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# Expression profiling reveals genes associated with transendothelial migration of tumor cells: A functional role for αvβ3 integrin

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**Transendothelial migration is a key step in the extravasation of tumor cells during metastasis formation. Here, we have classified 45 human tumor cell lines derived from various tissues according to their capacity for transendothelial migration *in vitro*. We could distinguish cell lines showing strong transmigration (TEM+ cell lines) from others that did not transmigrate (TEM− cell lines). By DNA microarray analysis we could cluster TEM+ and TEM− cell lines according to their gene expression pattern and identify genes differentially expressed between the 2 groups. Among these we found the integrin β3 subunit to be highly expressed in TEM+ cell lines as compared to TEM− cell lines. Cell surface localization of αvβ3 integrin receptors was exclusively found in TEM+ cell lines. Transendothelial migration of TEM+ cells but not their adhesion to the endothelial cells, or invasion into collagen gels could be blocked with an antibody against αvβ3 integrin and by RNAi mediated knock-down of the integrin β3 subunit. These data establishes αvβ3 integrin as one key component of the transendothelial migration process of tumor cells, and as a potential target for anti-metastatic therapy. Our gene expression analysis of a defined collection of tumor cell lines can be used as a starting point to identify further genes functionally involved in transendothelial migration.**


**Key words:** αvβ3 integrin; metastasis formation; transendothelial migration

Metastasis formation is a complex process, which involves the detachment of invasive cells from the tumor mass, their penetration into the surrounding stroma and blood or lymph vessels and finally their extravasation into the target organs. The metastatic cascade requires dynamic alterations of the interactions among the tumor cells and of tumor cells with other cell types or components of the extracellular matrix. In line with this, several cell surface adhesion receptors such as cadherins and integrins have been identified that act as either negative or positive factors of tumor invasion and metastasis.[1–4] Matrix adhesion mediated by integrins was shown to be essential for tumor cell migration in stromal tissues, and inhibition of this interaction using blocking antibodies or interfering peptides reduces metastasis formation in animal models.[3] Integrins also mediate interaction of tumor cells with platelets and leukocytes, which increases resistance to shear stress in the vasculature.[4]

One key step of metastasis is the extravasation of tumor cells across the endothelium. This process requires the adhesion of tumor cells to endothelial cells and their transmigration across the endothelial border into the stroma of the target organ. Although transendothelial migration is of considerable interest both from a cell and tumor biological perspective its underlying molecular mechanisms are rather ill defined. Molecular determinants have been mainly identified by candidate approaches based on analogies drawn from studies on the transendothelial migration of leukocytes during inflammation.[5] For instance, selectins, which play a crucial role in mediating rolling of leukocytes along an endothelial monolayer, also induce rolling of tumor cells *in vitro* and allow transendothelial migration, thus leading to lung arrest in experimental metastasis models.[6,7] Transgenic expression of E-selectin in the liver could redirect tumor cells to this target organ.[8] Furthermore, tumor cells frequently overexpress the Lewis Sialyl-X epitope recognized by selectins which correlates with poor prognosis.[9,10] However whether tumor cells need to roll prior to adhesion to the endothelium is unclear[11] as *in vivo* the bulk of tumor cells is predicted to get arrested by mechanical trapping within capillaries,[12] although colon carcinoma cells can also arrest in target organs without size restriction.[13]

Integrins are also good candidates for playing a role in tumor-endothelial cell interactions. In breast and colon carcinoma cells, activation of LFA-1 and the VLA-4 (α4β1) integrins by crosslinking of the hyaluronate receptor CD44 mediates adhesion of the tumor cells to the endothelium, thereby facilitating transendothelial migration.[14,15] The β3 integrin receptor was shown to be required for transendothelial migration of WM 239 melanoma and PC-3 prostate carcinoma cells.[16,17]

*In vitro* assays have been frequently used to analyze the passage of tumor cells across the endothelium. In a coculture system of melanoma cells with endothelial cells, rearrangements in the cytoskeleton and cell shape of both cell types were apparent, and tumor cells were shown to penetrate in between endothelial cells, probably by locally disturbing the integrity of endothelial cell junctions.[18,19] The endothelial cell adhesion molecules VE-cadherin and PECAM-1 became redistributed during this process coinciding with loss of cell–cell adhesion at the site of melanoma cell penetration. Similar results were obtained in studies of pancreatic cancer cells which demonstrated that tumor cells induce an increase in endothelial permeability.[20] Several studies showed that after intercalation of tumor cells, endothelial cells spread over the tumor cells and reform the endothelial monolayer.[19,21] Altogether, transendothelial migration of tumor cells is a complex process involving drastic changes in cellular activities of both tumor and endothelial cells.

As most of the previous studies on transendothelial migration examined only individual cell lines from specific tumor types the results are difficult to generalize. In particular, it is unknown whether molecular determinants of transendothelial migration exist that are common to different tumor cell types. In order to approach this problem we analyzed a large collection of tumor cell lines for their ability to transmigrate across a monolayer of human umbilical vein endothelial cells (HUVEC) cultured on collagen gels *in vitro*. We could distinguish cell lines that were able to transmigrate across the endothelium from others that did not. By using gene arrays we could identify genes whose expression correlates with the differential capacities of tumor cells for transendothelial migration. We have analyzed one of the candidates, the β3 integrin for a functional role in tumor cell transendothelial migration in more detail. We found that expression of β3 integrin on tumor cells is positively correlated with the capacity of cells to transmigrate, and that inhibition of αvβ3 integrin receptor function results in reduced transendothelial migration.

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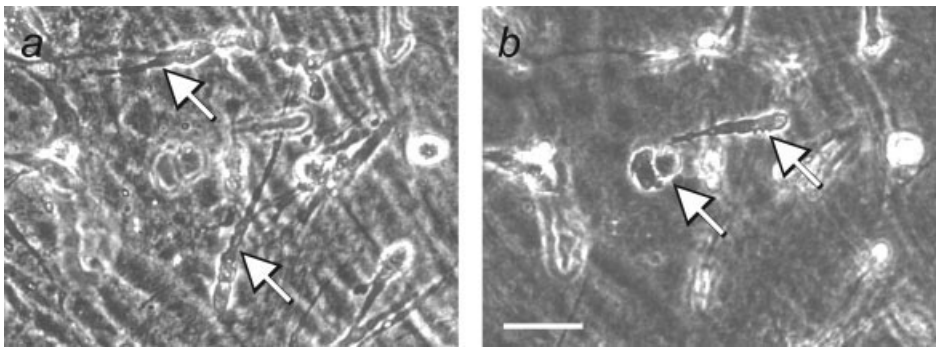



**FIGURE 1** – Transmigration of tumor cells across a HUVEC monolayer. 786.0 kidney cancer cells were seeded on HUVEC monolayers which had been cultured to confluency on collagen type I gels. Microphotographs were taken after 2 days of culture focusing either on top (*a*) or below (*b*) the HUVEC monolayer. Arrows in (*a*) denote tumor cells on top and in (*b*) tumor cells below the HUVEC monolayer which is out of focus in (*b*). Bar, 50 μm.

