Methods

Tissues and cells

RA and osteoarthritis (OA) synovium as well as OA cartilage was obtained during knee replacement surgery. Synovium was digested with Dispase-II-solution (2.4U/ml, PAN-Biotech, Aidenbach, Germany) in PBS at room temperature (RT) for 1h. The cell suspension was passed through a 70µm cell strainer (BD-Biosciences, Heidelberg, Germany). Cells were detached using trypsin-EDTA (PAA Laboratories, Cölbe, Germany) unless stated otherwise. RASF were used for experiments were used up to passage 6 (passage 9 in assays requiring high amounts of cells, SCID-mouse-experiments). RASF at 70% confluency were stimulated for 16 hours, afterwards cells were washed with PBS and lyzed for RNA isolation.

CD82 overexpression and knockdown

For lentiviral transduction of the *Cd82* gene (full length), Precision LentiORF individual clone (with native stop codon) and Precision LentiORF RFP (red fluorescent protein) control was used (GE Healthcare). The transduction efficiency (MOI, multiplicity of infection) was determined by serial dilutions of virus in the supernatants of RASF and MOI 5 selected for experiments. The day before transduction, cells were detached, counted, and $2x10^4$ cells/well distributed into 6-well plates in DMEM with 10% FCS. Virus working solution with MOI of 5 (in DMEM with 10% FCS, HEPES, 0.01mg/ml protamine sulfate in PBS) was added followed by incubation for 15min at 37°C and centrifugation for 30min at 1600xg. After 48h at 37°C in a 10% CO_2 incubator, 11µg/ml Blasticidin S hydrochloride (Roth, Karlsruhe, Germany) was added as selection medium, resulting in stable overexpression of CD82 after 2 weeks. Mock cells (same treatment but without virus) served as negative control.

For Amaxa nucleofection, 2.66µg or 0.2nm siRNA (ON-TARGETplus SMART pool CD82 or ON-TARGETplus siCONTROL non-targeting-pool (NTP) siRNA, PerbioScience, Bonn, Germany) for $4x10^5$ cells were used according to the manufacturer's instruction. Mock-treated cells without siRNA served as control.

Western blot

Cells were detached and 2.4-2.6x10^6 cells/ml were lysed. Lysates were mixed with 6x SDS-protein buffer (0.375M Tris pH 6.8, 12% SDS, 60% glycerol, 0.6M DTT, 0.06% bromophenol blue, non-reducing), denaturated at 95°C for 5min, centrifuged and the supernatant loaded on a 6%-12% SDS-polyacrylamide gel followed by gel electrophoresis (120V, 1.5 hours). Protein transfer was performed for 30min at 120mA to PVDF membranes (Merck Millipore, Darmstadt, Germany). After blocking with 5% milk powder in PBS or TBST (0,01M Tris Base, 0.15M NaCl, 0.1% Tween-20) for 1h at room temperature, the membrane was incubated with anti-human CD82 antibodies diluted 1:1000 in TBST over night at 4°C. Membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse, Agilent Technologies, Santa Clara, US) diluted 1:1000 in TBST for 1h at room temperature and then washed extensively. Detection was performed using an enhanced chemiluminescence (ECL) detection kit (Western blotting detection system, West Dura). All Western blots were probed for cyclophilin B (Abcam) diluted 1:2000 in TBST to ensure equal samples loading. Quantification of the signal intensity for each band was measured and the area below the respective histogram curve calculated using the imageJ software.

Immunohistochemistry and immunofluorescence

5µm tissue sections or glass chamber slides with cultured fibroblasts were acetone-fixed for 10min and blocked with 2% BSA for 1h. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in 100% methanol for 30min at 4°C. Sections were incubated with anti-human CD82 antibody (1:200) or monoclonal isotype control IgG (1:100) in PBS over night at 4°C in a humid chamber. As secondary antibody, a biotinylated goat anti-rabbit antibody (1:200 in PBS) for 1h combined with HRP-conjugated streptavidin (1:500 in PBS) for 30min was used.

Fluorescent double staining was performed using anti-human Integrin α6 (1:40), integrin αV (1:100), integrin β1 (1:200), and CD82 (1:200) antibodies. The secondary antibodies were either anti-mouse for integrins or anti-rabbit for CD82, 1:500 each. The blocking was performed using a combination of

fetal calf, chicken and BSA (each 10% in PBS) for 45min in a humid chamber. The first antibodies, diluted in 10% BSA in PBS, were incubated over night (4°C). After washing, secondary antibodies (in 10% BSA in PBS) were added for 1.5h at room temperature.

All tissues were evaluated for CD82 and integrin subunit signals regarding specific tissue areas followed by calculation of the percentage of positive tissues: the outer synovial lining layer, the whole lining layer, inflammatory infiltrates, the EC layer of the vessel wall, the whole vessel wall, the cartilage invasion zone. The presence of overlay (yellow signal) was evaluated for the respective areas.

SCID-mouse-model

Six weeks old Crl-scidBR mice were used. Cartilage from knee replacement surgery from OA patients was obtained and intact areas of cartilage cut for histological evaluation (H/E-staining). Only cartilage areas with homogeneous matrix and viable chondrocytes were used for co-implantation. $1.5x10^5$ RASF together were used for implants at the ipsilateral side. Invasion score: 0 = no or minimal invasion. 1 = visible invasion (≥ 2 cell depths), 2 = invasion (≥ 5 cell depths), 3 = deep invasion (≥ 10 cell depths).

