400, Cell Signaling Technology, #3195), Vimentin (1:400,

87 Cell Signaling Technology, #5741), and CD31 (1:400, Cell Signaling Technology,

88 #77699) at 4°C for overnight, next day washed with PBS and incubated with the

89 corresponding secondary antibody (PV-9001/PV-9002, ZSGB-BIO, Beijing, China)
90 for 30 min at 37°C. DAB kit (PV-9001/PV-9002, ZSGB-BIO, Beijing, China) was
91 applied to visualize the sections, followed by counterstaining with hematoxylin and
92 dehydrating. Terminal images of these sections were captured by a bright-field
93 microscope (Leica Microsystems).

94 **Collecting conditioned medium**

95 RHOJ knockdown cells (SGC7901, SNU-1) were seeded into 6-well plates
96 ($3\times10^5$ cells/well) and cultured with DMEM complete medium. After 36 h cultivation,
97 sterile syringes and 0.2 μm filters (4433, PALL) were used to collect the cells'
98 supernatant as a conditioned medium. Then its were stored at 4°C and used within a
99 week.

100 **Angiogenesis assay**

101 The conditioned medium of RHOJ knockdown cells (SGC7901, SNU-1) was
102 collected in advance and reserved at 4°C. 1 h prior to running angiogenesis assay, 500
103 μL diluted matrigel (40183ES08, Yeasen, Shanghai, China) was added to 24-well
104 plates and placed at 37°C for solidification. Then $4\times10^5$ HUVEC cultured with 500
105 μL conditioned medium were dripped in 24-well plates. After 16 h incubation, the
106 tube formation images of HUVEC were photographed by a microscope, and the tubes'
107 relative length and number were counted by Image J software.

108 **Colony-forming assay**

109 Cells were plated in 6-well plates ($1.5\times10^3$ cells/well) and cultured for 15 days. Then
110 cells were fixed with 4% paraformaldehyde for 40 min and stained with 0.1% crystal

violet for 40 min. After washing with water, the final figures were photographed and counted by Image J software.

**CCK-8 assay**

Approximately 2,000 cells per well with 100 μL DMEM complete culture medium were planted in 96-well plates. At 0 h, 48 h, 72 h, 96 h, and 120 h, respectively, 10 μL CCK-8 solution (40203ES76, Yeasen, Shanghai, China) was added to per well. After 1.5 h incubation, the absorbance was measured at 450 nm.

**Cell counting assay**

Cells were seeded in 12-well plates ($5\times10^5$ cells/well), and each group of cells had three independent wells at least. Then cells were separated from plates using trypsin at 0 h, 48 h, 72 h, and 96 h, respectively, and then blood counting plates were used for cell counting.

**Small interfering RNA (siRNA) transfection assay**

siRNAs were purchased from Genepharma (Shanghai, China), and the sequence of siSTAT3 was (forward) 5'-GCAGCAGCUGAACAACAUGTT-3'. According to the manufacturer's protocol, siRNAs were transfected into GC cells by siRNA-Mate Transfection Reagent (G04003, Genepharma, Shanghai, China). After 48 h incubation, total proteins were collected for further experiments.

130 **Supplementary figure legends**

131 **Figure S1. Upregulated in EMT-subtype GC, RHOJ is correlated with poor GC**

132 **prognosis, supplemented for Figure 1**

133 (A) In the GSE62254 dataset, RHOJ (labeled by molecular probes 235489_at)

134 expression levels in the four GC subtypes of ACRG.

135 (B) Kaplan-Meier analysis showed the OS in RHOJ high expression group and low

136 expression group GC patients, according to the TCGA database.

137 (C) The ingenuity pathway analysis (IPA) of EMT-subtype GC-related genes

138 identified the signaling of RHO Family GTPases.

139 *$P$<0.05, **$P$<0.01, ***$P$<0.001, ****$P$<0.0001. Data were expressed as Mean±

140 SD.

141 **Figure S2. RHOJ mediates EMT to regulate the migration and invasion of GC**

142 **cells, supplemented for Figure 2**

143 (A) Pearson correlation analysis assessed the links between the expression levels of

144 the EMT-related genes (CDH1, VIM, ZEB1, ZEB2, and FN1) with RHOJ in the

145 TCGA database.

146 (B) RHOJ relative expression levels of RHOJ knockdown cells (SGC7901, SNU-1)

147 were assessed by qPCR.

