

## Tumorigenesis and Neoplastic Progression

# Focal Adhesion Kinase Regulates Metastatic Adhesion of Carcinoma Cells within Liver Sinusoids

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**Organ-specific tumor cell adhesion to extracellular matrix (ECM) components and cell migration into host organs often involve integrin-mediated cellular processes that can be modified by environmental conditions acting on metastasizing tumor cells, such as shear forces within the blood circulation. Since the focal adhesion kinase (FAK) appears to be essential for the regulation of the integrin-mediated adhesive and migratory properties of tumor cells, its role in early steps of the metastatic cascade was investigated using *in vitro* and *in vivo* approaches. Human colon and hepatocellular carcinoma cells were used to study adhesive properties under static conditions and in a parallel plate laminar flow chamber *in vitro*. In addition, intravital fluorescence microscopy was used to investigate early interactions between circulating tumor cells and the microvasculature of potential target organs *in vivo*. Shear forces caused by hydrodynamic fluid flow induced Tyr-hyperphosphorylation of FAK in cell monolayers. Reduced expression of FAK or its endogenous inhibition by FAK-related non-kinase (FRNK) interfered with early adhesion events to extracellular matrix components under flow conditions. In contrast, tumor cell adhesion to endothelial cells under these conditions was not affected. Furthermore, down-regulation of FAK inhibited metastatic cell adhesion *in vivo* within the liver sinusoids. In summary, FAK appears to be involved in early events of integrin-mediated adhesion of circulating carcinoma cells under fluid flow *in vitro* and *in vivo*. This kinase may take part in the establishment of definitive adhesive interactions that enable adherent tumor cells to resist fluid shear forces, resulting in an organ-specific formation of distant metastases. (Am J Pathol 2005, 166:585–596)**

Most carcinomas often show organ preference of metastasis formation.<sup>1,2</sup> In particular, the liver and lung are sites often colonized by metastatic carcinoma cells. Organ-specific sites of metastatic lesions are determined, in part, by adhesive interactions of malignant cells with organ microvessel endothelial cells (EC) and underlying extracellular matrix (ECM).<sup>1,2</sup> Important in these interactions is the role played by integrin-mediated adhesion of malignant cells to ECM components.<sup>3</sup> For example, different adhesive and migration properties of poorly and highly metastatic human colon carcinoma cells to microvascular EC obtained from various organs have been described.<sup>4</sup> Recently, we found that subclones of HT-29 colon carcinoma cells with different metastatic properties possessed different patterns of integrin-mediated adhesion to various ECM components in static and dynamic adhesion assays.<sup>5,6</sup> However, these differences were not caused by differences in the expression of integrin subunits, because both cell lines expressed similar patterns of integrins.<sup>5</sup>

Under specific conditions, such as the presence of fluid flow within the microcirculation of host organs, distinct intracellular events during the adhesion of carcinoma cells to ECM components may be required for the adhesive process and its stabilization. For example, blockade of different integrins with antibodies can specifically inhibit metastatic tumor cell adhesion within the liver sinusoids *in vivo* suggesting direct interactions with ECM components, such as collagens, in the space of "Disse". These experiments indicated that metastasizing colon or hepatocellular carcinoma cells can arrest in target organs without size restriction. Cell adhesion of circulating tumor cells occurred in metastatic target organs only, where specific interactions appear to be required for successful arrest. Furthermore, invasion of colon carcinoma cells into target organs correlated with their metastatic potential. (Schlüter et al, submitted)

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Moreover, the integrity of microtubules and actin filaments can modulate this tumor cell adhesion within the hepatic microcirculation.<sup>7</sup> Since integrins appear to be directly involved in early steps of metastasis formation, cell signaling and regulatory processes that modulate their affinity and/or avidity may also influence metastatic tumor cell adhesion or migration into host organs.