## Material and methods

### *Material*

The function-blocking mouse monoclonal antibody anti-αvβ3 integrin LM609 (clone MAB1976Z) was purchased from Chemicon Europe (Hofheim, Germany) and goat anti-β3 integrin polyclonal antibody (N-20, pAb) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Carboxyfluorescein diacetate, succinimidyl ester (CFDA/SE) was purchased from Molecular Probes (Invitrogen, Karlsruhe, Germany). Rat tail type I collagen R (4 mg/ml) was from Biochrom (Berlin, Germany) and calf skin type I collagen G (2 mg/ml) from Serva (Heidelberg, Germany).

Cell lines and cell culture. The used tumor cell lines were obtained from the American Type Culture Collection (ATCC, Middlesex, UK), except of DANG, KS, EJ-28, LX-1 and CX-1, which were derived from the tumor bank of the German Cancer Research Center (DKFZ, Heidelberg, Germany), A125 from Peter Altevogt (Heidelberg), A875 from Stuart Aaronson (New York) and CX-2 from the Cell Line Service (CLS, Eppelheim, Germany).

Tumor cell lines were cultured in DMEM supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Human umbilical vein endothelial cells were harvested from human umbilical cords. For this, fresh umbilical vein was washed with PBS, and endothelial cells were released with trypsin 0.25%/EDTA 0.05% for 20 min at 37°C, and maintained in endothelial cell basal medium 2 (Promocell, Heidelberg, Germany). All cell cultures were kept at 37°C in a humidified atmosphere of 10% $CO_2$.

### *RNA isolation and reverse transcription*

Total cellular RNA was isolated using peqGold Trifast® reagent (Peqlab, Erlangen, Germany) following the manufacturer's protocol. cDNAs were generated from 1 μg RNA by reverse transcriptase reaction with Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany) using random hexamer primer, according to the recommendations of the manufacturer. In addition 40 units RNAse inhibitor were added (RNaseOUT™, Invitrogen, Karlsruhe, Germany).

### *RT-PCR analysis*

Reverse transcription-PCR (RT-PCR) was performed using specific sense and antisense primers (Supplementary Table I) in a 10 μl reaction volume containing 1 μl 10× Taq-buffer, 1 μl of cDNA, 0.4 μl of each primer (10 mmol/l), 0.3 μl of deoxynucleotide triphosphates (10 mmol/l), 0.3 μl $MgCl_2$ (50 mM), 0.5 units of Taq polymerase (Invitrogen, Karlsruhe, Germany), and 6.5 μl of water. For amplification the number of cycles according to Supplementary Table I were applied for 30 sec at 96°C, 1 min at the given annealing temperature, and 30 sec at 72°C, with an initial step of 96°C for 2 min and a final step of 72°C for 10 min.

### *Western blot analysis*

Subconfluent cells were lysed in 100 μl lysis buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mM DTT) for 10 min on ice. Equal amounts of protein as determined by Bradford assay were separated by 6% nonreducing SDS-PAGE. After electrotransfer of proteins to nitrocellulose membranes and blocking, blots were incubated with the goat anti-β3 integrin polyclonal antibody (final dilution, 1:500) overnight at 4°C. Subsequent incubation with the peroxidase-conjugated donkey anti-goat IgG for 1 hr at room temperature was followed by detection using enhanced chemiluminescence Western blot detection reagents (Amersham Biosciences).

### *Flow cytometry*

Subconfluent tumor cells were washed with PBS, harvested with Accutase (PAA, Cölbe, Germany), and resuspended in buffer (20 mM Hepes, 125 mM NaCl, 45 mM glucose, 5 mM KCl, 0.1% albumin, pH 7.4). Cells were incubated with mAb LM609 at a dilution of 1:50 for 30 min at 4°C, washed and incubated with phycoerythrin-labeled goat anti-mouse IgG at a dilution of 1:50 for 30 min at 4°C. The cells were then analyzed by fluorescence-activated cell sorting. Cell fluorescence was measured with a FACScan flow cytometer (BD Biosciences, Heidelberg, Germany). Population gates were set using cells without primary antibody.

### *Cell adhesion assay*

HUVEC were seeded at $2 \times 10^5$ cells/well in 12-well plates and cultured overnight at 37°C in 10% $CO_2$, after which cells were washed with a buffer containing 20 mM Hepes, 125 mM NaCl, 45 mM glucose, 5 mM KCl, 0.1% albumin, pH 7.4. Tumor cells were stained with CFDA/SE (15 nM) for 15 min at room temperature, washed with PBS, and added in Endothelial Cell Basal Medium 2 at $5 \times 10^4$ cells/well to the HUVEC monolayers. After 3 hr, nonadhering tumor cells were removed by 2 washes with the same buffer. The number of adherent cells was determined by counting cells in 10 optical fields in a fluorescence microscope.

### *Invasion and transendothelial migration assay*

Collagen G and collagen R were mixed at a ratio of 1:1, 0.1 vol of sodium bicarbonate (22 mg/ml) and 0.1 vol of 10× DMEM was added, and the solution was neutralized with sodium hydroxide. Aliquots of 1.2 ml/well were allowed to gel in 6-well culture dishes at 37°C.

