Supplementary material

Materials

siRNA

siRNAs were from Qiagen. A BLAST search of the sequences was carried out against GenBank using the NCBI website. Only siRNA duplexes with a similarity region to other human sequences shorter than 15 base pairs were used. The following target sequences were used in this study: IMP1(1), 5'-AAGCTGAATGGCCACCAGTTG-3'; IMP1(2), 5'-AACACCTGACTCCAAAGTTCG-3'; IMP3(1), 5'-AATCGATGTCCACCGTAAAGA-3'; IMP3(2), 5'-AATCCAGAACGCACTATTACA-3'; Scr siRNA exhibiting a scrambled IMP1 sequence, 5'-AAGCATGCTCATCGACGGGAC-3'; and CD44 (3'UTR) 5'-CACCTGTTCTCTCCTGTGAAA-3`. The IMP siRNAs were used in sets to knock-down both IMP1 and IMP3, and were denoted IMP(1,3)A consisting of the IMP1(1) and IMP3(1) siRNAs and IMP(1,3)B consisting of the IMP3(2) + IMP3(2) siRNAs. The following siRNAs were ordered from the Qiagen siRNA library: A fluorescein-conjugated control siRNA (non-silencing), 5'-AATTCTCCGAACGTGTCACGT-3'; lamin A/C, 5'-AACTGGACTTCCAGAAGAACA-3'; and CD44 coding region siRNA (Catalogue #1022560)/(#SI00299705).

Antibodies

Polyclonal rabbit anti-IMP1, anti-IMP2 and anti-IMP3 antibodies were generated as previous described (Nielsen *et al*, 1999). Rabbit anti-TBP (sc-204), mouse anti-MCAM (sc-18837), rabbit anti-Arp2 (sc-15389), rabbit anti-paxillin (sc-5574) and mouse anti-CD44 (sc-7297) antibodies were obtained from Santa Cruz Biotechnology. Rabbit anti-Arp3 (07-272), mouse anti-phospho-tyrosine (05-321), mouse anti-lamin A/C (05-714), rabbit anti-p34-Arc (07-227), mouse anti-Rac (05-389), mouse anti-Rho(-A, -B, -C) (05-778) and mouse anti-Cdc42 (05-542) antibodies were purchased from Upstate. Rabbit anti-β-actin (A5316) and mouse anti-β-tubulin (T4026) were from Sigma.

Mouse anti-α3 integrin (17C6), mouse anti-α5 integrin (JBS5), mouse anti-α6 integrin (450.30A) and mouse anti-β1 integrin (12G10) was purchased from Serotec. Mouse anti pan-cadherin (ab6528) was from Abcam. Mouse anti-β1 integrin (MAR4) (555442) and mouse isotype control (555746) was purchased from BD bioscience. The integrin β1 function-blocking mAbs rat IgG_1 AIIB2 developed by C.H. Damsky was obtained from the Developmental Studies Hybridoma Bank. Mouse anti-BrdU was from DakoCytomation. Texas-Red anti-mouse IgG (150-075-150), Texas-Red anti-rabbit IgG (711-075-152), fluorescein anti-mouse IgG (715-095-150) and fluorescein anti-rabbit IgG (711-095-152) were obtained from Jackson Immunoresearch, while HRP-conjugated goat anti-mouse (M15345), HRP-conjugated goat anti-rabbit (R14745) were purchased from BD Transduction Laboratories. HRP-conjugated rabbit anti-digoxigenin (P5104) was obtained from DakoCytomation. HRP-conjugated sheep anti-digoxigenin (11 207 733 910), Rhodamine-conjugated sheep anti-digoxigenin (11 207 750 910) and AP-conjugated sheep anti-digoxigenin (11 093 274 910) were from Roche. Finally, Alexa Flour 488 phalloidin, Alexa Flour 660 phalloidin and Alexa Flour 660 goat anti-mouse (A-21055) were obtained from Molecular Probes.

Plasmids

Mouse *Imp1* plasmid was obtained from MRC (accession number BC051679) and cloned into the pcDNA3.1 vector (Invitrogen), while the pEYFP-actin and pEYFP-C2 plasmids were obtained from BD Biosciences Clontech.

Primers

The following primers were used in RT-PCR and quantitative RT-PCR: IMP1, 5'-CAGGAGATGGTGCAGGTGTTTATCC-3' and 5'-GTTTGCCATAGATTCTTCCCTGAGC-3'; IMP3, 5'-GGACGTCTTATTGGTAAAGAAGG-3' and 5'-CCCAAGGCGTTCAGATTTAATCC-3', β-actin, 5'-TCCTGTGGCATCCAC-3' and 5'-GAAGCATTTGCGGTG-3'; Col5A1, 5'-CATGGAAGAGATCTTCGGCTCTCTCAAC-3' and 5'-GCTGTGAAGTTGCAGTAA