Signal transduction is apparently required both for the adhesion event itself and for subsequent steps involved in host organ colonization.<sup>8</sup> These cell-ECM interactions that lead to signal transduction are mediated mainly through integrins, which are intracellularly linked to cytoskeleton components<sup>9</sup> and other protein molecules.<sup>10,11</sup> The functional status of integrins is regulated by complex interactions with a number of these cytosolic, cytoskeletal, and membrane-bound proteins.<sup>12</sup> For example, cells can modulate integrin-binding affinities and kinetics of interactions between integrin receptors and their adhesive ligands through inside-out signaling.<sup>13,14</sup> After binding and clustering of integrins at the cell surface, interactions between integrins and intracellular proteins, often involving protein phosphorylation, are induced.<sup>10,15</sup> One of the most important components of the integrin-linked focal contacts is the focal adhesion kinase (FAK) that can bind directly to integrins and mediate various signal transduction events.<sup>16,17,18</sup>

In transformed cells and in clinical analyses of human tumors, elevated FAK expression and activity have been correlated with the progression to a highly malignant and metastatic phenotype (reviewed in<sup>19</sup>). In carcinoma cells, FAK-mediated signaling appears to be required for epithelial to mesenchymal transition, invasive properties, and the formation of  $\beta 1$ -integrin focal contact sites.<sup>20</sup> Additionally, studies using antisense to reduce FAK expression in these cells also found that FAK was a required component for optimal cell motility.<sup>21</sup> Notably, changes in cell invasion were independent from changes in either cell motility or transformation. FAK has been linked to the formation of focal contacts and FAK-null fibroblasts exhibited motility defects as a result of increased focal contact formation and the inability to remodel contact sites in response to various motility stimuli<sup>22</sup> suggesting an additional role of FAK in focal adhesion disassembly.<sup>23</sup> Besides its role in focal adhesion turnover, FAK has been implicated in adhesion stabilization and mechanotransduction. The interactions of the integrin receptors with ECM ligands can stabilize cell adhesions by recruiting signaling proteins involving FAK, among others, and cytoskeletal components.<sup>24,25</sup> The mechanistic role of FAK, however, seems to be highly context-dependent. For example, for integrin-stimulated cell motility the FAK Tyr397 site and its kinase activity were required.<sup>26</sup> In contrast, growth factor-stimulated cell motility of FAK-null fibroblasts was unaffected by the expression of kinase-inactive FAK.<sup>27</sup>

The majority of studies on adhesion-mediated signal transduction has been performed using assays where cell adhesion was conducted under static conditions, and the influence of fluid flow in the microcirculation was not considered.<sup>28</sup> However, it is known that shear forces alone can mediate various cellular events in endothelial

cells, leukocytes or platelets, such as phosphorylation,<sup>29</sup> cytoskeletal alterations, or secretion of soluble factors.<sup>30</sup> To counteract the shear forces of blood flow that appears to be required for cell adhesion of circulating tumor cells to vessel walls during metastasis formation several adhesion systems acting in parallel are probably necessary to form definitive adhesions that ultimately allow adherent tumor cell stabilization, migration, and invasion into the host organ.<sup>1</sup> Hydrodynamic adhesion assays are capable of mimicking hemodynamic conditions in the microcirculation.<sup>30,31</sup> Various studies have shown that tumor cells, such as colon carcinoma cells, can interact with EC and ECM under dynamic conditions of fluid flow.<sup>32,33</sup> Instead of measuring the complex of initial cell-surface interactions and adhesion stabilization using microtiter plate assays, we have used specific parallel plate laminar flow chambers for hemodynamic adhesion assays<sup>34</sup> to monitor the primary events of adhesion between circulating cells and vascular surfaces (EC or ECM) and their stabilization separately.<sup>35</sup> With this flow chamber assay we recently demonstrated that the initial dynamic interactions between colon carcinoma cells and various ECM components were mostly nonspecific, whereas definitive adhesion and its stabilization were specifically mediated, in part, by integrins.<sup>36</sup>

The C-terminal non-catalytic region of FAK (FRNK: FAK-related non-kinase) can be expressed as a separate RNA transcript in a variety of embryonic tissues,<sup>37</sup> in chicken fibroblasts,<sup>38</sup> and also in smooth muscle cells after vascular injury.<sup>39</sup> When expressed in various cell types by transfection methods FRNK exerts distinct effects on cell migration, cell adhesion, and cell spreading *in vitro*.<sup>39,40</sup> As a dominant-negative inhibitor of FAK, FRNK can negatively affect FAK-mediated cell functions, such as cell migration.<sup>41</sup> It has been suggested that FRNK disrupts associations of FAK with other intracellular proteins, such as pp60src,<sup>42,43</sup> prevents the localization of FAK to focal adhesions and thus antagonizes their formation.<sup>44</sup> FAK undergoes autophosphorylation as a reaction to cell adhesion and integrin-mediated stimulation on several Tyr residues, one of which is the crucial residue Tyr397.<sup>45</sup> This autophosphorylation can also be inhibited by FRNK overexpression.<sup>44</sup> In addition, there are different reports as to whether ectopic FRNK expression can induce apoptosis.<sup>44,46</sup>

