

Appendix S1

SUPPLEMENTARY MATERIAL AND METHODS

In vitro migration assays using digital holographic microscopy

In order to analyse the migration of the PACE4 transfectants and controls in a 3-dimensional environment, a collagen matrix was used. A cell suspension of 1×10^6 cells/ml cell culture medium was prepared and stored on ice. 1,000 μ l cell suspension and 200 μ l calf skin collagen type I (collagen G, Biochrom, Berlin, Germany) were mixed carefully at 4°C without producing air bubbles to a final concentration of 0.67 mg/ml, and incubated for 10 min at 37°C. Afterwards, the solution was mixed carefully again and 200 μ l were added to a Petri dish. After further incubation for 20 min at 37°C for complete polymerization 900 μ l HEPES buffered cell culture medium was added. Cells were observed with an inverted microscope (iMIC, Till Photonics, Gräfelfing, Germany) that had an attached module for digital holographic microscopy as described previously (38). The coherent light source was a frequency-doubled Nd:YAG laser (Compass 315M-100, Coherent, Lübeck, Germany, $\lambda = 532$ nm). A climate chamber (Solent Scientific Ltd, Segensworth, UK) was utilized for temperature stabilization at 37°C. In a time-lapse series, digital off-axis holograms of selected cells were recorded every 3 min over periods of 24 h, with a charge-coupled device sensor (CCD, 1,280 \times 960 pixels, The Imaging Source DMK 41BF02, Bremen, Germany). For imaging, a 20 \times microscope lens (Zeiss LD Acroplan 20 \times , 0.4 Korr, Zeiss Göttingen, Germany) was used. Numerical evaluation of resulting digital holograms was performed by spatial phase shifting-based reconstruction as described in detail previously (27, 28). Cell tracking on resulting quantitative phase contrast images was performed by determination of the coordinates related to the maximum cell thickness (39).

Immunofluorescence analysis

Cells were seeded into 8-well chamber slides and fixed on the next day with methanol at -20°C. Slides were then blocked with 5% donkey serum for 1 h, followed by incubation for 1 h with an anti-PACE4 antibody (Acris, San Diego, CA, USA) at a final concentration of 1 μ g/ml. Negative controls consisted of cells incubated with control IgG at the same concentration as the primary antibody. Bound antibodies were detected with a secondary donkey anti-rabbit antibody coupled to Texas-Red (Dianova, Hamburg, Germany, 1:1000). Cells were imaged by fluorescence microscopy (Carl Zeiss Inc., Oberkochen, Germany).

Tissue was embedded in OCT (Thermo Fisher, Schwerte, Germany) and frozen in liquid nitrogen. For immunofluorescence staining tissues were cut into 4- μ m sections and fixed with ice cold acetone for 10 min. Subsequently, non-specific binding was blocked with 10% bovine serum albumin, primary antibodies (polyclonal rabbit anti PACE4 (Biorbyt, Cambridge, UK, Cat.-

No. 312626 or rabbit IgG (Jackson ImmunoResearch, Ely, UK, Cat.-No. 011-000-003) were added with 10 μ g/mL and incubated overnight at 4°C. Staining was visualized using a FITC coupled swine anti-rabbit antibody 1:200 (DAKO, Hamburg, Germany) and sections were embedded in NEG-50 (Thermo Scientific).

Immunohistochemistry

Paraffin sections were deparaffinized and treated in EDTA (pH 8.0) in a steamer for epitope unmasking. Sections were incubated for 45 min with a rabbit polyclonal anti-PACE4 antibody (1:900; Alexis, Lörrach, Germany) or rabbit IgG as control. After washing, sections were developed by an indirect immunoperoxidase technique using 3-amino-9-ethylcarbazole (DCS, Hamburg, Germany) as chromogen. Overall, PACE4 immunoreactivity in the melanoma cells was examined independently by 2 investigators (DM and MB) and scored as absent (0), weak (+), strong (++) or very strong (+++).

Western immunoblotting

Total cell lysates or concentrated conditioned media were obtained after melanoma cells were kept for 24 h in culture medium without FCS. Precipitated proteins were washed in acetone and solubilized in loading buffer. Identical amounts of cellular protein (50 μ g/lane) were separated by 8–10% SDS-PAGE, followed by blocking with 2% blocking reagent (Roche, Mannheim Germany) or 10% bovine serum albumin (BSA) for 1 h at room temperature (RT). Membranes were then incubated for 1 h at RT or overnight at 4°C with primary antibodies: anti-rat PACE4 (JH1475, 1:1000) directed against the amino acids 570 to 656 of rat PACE4 (26) anti-human PACE4 (Acris, San Diego, CA, USA; 1:250) or directed against the C-terminal region of human PACE4 and anti-MMP-2 (1:2000; Merck, Darmstadt; Germany). After washing, membranes were incubated with the respective secondary anti-rabbit or anti-mouse IgG-horseradish peroxidase conjugated antibodies (Amersham-Pharmacia, Freiburg, Germany; 1:10.000) for 20 min at RT. Antigen-antibody complexes were visualized by enhanced chemiluminescence (Amersham-Pharmacia) following the manufacturer's instructions.

Zymography

Aliquots of conditioned media from PACE4 transfectants and controls were mixed with equal volumes of sample loading buffer without β -mercaptoethanol and separated by SDS-PAGE 10% gels containing 0.5 mg/ml gelatin (Biomol, Munich, Germany). Gels were washed twice with 2.5% Triton X-100 for 20 min, followed by overnight incubation at 37°C in reaction buffer (50 mM Tris-HCL, pH 7.6, 10 mM CaCl₂ and 200 mM NaCl). Gels were finally stained with Coomassie brilliant blue R250 for 2 h and destained with 10% acetic acid for 2–3 h to visualize bands with gelatinolytic activity.