For transendothelial migration, $5 \times 10^5$ HUVEC in endothelial cell basal medium 2 were plated per well onto collagen surfaces and allowed to form confluent monolayers overnight at 37°C in 10% $CO_2$. Then $1 \times 10^5$ tumor cells resuspended in endothelial cell basal medium 2 were added per well. For invasion assays, tumor cells were directly seeded onto the collagen surfaces. For in-

**TABLE I** – TRANSENDOTHELIAL MIGRATION AND αvβ3 EXPRESSION OF HUMAN TUMOR CELL LINES

| Cell line | Tissue | TEM[1] | αvβ3[2] |
|---|---|---|---|
| TEM+ | | | |
| MDA-MB-231 | Breast | 8224 ± 1853 | +++ |
| 786.0 | Kidney | 6799 ± 2006 | ++ |
| EJ-28 | Bladder | 4977 ± 1298 | + |
| PC-3 | Prostate | 3476 ± 1522 | + |
| A375 | Melanoma | 2417 ± 1675 | +++ |
| A125 | Lung | 2262 ± 1099 | +++ |
| DU145 | Prostate | 1775 ± 625 | + |
| A172 | Glioblastoma | 1603 ± 820 | − |
| HS578T | Breast | 1270 ± 512 | ++ |
| MDA-MB-436 | Breast | 749 ± 629 | − |
| A875 | Melanoma | 419 ± 261 | + |
| CAKI-I | Kidney | 291 ± 45 | ++ |
| FADU | Hypo pharynx | 223 ± 111 | − |
| A427 | Lung | 121 ± 3 | −/+ |
| BT549 | Breast | 117 ± 21 | + |
| TEM− | | | |
| LoVo | Colon | 83 ± 33 | − |
| A431 | Vulva | 78 ± 94 | − |
| RT112 | Bladder | 74 ± 81 | − |
| A549 | Lung | 67 ± 38 | − |
| T47D | Breast | 51 | − |
| Me180 | Cervix | 51 | − |
| CX-1 | Colon | 51 | − |
| MDA-MB-468 | Breast | 47 ± 66 | − |
| HT-29 | Colon | 44 ± 76 | − |
| Skbr3 | Breast | 40 ± 57 | − |
| MiaPaca2 | Pancreas | 39 ± 54 | − |
| MCF-7 | Breast | 32 ± 41 | − |
| SW620 | Colon | 25 ± 24 | − |
| MDA-MB-453 | Breast | 17 ± 12 | − |
| SW948 | Colon | 25 | − |
| MDA-MB-361 | Breast | 21 ± 6 | − |
| MS751 | Cervix | 22 ± 14 | − |
| LX-1 | Lung | 17 | −/+ |
| HCT116 | Colon | 54 ± 48 | − |
| SW480 | Colon | 13 ± 13 | − |
| C33A | Cervix | 8 | − |
| CaCo-2 | Colon | 8 | − |
| CX-2 | Colon | 8 | − |
| DANG | Pancreas | 4 | − |
| KS | Breast | 7 ± 7 | − |
| DLD-1 | Colon | 0 ± 0 | − |
| Capan I | Pancreas | 0 | − |
| SW48 | Colon | 0 | − |
| Colo201 | Colon | 0 | − |
| Colo205 | Colon | 0 ± 0 | − |

[1]TEM (transendothelial migration) across a HUVEC monolayer. Number of cells per $cm^2$ entering the collagen gel after 48 h. Results represent the mean value ± s.d.–[2]Cell surface expression of αvβ3 integrin as determined by flow cytometry. +++, >20 fold; ++, 5–20 fold; +, 2–4 fold more than isotype control; −/+, heterogeneous population; −, equal to isotype control.

hibition experiments, antibodies were added to the tumor cells directly before the addition to the HUVEC, or HUVEC were preincubated with antibodies for 30 min, washed and then incubated with tumor cells. Cocultures were carried out for 48 hr at 37°C before fixation with 3% paraformaldehyde. Cells invading the collagen gel were detected by focusing down into the matrix, and quantified by counting 30 optical fields per well.

*Transfection experiments*

For generation of 786.0 cells stably expressing pSUPER shRNAi targeting β3 integrin, $7.5 \times 10^5$ cells were seeded in 10 cm cell culture dishes and transfected with 10 μg of the pSUPER shβ3 integrin plasmid using Lipofectin (Invitrogen) according to manufacturer's specifications. One day after transfection, cells were placed into selection medium containing 1 mg/ml G418. After 14 days of selection, individual G418-resistant colonies were subcloned and tested for Integrin β3 expression by Western blot analysis.

*Cloning of shRNAs for β3 integrin into pSUPER*

The oligonucleotides for the shRNA insert for β3 integrin were: 5′-GATCCCCCTCTGCCTCCACTACCATGTTCAAGAGACATGTAGTGGAGGCAGAGTTTTTGGAAA-3′ (shRNA sequence coding strand) and 5′-AGCTTTTCCAAAAACTCTGCCTCCACTACCATGTCTCTTGAACATGGTAGTGGAGGCAGAGGGG-3′. They contain a unique 19-nt human Integrin β3 target sequence (β3 integrin open reading frame 921–939) present as an inverted repeat, separated by a 9-nt spacer. For annealing, equimolar amounts of the two oligonucleotides were incubated at 95°C for 5 min in 50 mM Tris/HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and 25 μg/ml BSA, at pH 7.5, and were allowed to cool gradually to room temperature. The inserts thus produced were directionally cloned into the BglII/HindIII digested pSuper vector downstream of a human RNA polymerase-III H1-gene promoter. The shRNA clones were confirmed by DNA sequencing and tested in transient transfections.

*Affymetrix GeneChips arrays*

Reverse transcriptions, second strand synthesis and probe generation were all accomplished by the standard Affymetrix protocol (Affymetrix, Santa Clara, CA). Human genome HG-U133 GeneChips (Affymetrix, Santa Clara, CA) were hybridized, washed and scanned with the G2500A GeneArray scanner (Affymetrix, Santa Clara, CA) in cooperation with Dr. Möröy and Dr. Klein-Hitpass, Institute of Cell Biology (Tumor Research), University of Essen Medical School.

*Data analysis*

Microarray data were analyzed using the MicroArray Suite 5.0 (MAS 5, Affymetrix, Santa Clara, CA), Data Mining Tool 3.0 (DMT 3, Affymetrix, Santa Clara, CA) and Spotfire Decision Site for Functional Genomics 7.0 (Spotfire, Somerville, MA). To compensate for variations in experimental variables of nonbiological origin, the mean signal intensity for each array was calculated and scaled globally (across all probe sets) to a mean target intensity of 1,000. For further analysis the signals of only those genes were used which were classified "present" at least on one gene array. The median signal was calculated for the resulting 15,255 genes and the log values calculated for each signal. These log values were used to perform a two-dimensional hierarchical cluster analysis (Unweighted Pairgroup Method with Arithmetic Mean (UPGMA), correlation) based on the similarity of the gene expression profiles of the different samples (red colour, upregulated compared to median; black, not regulated and green, downregulated compared to median). To identify genes differentially expressed between TEM+ and TEM− tumor cell lines a significance filter of $p < 0.014$ (Mann-Whitney test) was applied in a second supervised cluster analysis.