Furthermore, most studies investigating the role of FAK in adhesive interactions have been carried out on fibroblasts,<sup>40-42</sup> myocytes,<sup>39,44</sup> and embryonic tissues.<sup>37</sup> Since the ability of various carcinoma cells to migrate and adhere on ECM components appears to correlate with their invasiveness and organ-specific metastatic potential, biophysical forces can modulate cell signaling in circulating tumor cells, and integrins can directly mediate metastatic tumor cell adhesion within host organs, we focused on adhesive and migratory properties under these conditions and the regulation of these processes. We hypothesized that FAK is involved in these early integrin-dependent steps of metastasis formation. The purpose was to investigate the involvement of FAK in the regulation of static and dynamic cell adhesion to ECM components *in vitro* and in metastatic tumor cell adhesion

within the hepatic sinusoids *in vivo*. First, we found that hydrodynamic shear forces can increase Tyr-phosphorylation of FAK in these cells. In addition, we demonstrated that reduced expression of FAK or its endogenous inhibition using FRNK can interfere with integrin-mediated cell adhesion to collagen and adhesion stabilization under the influence of hydrodynamic shear forces, but not under static conditions or to EC as adhesive substrate. Finally, blockade of FAK resulted in a loss of metastatic tumor cell adhesion within the liver microcirculation *in vivo*.

## Materials and Methods

### Materials and Cells

Fetal bovine serum (FBS), RPMI 1640, and minimal essential medium (MEM) were purchased from Life Technologies (Karlsruhe, Germany); type I collagen (C I) and type IV collagen (C IV) were obtained from Collaborative Biomedical Products (Bedford, MA). Genistein is a broad range inhibitor of protein tyrosine kinase (PTK) ( $IC_{50} = 1$  to  $100 \mu\text{mol/L}$ ). Erbstatin analog (inhibitor of EGFR-kinase:  $IC_{50} = 0.78 \mu\text{mol/L}$ ), Tyrphostin25 ( $IC_{50} = 3$  to  $150 \mu\text{mol/L}$ ) and Tyrphostin47 (broad-range PTK inhibitor with a different spectrum of inhibitory activity compared to Genistein:  $IC_{50} = 3$  to  $640 \mu\text{mol/L}$ ) served as control substances. All inhibitors were purchased from Calbiochem (Bad Soden, Germany) and reconstituted in dimethyl-sulfoxide (DMSO). The monoclonal antibodies (mAb) against P-Tyr (Py20), paxillin and FAK were obtained from Transduction Laboratories, and CalceinAM from Molecular Probes (Eugen, Netherlands). All other chemicals were purchased from Sigma (Taufkirchen, Germany).

We used highly metastatic HT-29LMM colon carcinoma cells or liver-metastatic Hep-G2 cells (hepatocellular carcinoma) to study cell adhesion properties. Cells were cultured in RPMI medium containing 10% FBS (HT-29LMM) or MEM medium containing 20% FBS (Hep-G2), respectively, without antibiotics in humidified 5%  $\text{CO}_2$ /95% air at  $37^\circ\text{C}$ . Only cells of less than 20 passages were used, and they were harvested with Trypsin/EDTA during the log-phase of growth. After trypsinization, the cells were resuspended in adhesion medium (RPMI [1:1], bovine serum albumin [BSA] 1%) at room temperature for 45 minutes for reconstitution of surface proteins and washed extensively in calcium-magnesium-free phosphate-buffered solution (CMF-PBS).<sup>47</sup> HUVEC (Cambrex Bioproducts, Apen, Germany) were grown on glass slides or in microtiter plates in endothelial cell medium to confluence.

### Static Adhesion Assays

Microtiter plates (96-well, Corning) were coated with C I ( $50 \mu\text{g/ml}$ ), C IV ( $50 \mu\text{g/ml}$ ), or 1% BSA (negative control) at  $37^\circ\text{C}$  for 3 hours ( $150 \mu\text{l/well}$ ). Effective concentrations to completely coat the wells were determined in previous experiments.<sup>6</sup> Blocking of nonspecific binding sites was

performed using 1% BSA ( $37^\circ\text{C}$ , 30 minutes,  $200 \mu\text{l/well}$ ). During reconstitution of surface proteins in adhesion medium, cells were fluorescence-labeled with CalceinAM ( $20 \mu\text{g}/15 \text{ ml}$ ). After washing, cells were resuspended in adhesion medium at a final concentration of  $0.5 \times 10^6$  cells/ml.