Laser-mediated microdissection (LMM)

Cryosections of human tissue or SCID-mouse implants were fixed in 5% acetic acid in ethanol, nuclei stained and dehydrated in 70%/96%/100% ethanol, twice each for 2min. Sections were dried and immediately used for dissection or stored at -80°C (for a maximum of 2 days). Areas of interest were cut using a focused, pulsed laser beam. Dissected areas (in total 3000 cells) were collected after catapulting in microcentrifuge tubes containing RLT lysis buffer. Cells from different tissues or implants were pooled before RNA isolation as indicated for the respective experiments.

Real-time PCR

Reverse transcription after denaturation (2min at 70°C) and immediate cooling on ice was performed for 15min at 25°C, 5min at 37°C, 60min at 42°C, 30min at 55°C and 5min at 72°C.

LightCycler real-time PCR mixture contained 2µl cDNA or distilled H2O (for negative control), 0.5µM sense and antisense primers each, 10µl of 2× QuantiTect SYBR Green PCR Master Mix (including HotStar Taq DNA polymerase, reaction buffer, dNTP mix, and SYBR Green I, Qiagen), and 4.5mM MgCl2. Real-time PCR cycling conditions were 15min at 95°C, 40 cycles of 15sec at 95°C, 25sec at 60°C and 30sec at 72°C, and finished by a melting curve analysis. The reference gene for normalization was 18S ribosomal RNA (18S rRNA). Relative gene expression was determined by ΔΔCt method.

Migration assay

Migration assessed in a Boyden chamber was performed with 8-µm pore size polycarbonate membranes coated with fibronectin (Sigma Aldrich) 1:10 in PBS. The lower compartment was filled with DMEM with 10% FCS (serving as chemoattractant), and the membrane was placed on top. Cells were harvested with accutase (Capricorn, Ebsdorfergrund, Germany) and $3x10^4$ cells were resuspended each in DMEM with 2% FCS per well. The cell suspension was placed in the upper compartment of the chambers (3 replicates each). After incubation at 37°C for 16 hours, the membrane was removed and the cells on the upper side of the membrane were scraped off. The membrane was then fixed with cold methanol, stained with hematoxylin and the stained nuclei of the migrated cells on the lower membrane side were counted. For the transwell migration assays, 6.5mm polycarbonate transwell filters with 8µm pores in 24well plate format were used. DMEM with 10% FCS was added to the lower compartment as chemoattractant. $1×10^5$ cells in 100µl DMEM with 2% FCS were added to the upper compartment. All settings were performed in triplicates. Cells were incubated for 16h in a 10% CO_2 incubator at 37°C. Migrated cells attached to the lower side of the filter were detached with trypsin, washed, and counted (Casy CellCounter or Neubaur Cell Chamber).

Cell motility assay

Fibroblasts were grown to a 100% confluent monolayer on uncoated 24-well plates. Cell layers were then wounded and supernatants replaced by with fresh medium. Cells were incubated for 17h at 10% CO_2 and photographed in three representative areas by phase contrast microscopy. The gap closure was evaluated microscopically for 3 representative areas per experimental replicate. The mean for each biological sample from different donors were used for statistical evaluation.

Adhesion assay

Early adhesion was analyzed in uncoated 24well plates or plates coated with Matrigel according to the manufacturer (BD Biosciences). Synovial fibroblasts were detached with accutase (Capricorn) and centrifuged. $1x10^4$ fibroblasts per well were incubated for 1h at 37°C. Then, the plate was shaken for 5min full speed. Supernatants containing non-adherent or loosely attached cells were removed and the shaking repeated twice. Fibroblasts were stained for 20min. Stained cells counted in 3 representative areas of 3 wells per cell population (means per patient used for statistics).

Cell-to-cell binding assay

Human Umbilical Vein Endothelial Cells (HUVEC) obtained commercially or primary EC isolated from human varicose veins were used. Briefly, varicose veins were flushed with sterile PBS to remove blood from the vascular lumen. One end of the vessel was clamped. The lumen was filled with collagenase H (1mg/ml; Roche). Then, the other vessel end was clamped, and incubated for 1 hour at 37°C in PBS. The detached endothelial cells were collected by withdrawing the cell suspension using tweezers and a pipette, and flushing the lumen twice with culture medium. The cell suspension was centrifuged, and the cells were resuspended in culture medium for further culture. Endothelial cells were characterized morphologically and by positive immunocytochemical staining for CD31 without fibroblast contamination.

EC were cultured on rat tail collagen-I coated plates (3mg/ml 1:40 in PBS). RASF were stained with Calcein-AM solution according to the manufacturer's instructions for 30min followed by washing in

PBS. $5x10^3$ RASF were added to the confluent EC-layer and incubated for 30min at 37°C. After shaking, cells were washed to remove non-adherent or loosely attached cells and the shaking repeated twice. Attached fibroblasts (stained fluorescent green easily distinguishable from non-stained EC) were evaluated in 3 representative areas of 3 different wells (experimental triplicates) per cell population and the number of fluorescent RASF was quantified. Using bright field microscopy, the confluency of the non-stained EC layer was confirmed. For statistical evaluation, means for each cell population (biological replicates) were used.

Proliferation assay

A colorimetric cell proliferation 5-bromo-2'-deoxyuridine (BrdU)-ELISA (Merck Millipore) assay was performed according to the manufacturer's instructions. Cells were seeded at $3×10^4$ cells/well in 96well plates. BrdU labeling in DMEM was added followed by incubation for 24h, denaturation of the cells and staining with anti-BrdU antibodies. The level of proliferation was quantified by light emission detected using a TECAN reader.

Statistical analysis

Results were evaluated using GraphPad Prism 6.0 (Graph Pad Software Inc., San Diego, USA).