## Results

*Classification of tumor cell lines according to their transendothelial migration capacity*

In order to study transendothelial cell migration of tumor cells *in vitro*, human umbilical vein endothelial cells (HUVEC) were cultured on top of three-dimensional collagen gels until they reached confluency, tumor cells were added, and cells that had penetrated through the endothelial monolayer and invaded into the underlying collagen gel after 48 hr were counted in the light microscope (Fig. 1). After attachment to the endothelial cells tumor cells readily spread on top of the endothelium, and started to intercalate with the endothelial cells within a few hours. There was no indication of increased cell death or major rearrangements of the endothelial monolayer. Within the collagen gel most transmigrating tumor cell lines acquired a spindle-like shape (Fig. 1). Electron microscopy of

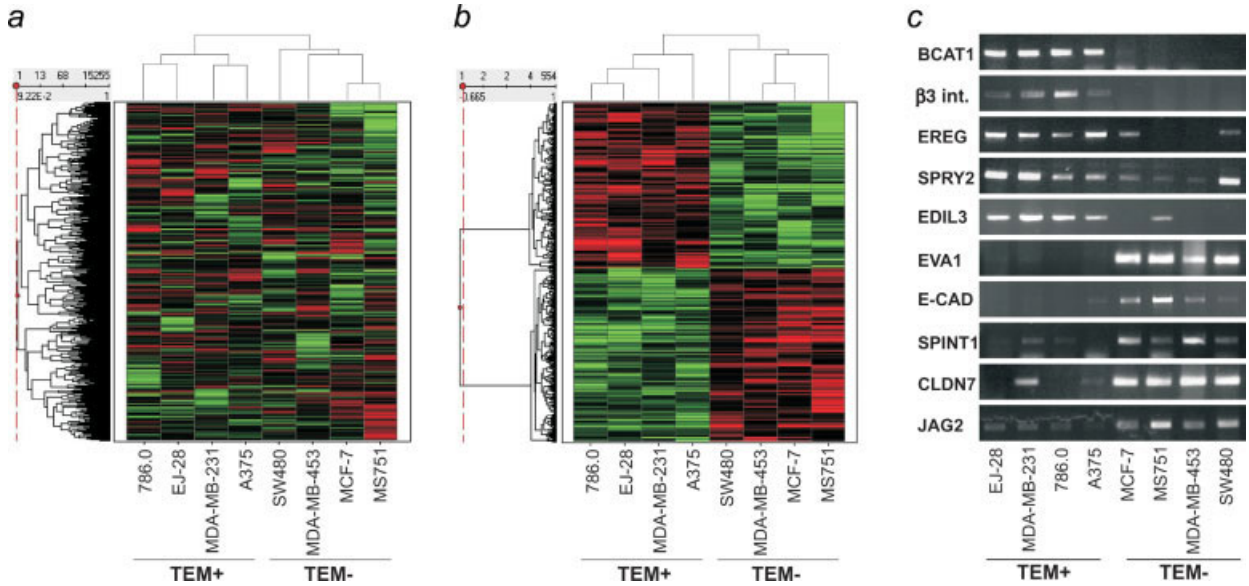



**FIGURE 2** – Cluster analysis of DNA microarray gene expression data of different tumor cell lines from the TEM+ and TEM− group. (*a*) Unsupervised clustering based on the expression of 15,255 informative genes. Red color, upregulated compared to median; black, not regulated; green, down-regulated compared to median. (*b*) Supervised clustering for identification of genes differentially expressed between TEM+ and TEM− cell lines. Color code as in (*a*). (*c*) Expression analysis of selected genes by RT-PCR. BCAT1, branched chain amino transferase 1, β3 int., β3 integrin, EREG, Epiregulin, SPRY, sprouty homolog 2, EDIL3, EGF-like repeats and discoidin I-like domains, EVA1, epithelial V-like antigen 1, E-CAD, E-cadherin, SPINT, Serine protease inhibitor, Kunitz type 1, CLDN7, claudin-7, and JAG2, jagged 2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ultra thin sections indicated that the tumor cells entered the collagen by squeezing in between endothelial cells rather than by a transcellular route or by destroying the monolayer. Moreover, the tumor cells appeared to remain in contact with endothelial cell processes in the course of transmigration (data not shown).

Using this assay we evaluated the transmigration capacity of 45 human tumor cell lines originating from different tissues. We could classify the tumor cell lines into 2 groups, termed TEM+ (transendothelial migration positive) and TEM− (transendothelial migration negative). By definition, cell lines were considered TEM+ when at least 100 cells per $cm^2$ were detected inside the collagen gel after 48 hr (Table I). This cut-off represents the maximal background of invasive endothelial cells or contaminating fibroblasts that was occasionally observed in HUVEC preparations. There was no correlation between the tissue of origin and the classification of cell lines into 1 of the 2 groups, except that colon carcinoma cell lines were absent from the TEM+ group. We have also analyzed 24 tumor cell lines from both groups for transmigration on human microvascular endothelial cells (HMVEC) from the lung (Supplementary Table II). We found that all TEM+ cell lines from the HUVEC analysis were also TEM+ in the HMVEC assay. Except of 2 cell lines (MDA-MB-453, LoVo), which showed transmigration just above the background, all other TEM− cell lines from the HUVEC analysis remained TEM− in the HMVEC analysis.

Furthermore we analyzed our cell lines for invasion into collagen gels and found that most of the TEM+ cells were invasive whereas the TEM− cells were not (Supplementary Table III). Thus our grouping of cell lines reflects general differences of motility between the 2 groups. Using video microscopy and long working distance objectives, we made sure that TEM+ cell but never TEM− cells were located underneath the endothelium indicating that the lack of transendothelial migration by TEM− is not simply due to their inability to invade.