Adhesion experiments were performed with  $150\text{-}\mu\text{l}$  single-cell suspension/well for different times (10 to 120 minutes) as previously described.<sup>5</sup> In some experiments, cells were incubated with Genistein (1 to  $100 \mu\text{mol/L}$ ), Erbstatin analog (0.1 to  $1 \mu\text{mol/L}$ ), Tyrphostin25 (1 to  $10 \mu\text{mol/L}$ ), Tyrphostin47 (1 to  $10 \mu\text{mol/L}$ ), or with the same amount of DMSO alone ( $10 \mu\text{l/ml}$ ). Pretreatment was done at  $37^\circ\text{C}$  for 1 hour before adhesion assays. Cell viability was assessed by the trypan blue exclusion method, and pretreatment or CalceinAM labeling did not affect the number of viable cells ( $>95\%$ ). The effects of PTK inhibitors were calculated by comparing adhesion of untreated (set to 100% adhesion) and treated cells. All experiments were performed in triplicate and repeated twice.

### Flow Experiments

The laminar flow chamber was constructed as previously described.<sup>36</sup> Briefly, using an ECM-coated glass support and a polycarbonate shear deck (CoverWell Grace Bio-Labs) to form a uniform channel with a small height-to-width ratio a parallel plate chamber was formed that allowed an approximation of laminar flow. The distance between shear deck and collagen- ( $0.5 \mu\text{g}/\text{cm}^2$ ) or EC-coated glass slide was ensured by a medical-grade silicon gasket. The flow chamber was mounted on the stage of an upright microscope (magnification 1:63.5). BSA-coated glass slides were used as negative controls. A uniform fluid supply containing a single-cell suspension was maintained using a standard syringe pump (Frese-nius, Germany). The temperature of the cell suspension was kept at  $37^\circ\text{C}$  using a water bath. The fluid flow  $Q$  could be varied between 0.17 to  $0.83 \text{ ml/min}$  (shear rates 1.6 to  $5.6 \text{ dynes}/\text{cm}^2$ ) as a laminar flow with a parabolic velocity within the chamber (Reynolds number  $<2$ ). Therefore, the wall shear stress  $\tau$  was calculated using the equation for Newtonian fluids. The viscosity for  $\text{H}_2\text{O}$  at  $37^\circ\text{C}$  was assumed to be 0.7 cP.

Analysis of dynamic cell adhesion was performed as described previously.<sup>36</sup> Briefly, tumor cells were resuspended in adhesion medium at a concentration of  $1 \times 10^5$  cells/ml. In some experiments cells were pretreated with different concentrations of PTK inhibitors as described above. Untreated cells were allowed to adhere to BSA-coated surfaces (negative control) or collagen-coated slides (positive controls) and the effects of the inhibitors were assessed using C I or C IV as specific adhesive substrate. Alternatively, confluent EC were used. Flow experiments were performed with variable fluid flow starting at a wall shear stress (WSS) of  $5.6 \text{ dynes}/\text{cm}^2$  which is above flow conditions in the normal microcirculation ( $<3 \text{ dynes}/\text{cm}^2$ ). Flow rates were decreased in 30 seconds intervals so that the WSS equaled

4.8, 4.0, 3.2, or 2.4 dynes/cm<sup>2</sup>. The WSS at the point of initial cell adhesion events was used to measure wall shear adhesion threshold (WSAT). The dynamic adhesion rate (DAR) represented the total number of adherent cells after 1 minute at a low flow rate of 2.4 dynes/cm<sup>2</sup>, which was approximately 50% of WSAT and in the range of physiological conditions in the microcirculation (1.6 dynes/cm<sup>2</sup> for EC-coated slides). Following this low-flow interval, the flow rate was increased immediately to 4.8 dynes/cm<sup>2</sup> in an attempt to detach adherent cells that had not achieved adhesion stabilization during the low-flow interval. The relative percentage of cells remaining adherent after 60 seconds in relation to DAR was evaluated as adhesion stabilization rate (ASR). All experiments were repeated five times and results were shown as mean  $\pm$  SD.