ACCTTGAAGG-3'; Amigo2, 5'-AACAGTGAAGAGGCAACACAGAGCTCC-3' and 5'-GTGGGCAGAGTGTGTACACGTAACGAC-3'; Lumican, 5'-TGGTTGAGCTGGATCTGTCCT-3' and 5'-GGAAGACTGGTTTCTGAGATGG-3'; Alcam, 5'-TGAGGCACCTACAATAG TCAAGG-3' and 5'-TATAGAGCTGAGTCACTGGGTCC-3'; Syncam, 5'-GTTGCGACCATCAGTTGCCAAGTC-3' and 5'-GAGCTGGCAAAAGTATCTTCCTTC-3'; CD44, 5'-CATCAGTCACAGACCTGCCCAATGC-3' and 5'-ATGTAACCTCCTGAAGTGCTGCTCC-3'; CD24, 5'-ACTGGAACTTCAAGTAACTCCTCC-3' and 5'-GTTTAGAAGACGTTTCTTGGCCTG-3'; GAPDH, 5'-TCACCAGGGCTGCTTTTAAC -3' and 5'-GACAAGCTTCCCGTTCTCAG-3'. The following primers were used in electrophoretic mobility-shift and cross-linking analysis and all contain the T7 promoter (5'-TAATACGACTCACTATAGGG-3') on the forward primer (F): CD44-1F, 5'-GCAGAAGAAAAAGCTAGTGATCAAC-3'; CD44-1R, 5'-GAAACAATCAGTAGCACATTGCATC-3'; CD44-2F, 5'-GATGCAATGTGCTACTGATTGTTTC-3'; CD44-2R, 5'-CCATTCAAATTTCCTCCCAGGGACC-3'; CD44-3F, 5'-GGTCCCTGGGAGGAAATTT GAATGG-3'; CD44-3R, 5'-GTTTAAACCCTTTTGTGCCTCTGAC-3'; CD44-4F, 5'-GTCAGAGGCACAAAAGGGTTTAAAC-3'; CD44-4R, 5'-GGATGGAAAACCTTTGG ACAGTGTC-3'; CD44-5F, 5'-GACACTGTCCAAAGGTTTTCCATCC-3'; CD44-5R, 5'-CAAACAGGATCTACATGGCATCATC-3'; CD44-6F, 5'-GATGATGCCATGTAGAT CCTGTTTG-3'; CD44-6R, 5'-GGAAGTTCTAGGACCAATCGGACAC-3'; CD44-7F, 5'-GTGTCCGATTGGTCCTAGAACTTCC-3'; CD44-7R, 5'-GAGGAGCAGAGGCTGGG AATAGTGC-3'; CD44-8F, 5'-GCACTATTCCCAGCCTCTGCTCCTC-3'; CD44-8R, 5'-CCTGGAGTGGCTTGTTGCTTTTCAG-3'; CD44-9F, 5'-CTGAAAAGCAACAAGCCAC TCCAGG-3'; CD44-9R, 5'-GACAATGGCCAAGGAATAGAAGCTC-3'; CD44-10F, 5'-GAGCTTCTATTCCTTGGCCATTGTC-3'; CD44-10R, 5'-GGAGACACAAAAGATTTAC AATTGC-3'; CD44-11F, 5'-GCAATTGTAAATCTTTTGTGTCTCC-3'; CD44-11R, 5'-GAATGGACCATGTTATTCTTATTC-3'; CD44-12F, 5'-GAATAAGAATAACATGG TCCATTC-3'; CD44-12R, 5'-CCTTTAGTCTTTTAATGTTA GCC-3'. The following primers were used to generate *in situ* probes: GAPDH-F: GTTCCAATATGATTCCACCCATGGC; T7-GAPDH-R: TAATACGACTCACTATAGGGGAGGCATTGCTGATGATCTTGAGGC and CD44-F:

GCAGAAGAAAAAGCTAGTGATCAACA T7-CD44-R:

TAATACGACTCACTATAGGGGAAACAATCAGTAGCACATTGCATC.

Methods

Cell culture, siRNA and plasmid transfections.

Human HeLa cells were obtained from the American Type Culture Collection and routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. One day before transfection 9000 cells/cm^2 were plated and siRNA transfections were performed using Oligofectamine (Invitrogen) and a final concentration of 20 nM siRNA duplex according to the manufacturer's instructions. The media were changed 24 hours after transfection and the cells were examined after another 48 hours. Transfection efficiencies were determined using a fluorescein-conjugated non-silencing siRNA duplex. Transient plasmid transfections were performed using FuGENE 6 (Roche) transfection reagent according to manufacturer's instructions.

Cell proliferation and TUNEL analysis

Seventy-two hours after siRNA transfection, cells were incubated with 50 μM bromodeoxyuridine (BrdU) for 2 hours, fixed with methanol for 5 min, washed with 0.5% tween-20 in PBS, and permeabilized with 1.5 M HCl for 20 min. After washing with 0.5% tween-20 in PBS, the cells were incubated with mouse anti-BrdU for 1 hour and subsequently with Texas-Red conjugated anti-mouse IgG for 1 hour. Fluorescent images of fixed cells were examined on a Zeiss LSM 510 confocal laser-scanning microscope. Cells with staining in the nucleus were scored as a cell in S-phase. A total of 500 cells throughout each dish were counted and 3 independent experiments were performed. Apoptotic cells were depicted by terminal deoxynucleotidyl transferase-mediated

dUTP nick-end labeling (TUNEL) staining with the Fluorescein-FragEL DNA fragmentation detection kit (Oncogene) according to the manufacturer's instructions.