*Identification of candidate genes involved in tumor cell TEM*

To identify genes possibly involved in the process of transmigration we performed expression profiling of subsets of cell lines of the 2 groups, *i.e.* of MBA-MB-231, 786.0, EJ-28, and A375 cells from the TEM+ group which showed the strongest transendothelial migration capacity, and MCF-7, MDA-MB-453, MS751, and SW480 cells from the TEM− group by DNA microarray analysis. Interestingly, unsupervised clustering of cell lines according to the expression pattern of 15,255 informative genes could correctly distinguish the TEM+ from TEM− cell lines (Fig. 2*a*), indicating that cells within each group exhibit a similar transcriptional program. To identify genes specifically associated with the transmigration phenotype, a supervised clustering analysis was performed. This revealed 336 genes as being differentially expressed by an average factor $\geq 3$ between TEM+ and TEM− tumor cell lines (Fig. 2*b*), including genes involved in signal transduction, extracellular matrix biology, and cell–cell adhesion (see Table II for a list of selected genes). It is of note that genes for matrix degrading enzymes were more highly expressed in TEM+ cell lines whereas genes for several cell–cell adhesion molecules were more prevalent in the TEM− group. The expression patterns for selected genes could be verified by RT-PCR analyses (Fig. 2*c*).

*Role of integrin β3 in tumor cell TEM*

For further analysis we focused on β3 integrin as this protein has been implicated before in metastasis formation in various experimental settings.[22] We analyzed the expression of β3 integrin on a larger panel of 41 of our functionally characterized tumor cell lines by RT-PCR (Fig. 3). It correlated well with the transmigration capacity of the cell lines, *i.e.* β3 integrin was highly expressed in the majority of TEM+ cells but showed low or undetectable expression in TEM− cells. By Western blotting the β3 integrin protein was detected in most of the TEM+ cell lines but was absent from TEM− cell lines despite of some mRNA expression of β3 integrin being detectable in these cells (Fig. 4*a*). A common heterodimerization partner of β3 integrin, the αv integrin subunit was ubiquitously expressed in all tumor cell lines as determined by RT-PCR (Fig. 3). By flow cytometry, cell surface expression of the αvβ3 integrin receptor was detectable in nearly all of the TEM+, but in none of the TEM− cell lines (Fig. 4*b*,

**TABLE II –** GENES DIFFERENTIALLY EXPRESSED BETWEEN TEM+ AND TEM− CELL LINES

| Regulation[1] | Genes | Fold difference[2] |
|---|---|---|
| Signal transduction | | |
| Up | IL-8 | 36 |
| | Epiregulin | 24 |
| | Insulin-like growth factor binding protein 1 | 21 |
| | Toll-like receptor 2 | 9 |
| | Serine/threonine phophatase 2A | 9 |
| | Transforming growth factor β receptor II | 7 |
| | IL-6 | 7 |
| | Oncostatin M receptor | 6 |
| | Sprouty homolog 2 | 6 |
| | IRS2 | 6 |
| | Homeodomain-interacting protein kinase 2 | 5 |
| | Jagged | 4 |
| Down | Interferon regulatory factor 6 | 28 |
| | ErbB3 | 19 |
| | EphA1 | 10 |
| | c-myb | 6 |
| | Jagged2 | 5 |
| | DDR1 | 4 |
| | SOX-13 | 3 |
| Extracellular matrix/proteases | | |
| Up | Integrin β3 | 33 |
| | Lysyl oxidase | 29 |
| | Plasminogen activator, urokinase | 12 |
| | EDIL3 | 8 |
| | Integrin α5 | 4 |
| Down | Serine protease inhibitor, Kunitz type 1 | 37 |
| | Matriptase | 12 |
| | Ladinin | 10 |
| Cell–cell contact | | |
| Up | Nectin-3 | 16 |
| Down | Epithelial V-like antigen | 138 |
| | E-cadherin | 96 |
| | P-cadherin | 76 |
| | Claudin-7 | 17 |
| | Discs large homolog 3 | 8 |
| | Epiplakin 1 | 6 |
| | Plakophilin 3 | 3 |
| | Flamingo homolog | 3 |
| Miscellaneous | | |
| Up | Aldehyde dehydrogenase 3 | 194 |
| | Aldo-keto reductase family 1 member B10 | 97 |
| | Branched chain aminotransferase 1 | 56 |
| Down | Microtubule-associated protein 7 | 33 |
| | Tumor-associated calcium signal transducer 1 | 18 |
| | Argininosuccinate synthetase | 7 |
| | Potassium channel subfamily K member 1 | 4 |

[1]Up, down: Genes upregulated or downregulated in TEM+ compared with TEM− cell lines.–[2]Ratio between the mean signal intensities of TEM+ and TEM− cell lines.

representative examples are shown, see also Table I). Thus, these data show a close correlation between the expression of β3 integrin, as well as cell surface localization of the αvβ3 integrin receptor, and the capacity of tumor cells for transendothelial migration.

We next examined whether TEM+ cells require αvβ3 integrins for transendothelial migration. For this we quantified transmigration of MDA-MB-231 cells from the TEM+ group in the presence or absence of a blocking antibody directed against αvβ3 integrin. Addition of this antibody but not of a control IgG reduced transmigration of MDA-MB-231 cells by 40% at 5 μg/ml and 55% at 20 μg/ml (Fig. 5*a*, data not shown). When tested with other TEM+ cell lines this inhibition was also apparent, with a reduction of transmigration of 50% for CAKI-I, 40% for EJ-28, 30% for A375 and 20% for 786.0 cells (data not shown). To dissect at what stage of the transmigration process αvβ3 integrin might function, we also examined the effects of the blocking antibody on the invasion of MDA-MB-231 cells into the collagen gel in the absence of a HUVEC monolayer, and on the adhesion of these cells to the endothelial cells after 3 hr of incubation (see Material and Methods). Neither collagen invasion nor adhesion to the endothelium was blocked by the antibody (Figs. 5*b* and 5*c*), indicating that αvβ3 function is mainly required for the proper passage of tumor cells across the endothelium rather than for the invasion of tumor cells into the collagen gel or the initial tumor-endothelial cell interaction.

Since αvβ3 integrin is reported to be expressed also on the surface of HUVEC we preincubated either tumor cells or endothelial cells with the blocking antibody. Preincubation of MDA-MB-231 or 786.0 cells reduced TEM to a similar degree as antibody treatment without preincubation, whereas preincubation of endothelial cells had no effect (data not shown), indicating that it is the αvβ3 integrin on tumor cells that is crucial for transendothelial migration.