### *Western Blotting of Phosphotyrosine, Paxillin, and Focal Adhesion Kinase*

Adhesion assays were performed as described above in microtiter plates (6-well, Corning) using a final concentration of  $1 \times 10^6$  cells/ml. After different adhesion time periods, the plates were placed on ice and adhesion medium was carefully removed by aspiration. The cells were lysed in ice-cold modified RIPA buffer (10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1.0% NP40, 0.25% deoxycholate, 0.1 mmol/L PMSF, 0.1 mmol/L TLCK, 0.1 mmol/L iodoacetamide, 10  $\mu$ g/ml leupeptin, and 10  $\mu$ g/ml aprotinin; pH 7.4) at 4°C for 30 minutes. Cell lysates were centrifuged at  $13,000 \times g$  for 10 minutes to remove insoluble material and lysates were added to sodium dodecyl sulfate (SDS) sample buffer and boiled at 95°C for 5 minutes. Twenty  $\mu$ g of protein was added to each lane. After SDS-PAGE electrophoresis (10% gel), proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (100 V for 1 hour), and the membranes were blocked in incubation buffer (10 mmol/L Tris-HCl, 50 mmol/L NaCl, 0.05% Tween 20, pH 7.5 containing 3% BSA) overnight at 4°C. Membranes were incubated with anti-P-Tyr, anti-paxillin, or anti-FAK mAb in incubation buffer containing 1% BSA at room temperature for 1 hour while rocking, and after washing three times they were incubated with anti-mouse specific anti-IgG-alkaline phosphatase for 1 hour at room temperature. For detection of bound antibodies, the membranes were incubated with BCIP/NBT solution (Zymed, Heiden, Germany) and the color reaction was stopped with distilled H<sub>2</sub>O. Paxillin or total FAK were used as control blots for equal loading.

### *Immunoprecipitation of Phosphorylated Proteins*

Cell lysates were obtained as described above. Pre-clearance was performed by adding 15- $\mu$ l anti-mouse-IgG-agarose to the lysate and incubating at 4°C for 2 hours. Supernatants were then incubated with 5- $\mu$ l anti-P-Tyr mAb at 4°C overnight. Immunoprecipitation was carried out using 15- $\mu$ l anti-mouse-IgG-agarose (4°C for 2 hours). After washing three times with RIPA-buffer, SDS sample buffer was added to the agarose-beads, and the

mixture was boiled at 95°C for 5 minutes. The beads were removed by centrifugation, and the supernatants were subjected to SDS-PAGE electrophoresis (10% gel). Proteins were subsequently blotted onto PVDF membranes. Phosphorylated proteins were identified using Western blotting as described above.

### *Suppression of FAK Expression with Antisense-Oligonucleotides*

For inhibition of FAK expression phosphorothioate oligonucleotides (ODN) against sequences of human pp125<sup>FAK</sup> were used. Antisense-FAK (FAK-AS2: 5'-ATAATCCAGCTTGAACCAAG-3') representing the nucleotides 1016 to 1035 of the human FAK sequence and a mismatch control ODN (FAK-AS control: 5'-ATAATC-GACTGTCAAGCAAG-3') were checked for sequence homologies using Blast Search and the GenBank. There were no cross-reactions to other genes, and FAK-AS control did not show any significant sequence homologies. The ODN were synthesized and HPLC-purified by Life Technologies. HT-29LMM cells were grown to 80% confluence. After removal of medium and washing with CMF-PBS, cells were incubated with serum-free DME/F12 medium at 37°C for 12 hours. Using fresh serum-free medium, 0.5 to 1  $\mu$ g ODN/ml medium were mixed with 10  $\mu$ l/ml Lipofectin (Life Technologies) according to the manufacturer's instructions. ODN containing liposomes were added to the cells; and HT-29 cells were incubated with FAK-AS2, FAK-AS control, or Lipofectin without ODN at 37°C for 12 to 36 hours. For further experiments, cells were lysed in modified RIPA-buffer or harvested using trypsin-EDTA as described above. Quantification of FAK expression was performed using Western blotting as described above. Detection of paxillin was used as an internal control. Toxic effects of ODN and serum-free conditions were evaluated using the Trypan blue exclusion method.