148 (C) Morphological observation showed morphological variance between parental

149 control and RHOJ knockdown cells (RHOJ), magnification, 100×, 200×, scale bar,

150 200 μm, 100 μm, respectively.

151 (D) Cultured with a serum-free medium, the CCK-8 assay assessed the viability of

152 RHOJ knockdown SGC7901 and SNU-1 cells at 48 h and 24 h, respectively.

153 (E) Cultured with a serum-free medium, the CCK-8 assay assessed the viability of

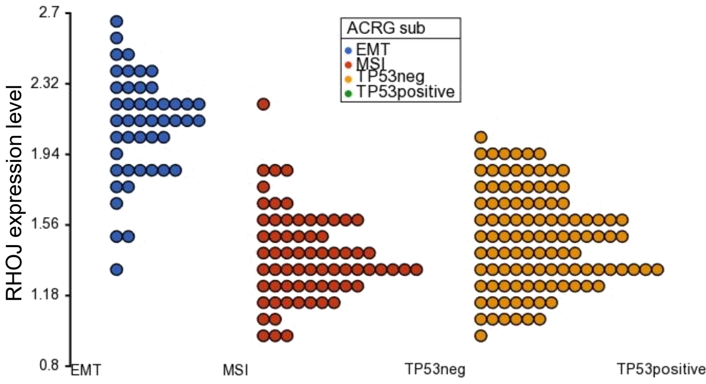
154 RHOJ overexpression SNU-1 and MKN-45 cells at 24 h and 36 h, respectively.

155 *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. Data were expressed as Mean ±

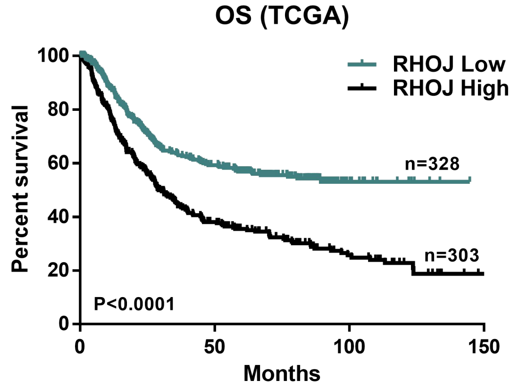
156 SD.

**Figure S1**

**A**




**B**




**C**

Signaling by Rho Family GTPases : GSE66229-EMT vs other 3 subtypes-FC1.5Q-single probe : Expr Fold Change