As an additional approach to interfere with αvβ3 integrin function we used the RNAi technique to downregulate β3 expression in tumor cells. 786.0 cells from the TEM+ group were stably transfected with a pSUPER derived expression vector for siRNA targeting human β3 integrin, or with the empty pSUPER vector, or pSUPER containing a scrambled control sequence. We obtained a consistent and significant downregulation of β3 integrin mRNA in the RNAi β3 clones as compared with the pSUPER controls (Fig. 6*a*). By Western blotting we observed an average reduction of 75% of β3 integrin protein in the RNAi β3 transfectants although expression levels varied to some extend between individual clones (Fig. 6*b*). When we examined transendothelial migration of these clones, we observed a significant decrease of transmigration by about 60% of the RNAi β3 clones as compared to pSUPER or scrambled controls (Fig. 6*c*, data not shown). In line with the results from the antibody blocking experiments, invasion of 786.0 cells into collagen or adhesion to endothelium was not affected by RNAi knock down of β3 integrin (Figs. 6*d* and

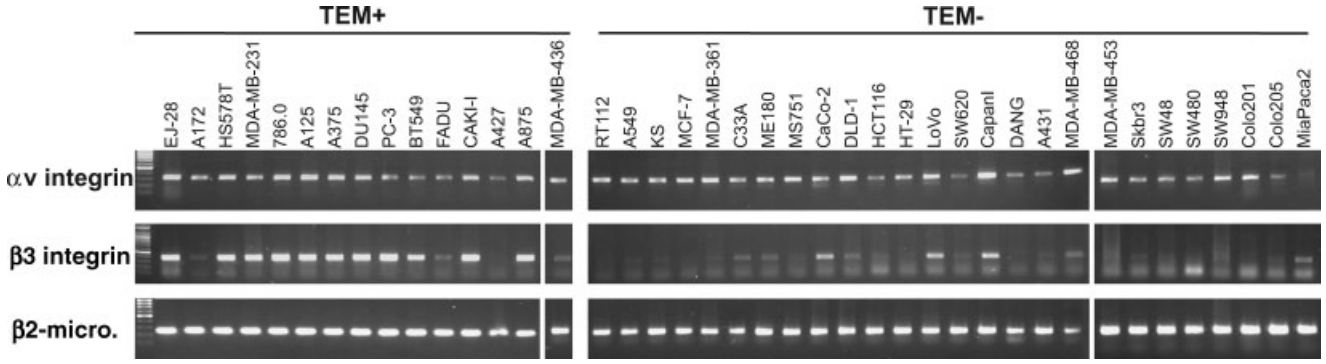



**FIGURE 3** – Expression analysis of αv and β3 integrins on the large panel of tumor cell lines by RT-PCR. Grouping of cell lines corresponds to Table I. β2-Micro, β2-microglobulin control.

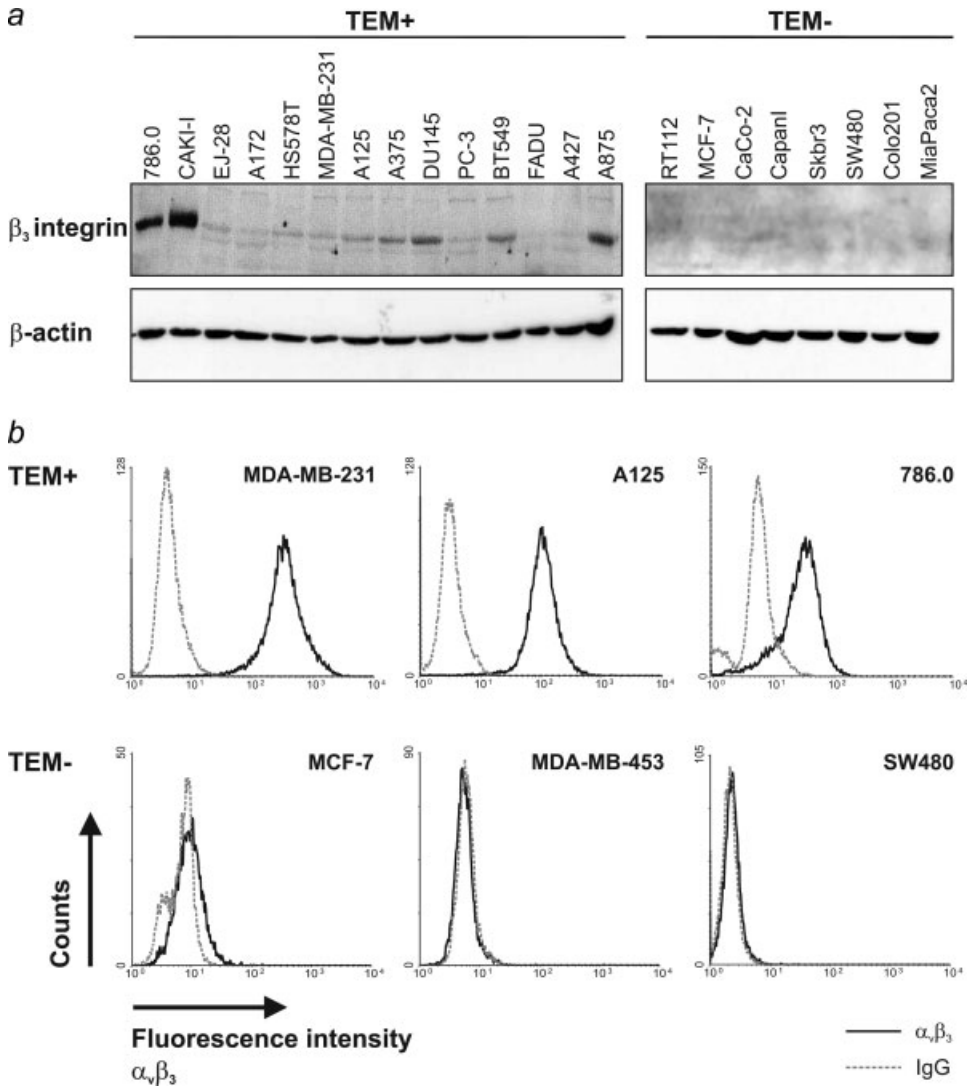



**FIGURE 4** – β3 integrin protein expression in different tumor cell lines. (*a*) Western blot analysis of β3 integrin. (*b*) Cell surface localization of αvβ3 integrin in TEM+ and TEM− tumor cell lines determined by FACS. Each panel shows a representative flow-cytometric histogram using anti αvβ3 integrin antibodies or isotype matched control IgG.