### *Overexpression of FRNK*

Total mRNA was isolated from HT-29 cells (RNeasy Mini Kit, Qiagen, Hilden, Germany) and transcribed into cDNA (Omniscript RT Kit, Qiagen). The FRNK gene sequence was amplified and restriction sites were added via PCR. A Kozak sequence was added as well. The resulting fragment was sequenced and cloned into the mammalian expression vectors pcDNA3.1 (Invitrogen, Karlsruhe, Germany) and pEGFP-C3 (Clontech, Heidelberg, Germany) using TaqPCR Core Kit (Qiagen), TOPO TA Cloning Kit (Invitrogen) and appropriate restriction enzymes (Promega, Heidelberg, Germany). The constructed expression vectors were transformed into the bacterial strain XL Blue (Stratagene, Amsterdam, Netherlands). From overnight cultures the plasmids were isolated and purified using a plasmid preparation system (QIAfilter Plasmid Midi Kit, Qiagen).

For transfection, Hep-G2 or HT-29LMM cells were used. The constructed plasmids were transiently transfected into the cells using a liposome-based method

(Lipofectamine 2000, Invitrogen) according to manufacturer's instructions. The genuine vectors pcDNA3.1 (Promega) or pEGFP-C3 (Clontech) were used as negative control. The day after transfection cell proliferation rates were determined using a BrdU test (Boehringer, Mannheim, Germany). Apoptosis rate was determined 3 days after transfection using an Annexin assay (Coulter-Immunotech, Krefeld, Germany) and evaluated by FACS analysis.

### *In Vivo Observation of Metastatic Tumor Cell Adhesion*

Male Sprague-Dawley rats (200 to 250 g, Charles River) were cared for in accordance with standards of the German Council on Animal Care, under an approved protocol of the local Animal Welfare Committee. Rats were anesthetized using inhalation of isoflurane (Curamed, Karlsruhe, Germany) and N<sub>2</sub>O and prepared as previously described.<sup>7,48</sup> For intravital observation of adhesive interactions between circulating tumor cells and the hepatic microcirculation, single-cell suspensions (1 × 10<sup>6</sup> cells) were injected intra-arterially over 60 seconds. In some experiments cells were pretreated as described above. This technique did not interfere with cardiocirculatory or pulmonary functions of the animals.

Various parameters were used for further investigation and semi-quantitative analysis of these interactions. The localization of stable tumor cell adhesions within the vascular tree and in relation to the diameter of the involved vessels were evaluated. If tumor cells were able to arrest within the sinusoids, the diameter of the involved vessel was determined compared to the diameter of the adherent tumor cell. Furthermore, remaining blood flow within this vessel or its occlusion was investigated. A semi-quantitative analysis of tumor cell adhesions and extravasation was performed over a 30-minute observation period and the numbers of adherent cells were counted for each of the 5-minute intervals. Using a standardized procedure, all fields were analyzed in each observation period and the average number of adherent cells, migrated cells, and total cells observed in 30 microscopic fields was counted. Relative migration rates were calculated as percentage of cells within the hepatic parenchyma in relation to the total number of arrested cells.

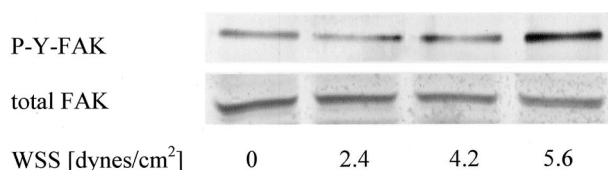
### *Statistical Analysis*

Statistical analysis was performed using the StatMost32 statistical program (DataMost Corp., Los Angeles, CA). *P* values were calculated according to Student's *t*-test. Significant differences were accepted for *P* < 0.05.

## **Results**

### *Fluid Flow Induces Tyr-Phosphorylation of FAK*

To investigate the influence of fluid flow on the phosphorylation status of immobilized cells we used the laminar flow chamber to expose cells grown on tissue culture



**Figure 1.** Stimulation of Tyr-phosphorylation by hydrodynamic shear forces. HT-29 cells grown on glass slides were subjected to different rates of WSS in a laminar flow chamber. Tyr-phosphorylated FAK were measured after 60 minutes of flow exposure. Experiments were performed three times and one representative example is shown.