Western blot analysis

Protein extracts from HeLa cells were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred to Hybond-P membranes (Amersham). After blocking, membranes were incubated overnight with primary antibody in blocking solution at 4°C, before they were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-mouse or anti-goat IgG for 1 hour at room temperature. Immunoreactive proteins were detected with Supersignal chemiluminescence reagents (Pierce) according to the manufacturer's instructions. Quantitation was performed on a LAS-1000 luminescent imager (Fuji) using Image Gauge 4.0 software (Fuji).

DNA microarray analysis

Two micrograms of purified RNA was biotin-labelled and hybridized to Affymetrix HG-U133A arrays, that contains 22.283 probe sets according to manufacturers instructions. Three independent experiments with RNAs from mock transfected cells and from cells treated with IMP(1,3)A and IMP(1,3)B siRNA sets, respectively, were performed. The individual samples were normalized using the invariant sets normalization method and the expression index computation and outlier detection were performed using the perfect-match only model implemented in the dChip software package, as described (Li and Wong, 2003). Transcripts were identified as being differentially expressed if the comparison of groups yielded a fold change higher than 1.5 (using the 90% lower confidence interval of the fold change) and a P-value below 0.05 (two-tailed, two-sample unequal variance t-test, Welch t-test).

Rho, Rac and Cdc42 activation assay

Rho, Rac and Cdc42 activation assay kits (Upstate) were used to measure the amount of the proteins in their active GTP-binding conformation. About 3 x 10^6 cells were seeded in a 150 mm dish and transfected with siRNA. Seventy-two hours later the cells were harvested and pull-down of the active GTP-binding conformation was performed according to the manufacturer's instructions, except for a change in lysis buffer (125 mM HEPES (pH 7.5), 750 mM NaCl, 50 mM $MgCl_2$, 5% Igepal, phosphatase inhibitors (P2850 and P5726, Sigma) and EDTA-free protease inhibitor cocktail (1.873.580, Roche)) The amounts of the precipitated G-proteins were quantified by western blot analysis using the anti-Rho, -Rac and -Cdc42 antibodies.

β1 integrin expression and activity

To characterize the role of β1 integrin during seeding, HeLa cells were trypsinized and washed once in culture media. The cells were resuspended and incubated at 37°C for 10 min in media to allow cell surface proteins to regenerate. The cells were preincubated for 15 min at room temperature with or without the function-blocking monoclonal antibodies to β1 integrin AIIB2 (10 μg/ml) before they were seeded on a laminin-1 coated glass-surface (2 μg/cm^2). After 30-60 min the cells were gently washed and fixed in 4% formaldehyde. Following staining with 0.1% crystal violet the cells was examined by microscopy. The cell surface expression and activation state of β1 integrin was examined by FACS analysis using a FACSCalibur flow cytometer (Becton Dickinson). Seventy-two hours after siRNA treatment HeLa cells were detached with cell dissociation buffer (Invitrogen) and restored for 5 min at 37^{o}C in culture medium supplemented with 10% FBS and washed in ice cold PBS supplemented with 1% BSA. Mouse anti-human 12G10, MAR4 and an isotype control antibody were diluted according to manufactures description and added to the cell suspension. After 20 min at 4^{o}C, cells were washed twice with washing buffer, incubated with FITC-conjugated goat anti-mouse IgG for 20 min at 4^{o}C and rinsed twice before analysis.

Polysome isolation

For polysome isolation, cells were harvested in 10 μg/ml cycloheximide and lysed in 500 μl lysis buffer (20 mM Tris-HCl (pH 8.5), 1.5 mM $MgCl_2$, 140 mM KCl, 0.5 mM dithiothreitol, 0.5% NP-40, 250 units of RNasin (Promega) and 0.1 mM cycloheximide). The lysates were centrifuged at 10,000 *g* for 10 min, and the supernatant was applied to a linear 20 to 47% sucrose gradient in 20 mM Tris-HCl (pH 8.0), 140 mM KCl, 5 mM $MgCl_2$. Centrifugation was carried out at 40.000 rpm for 2.25 hours in a Beckman SW41 rotor. Fractions of 1 ml were collected, followed by isolation of RNA and northern blot analysis.

Statistical analysis

Data in Figures 2, 4, 5 and 6 are expressed as mean ± s.d. Statistical comparisons were performed using a two-tailed unpaired students t-test with Welch correction, since we do not assume equal variance. The data in Figures 1 and 3 are presented as individual data points with the median indicated. Statistical comparison was made by a Mann-Whitney test, since the data were non-parametric.