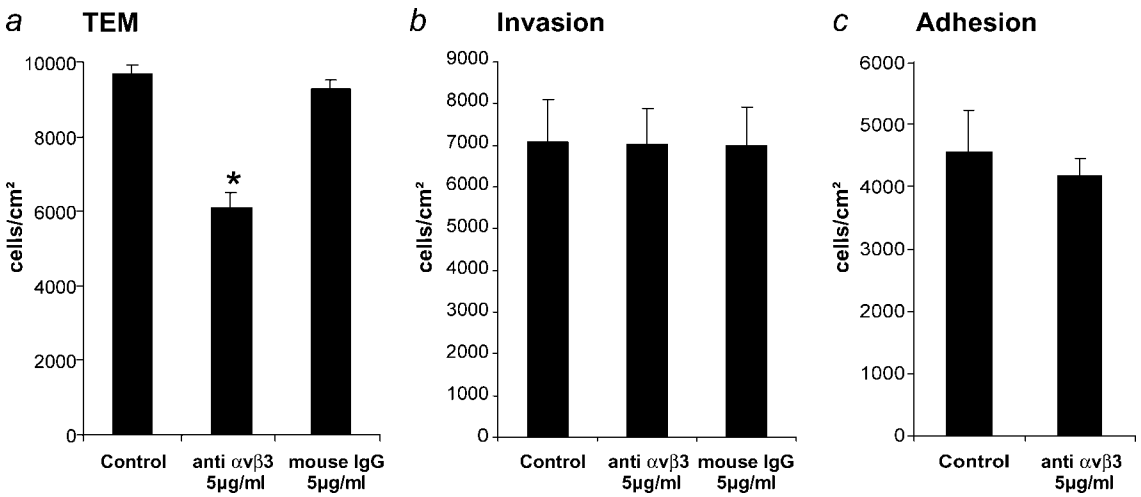
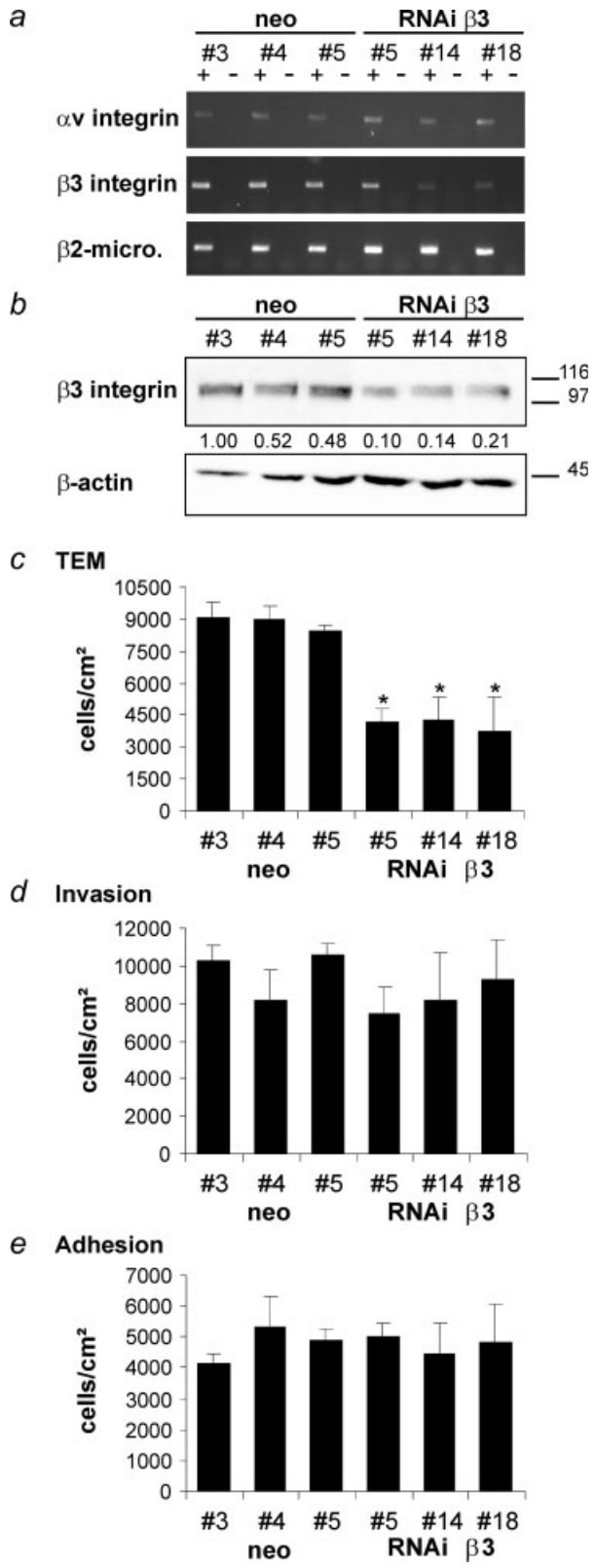



**FIGURE 5** – Antibody blocking experiments of αvβ3 integrin. (*a*) Effects of the anti-αvβ3 integrin blocking antibody on transendothelial migration (TEM), (*b*) invasion into collagen gels in the absence of HUVEC, and (*c*) adhesion to HUVEC of MDA-MB-231 cells. Transmigration and invasion were determined after 48 hr, whereas adhesion to HUVEC was measured after 3 hr. Lines represent the mean ± s.d. from 2 and 3 different experiments, respectively. *Significantly different from control ($p < 0.05$).

6*e*). Taken together the functional experiments indicate that αvβ3 integrin is required for efficient transendothelial migration of tumor cells.




## Discussion

Most of the previous studies analyzing transendothelial migration of tumor cells *in vitro* made use of individual cell lines derived from a particular tumor type, making it difficult to draw general conclusions on the cellular and molecular basis of this process. To our knowledge our study represents the first comprehensive analysis of transendothelial migration of a large panel of human tumor cell lines from various tissues. We observed drastic differences in the capacity of tumor cells to transmigrate *in vitro*, leading to a classification of cell lines into 2 groups, *i.e.* TEM+ and TEM−. The differences might reflect different stages of progression of the original tumors from which the cell lines originated. There was no clear correlation between the tissue of origin and transmigration capacity except that colon tumor cells did not transmigrate. Whether this is accidental or reflects a special mode of extravasation of this tumor type is not clear.