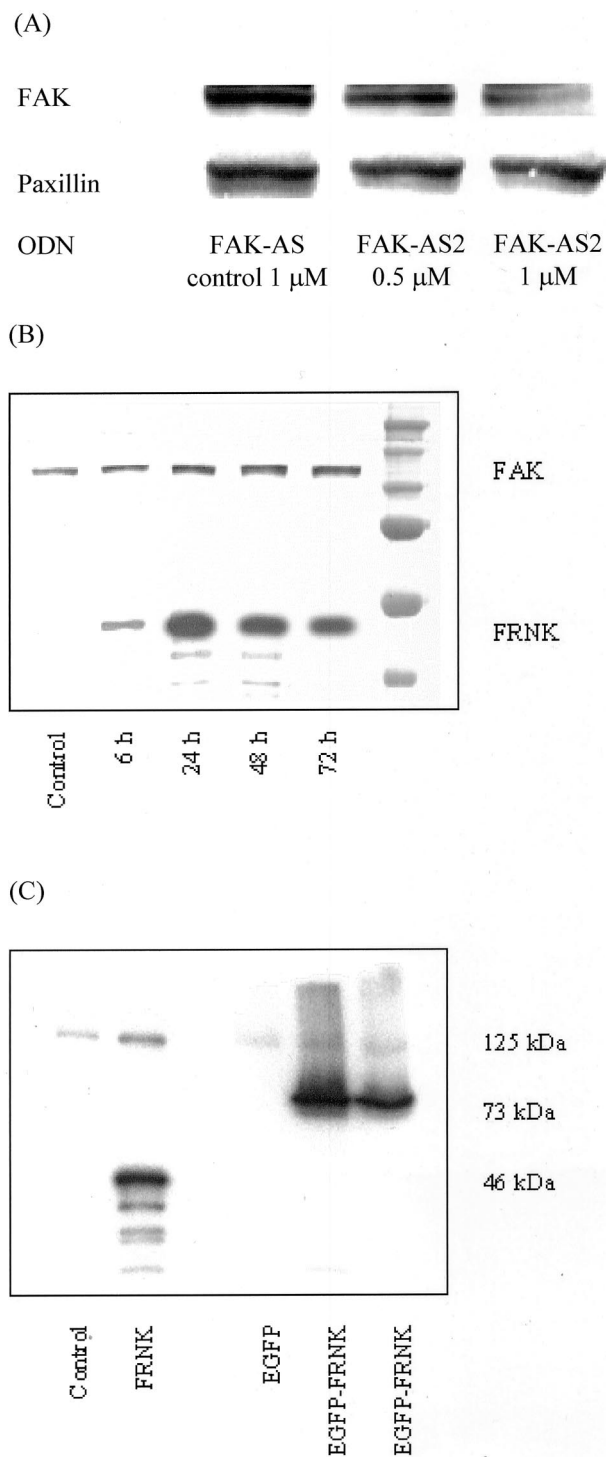
slides to hydrodynamic shear forces. After 60 minutes of different WSS, hydrodynamic shear forces induced an increase in Tyr-phosphorylation of FAK. Highest stimulation was observed if high wall shear stress (WSS = 5.6 dynes/cm<sup>2</sup>) was applied (Figure 1). Tyr-phosphorylation of FAK was increased 2.3-fold at high WSS compared to control cells that were kept under static conditions and not exposed to shear forces. Therefore, further experiments were performed to investigate the potential role of FAK for tumor cell adhesion under hydrodynamic flow conditions comparable to the conditions found in potential metastatic organs, such as within liver sinusoids. FAK was either blocked using antisense oligonucleotides or its competitive inhibitor FRNK.

### *Reduced Expression of FAK Using Antisense Oligonucleotides*

Transient transfection was performed with different concentrations of ODN and various incubation times. Using 1 μg/ml FAK-AS2, a decrease of FAK expression was found starting after 24 hours, but maximal effects were seen after 36 hours of incubation. Longer incubation (72 hours) resulted in an occurrence of cell death (>10%, determined by dye exclusion method) without further reduction of FAK expression. In contrast, FAK-AS control led only to minimal effects on FAK expression. Densitometrical comparison of FAK protein expression after FAK-AS2 treatment revealed protein expression of FAK was reduced to less than 30% after 36 hours, whereas FAK-AS control resulted in 88% of FAK expression. These effects were concentration-dependent (Figure 2A). As an internal control the expression level of the focal adhesion protein paxillin was not significantly influenced. For further experiments, cells were pretreated with 1 μg/ml ODN for 36 hours in serum-free medium using Lipofectin. This was consistent with antisense-mediated reduction of FAK using ODN against a different part of the FAK sequence.<sup>49</sup>