Figure S2

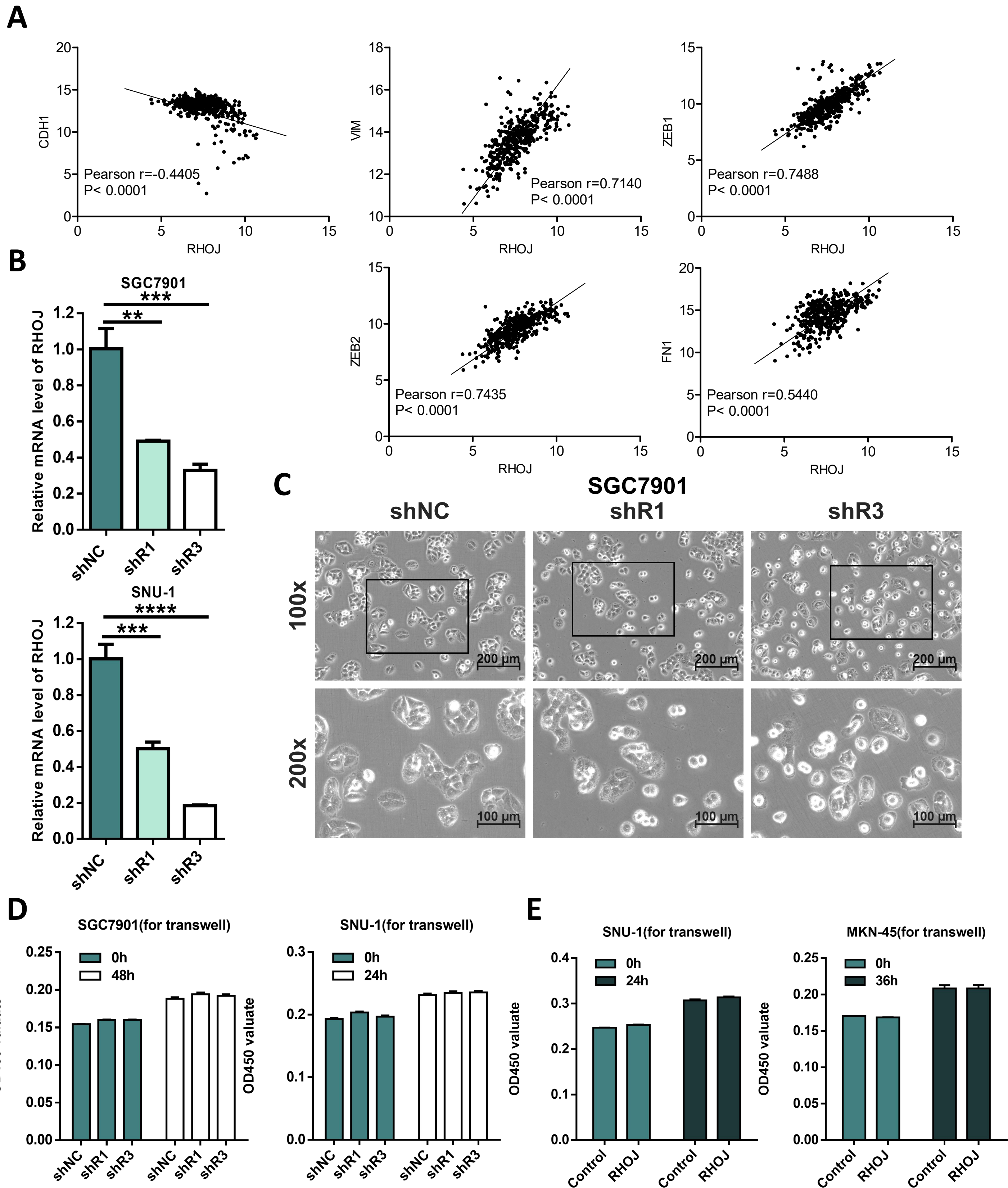


**Table S1 Correlation between the GC clinicopathological features and expression of RHOJ**

| Characteristics | Low Expression [N, (%)] | High Expression [N, (%)] | *P*-Value |
|---|---|---|---|
| **Molecular Subtype** | | | |
| EMT | 3 (2.0) | 43 (28.7) | |
| MSI | 47 (31.3) | 21 (14.0) | **<0.0001** **** |
| MSS/TP53- | 59 (39.4) | 48 (32.0) | |
| MSS/TP53+ | 41 (27.3) | 38 (25.3) | |
| **Age** | | | |
| Mean (SD) | 63.29 (10.64) | 60.59 (11.91) | **0.0398** * |
| Median [Min, Max] | 64.0 [31.0, 84.0] | 62.0 [24.0, 86.0] | |
| Not reported | 0 | 0 | |
| **Gender** | | | |
| Male | 107 (71.3) | 92 (61.3) | 0.0869 |
| Female | 43 (28.7) | 58 (38.7) | |
| **Status** | | | |
| Alive | 89 (59.3) | 59 (39.3) | **0.0008** *** |
| Dead | 61 (40.7) | 91 (60.7) | |
| **Stage (T)** | | | |
| T1+T2 | 117 (78.0) | 71 (47.3) | **<0.0001** **** |
| T3+T4 | 33 (22.0) | 79 (52.7) | |
| **Stage (N)** | | | |
| N0+N1 | 91 (60.7) | 78 (52.0) | 0.1623 |
| N2+N3 | 59 (39.3) | 72 (48.0) | |
| **Stage (M)** | | | |
| M0 | 142 (94.7) | 131 (87.3) | **0.0420** * |
| M1 | 8 (5.3) | 19 (12.7) | |
| **pStage** | | | |
| I | 20 (13.3) | 12 (8.0) | |
| II | 60 (40.0) | 36 (24.0) | **0.0029** ** |
| III | 38 (25.4) | 57 (38.0) | |
| IV | 32 (21.3) | 45 (30.0) | |
| **Recurrence** | | | |
| Yes | 46 (30.7) | 79 (52.7) | |
| No | 95 (63.3) | 62 (41.3) | **0.0004** *** |
| Unknown | 9 (6.0) | 9 (6.0) | |
| **Perineural Invasion** | | | |
| Positive | 35 (23.3) | 53 (35.3) | |
| Negative | 90 (60.0) | 69 (46.0) | **0.0364** * |
| Not reported | 25 (16.7) | 28 (18.7) | |