We have used HUVEC for our analysis which represent embryonic macrovascular cells. In metastasis formation it is assumed that tumor cells mainly interact with microvascular endothelial cells. Hence we also tested some of the cell lines for transmigration across human microvascular endothelial cells and found essentially the same behavior of TEM+ and TEM− cells. This ascertains that our model is valid and reflects the basic aspects of tumor endothelial interactions.

Our classification of tumor cell lines was used as a starting point to define molecular parameters that underlie transendothelial migration. DNA microarray analysis of a subset of tumor cell lines revealed a large number of genes that were differentially expressed between the transmigrating and non-transmigrating cells (cf. Table II for examples). It is possible that many of these differences are accidental and not causally linked to the transmigration process. However, transendothelial migration is a complex process that probably requires the coordinate activity of a variety of different gene functions. Indeed, several genes that we found have functional properties with a possible role for the execution, or prevention of transendothelial migration. For instance, transmigrating tumor cells showed elevated expression of cytokines such as interleukin-6 and -8 which play a role in stimulating neutrophil extravasation.[23,24] Other examples include the plasminogen activator protease which could aid in the digestion of extracellular matrix, and EDIL-3 (Del1) which is implicated in promoting angiogenesis and might have an impact on the structural integrity of the endothelial cell monolayer.[25] On the other hand, cell junction components such as cadherins, claudin-7, and plakophilin 3 which are characteristic for a differentiated, noninvasive epithelial phenotype, were more prominently expressed in the non-transmigrating cells, indicating that loss of epithelial differentiation is a prerequisite for transendothelial migration as it is for tumor cell invasion.[26]

For a detailed functional characterization we chose β3 integrin, which is implicated in a variety of cellular processes related to tumorigenesis such as adhesion to extracellular matrix, cell migration, cell signaling, and angiogenesis.[3,22] It was also noted previ-

**FIGURE 6** – Effects of RNAi mediated knock down of β3 integrin in 786.0 renal cell carcinoma cells. (*a*) RT-PCR analysis of expression of αv and β3 integrins in stable 786.0 pSUPER neo control clones (neo) or 786.0 clones expressing RNAi against β3 integrin (RNAi β3). Note the specific downregulation of β3 integrin as compared with αv integrin, which remained constant. +,−, with and without reverse transcriptase. β2-Micro, β2 microglobulin control. (*b*) Western blot analysis of β3 integrin of cell clones from (*a*). Numbers between panels indicate relative expression ratios of β3 integrin normalized to β-actin. (*c*) Transendothelial migration (TEM), (*d*) invasion into collagen I gels, and (*e*) adhesion to HUVEC of cell clones shown in (*a*, *b*). Transmigration and invasion were determined after 48 hr, whereas adhesion to HUVEC was measured after 3 hr. Lines represent the mean ± s.d. from 3 different experiments. *Significantly different from control ($p < 0.05$).

ously that β3 integrin expression correlates with aggressiveness of tumors.[22] A polymorphism at the extracellular domain of β3 integrin which alters its conformation and may increase its affinity towards ligands was associated with increased risk for cancer development.[27] For WM239 melanoma cells and PC-3 prostate cancer cells a functional role for β3 integrin in transendothelial migration was inferred from antibody blocking experiments.[16,17] Our functional studies of several tumor cell lines indicate a more general and widespread role of αvβ3 integrin in transendothelial migration, which is supported by the almost perfect correlation between β3 integrin mRNA expression, cell surface localization of αvβ3 integrin receptor, and transmigration of various tumor cell lines.

We found that transendothelial migration of various tumor cell lines could be reduced by a monoclonal antibody against αvβ3 integrin or by knock down of β3 integrin. The fact that transmigration was not completely nullified might be due to the incomplete inhibition of β3 function in both settings but might also indicate that these cells have various means for passing the endothelial monolayer. Since the knock down of β3 integrin in the tumor cells led to a similar inhibition of transendothelial migration as antibody treatment, and preincubation of HUVEC with the antibody had no effect on transendothelial migration (data not shown) we conclude that αvβ3 expressed on the tumor cells but not on HUVEC is of relevance. We also over-expressed β3 integrin in TEM− tumor cells that had low endogenous β3 integrin levels in order to determine whether this could promote transmigration. However, we did not observe a significant increase in transmigration as compared with controls (data not shown) suggesting that β3 integrin while being necessary for transmigration in our system does not suffice to convert a non-transmigrating cell to a strongly transmigrating one. This is not surprising if one takes into account that other characteristics, such as changes in the cytoskeleton, protease production *etc*. might be required to establish the full-blown TEM+ phenotype.

We showed that the αvβ3 integrin receptor is required for transendothelial migration but not for invasion of tumor cells into collagen matrices or adhesion to the endothelial cells, indicating that αvβ3 integrin exerts its function at a step after initial contact formation between tumor cells and endothelium and before matrix invasion. Whether this involves interaction of αvβ3 with a counter-receptor on endothelial cells or with extracellular matrix produced by the endothelial cells is currently not known. In a transendothelial migration analysis of WM239 melanoma cells (which were not included in our study) αvβ3 integrin was shown to interact with the L1 immunoglobulin superfamily protein on endothelial cells, and transmigration was partially blocked by both anti-αvβ3 and anti-L1 antibodies.[16] We could not detect L1 expression in HUVEC while it was readily detected in other cells, *e.g.* 293T cells, excluding this possibility in our system (data not shown). Another possible counter-receptor for αvβ3 integrin is the platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), which is a junction-associated protein of endothelial cells. Functional studies have demonstrated a requirement of PECAM-1 and of αvβ3 integrin for transendothelial migration of monocytes, and an enrichment of PECAM-1 at the region where transmigration takes place,[28] making PECAM-1 and αvβ3 integrin an attractive candidate receptor pair also for transendothelial migration of tumor cells.

Integrins have recently gained interest as potential drug targets for pharmacological interference with various diseases, and low molecular weight antagonists of αvβ3 integrins have been identified.[29] It will be of interest to determine the consequences of application of these inhibitors on the transendothelial migration of tumor cells. Our findings provide a further rationale for the possible application of such antagonists in the prevention of metastatic disease.

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