### *Overexpression of FRNK and EGFP-FRNK*

A transient transfection with a successful expression of FRNK and EGFP-FRNK in Hep-G2 or HT-29LMM cells was performed to avoid clonal selection for further experiments. For detection of transfection effectivity, an anti-FAK antibody was used that cross-reacts with FAK (125 kd) as well as FRNK (46 kd) and EGFP-FRNK (73 kd). In Figure 2B, the time-dependence of FRNK expression for 6 to 72 hours is shown for Hep-G2 cells. FRNK and



**Figure 2.** Inhibition of FAK function or expression. **A:** HT-29 cells were incubated with 0.5–1 μmol/L FAK-AS2 or FAK-AS control oligonucleotides using Lipofectin (10 μl/ml) in serum-free DME-F12 medium for 36 hours. Expression of FAK was determined using Western blotting. In antisense-treated cells expression of FAK was significantly reduced to less than 30% compared to untreated cells ( $P < 0.05$  from four experiments), whereas mismatch-control treatment had only slight effects (88%) on FAK protein amounts. In contrast, the focal adhesion protein paxillin was not affected. **B:** Transient transfection of Hep-G2 cells resulted in overexpression of FRNK after 24 to 72 hours as determined by Western blot using monoclonal antibodies against the C-terminal region of FAK. Endogeneous FRNK has not been detected in these cells. **C:** The 46 kD-FRNK fragment was found using pcDNA3.1 plasmids, where the EGFP-FRNK plasmid produced a 73-kD fragment. Control cells were transfected with genuine plasmids.

EGFP-FRNK were well expressed after 24 hours which was stable for approximately 48 hours. (Figure 2C). Transfection rates for EGFP-FRNK were 60 to 70% as verified by FACS analysis (data not shown). Endogenous FRNK was not detected in cell lysates.

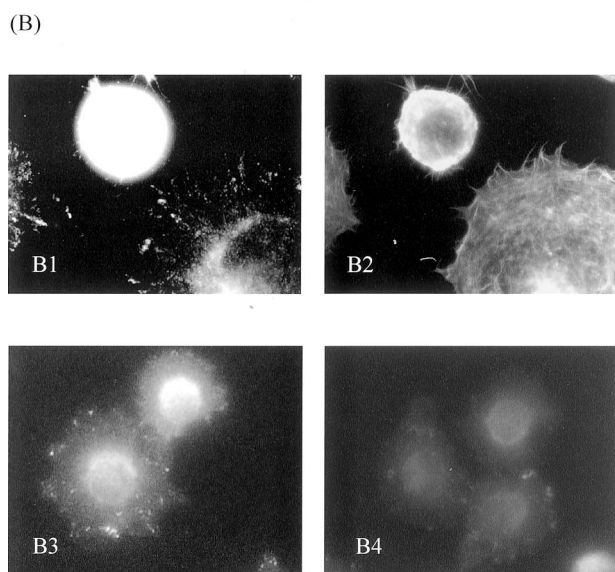
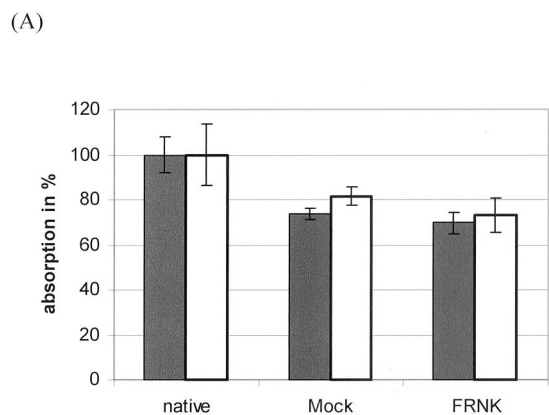
Toxicity of FRNK expression was determined using BrdU-proliferation assays and annexin-apoptosis detection after 48 and 72 hours. Transient transfection slightly reduced cell division, but differences between transfection with the genuine EGFP-plasmid and the EGFP-FRNK transfection were not found. Similarly, apoptosis was un-specifically affected by the plasmid ( $3.3 \pm 0.8\%$  versus  $12.9 \pm 1.7\%$ ), but overexpression of FRNK did not result in further effects ( $11.9 \pm 1.2\%$ ) (not shown).

### *FAK Is Not Required for Static Cell Adhesion to Collagens*

Using the microtiter plate assay, HT-29LMM cells showed substantial time-dependent adhesion to collagens under static conditions. Maximum values were reached after 60 to 90 minutes. After more than 120 minutes, adhesion began to decrease, probably because of the release of proteases and degradation of ECM.<sup>5</sup> Similar results were obtained using HT-29LMM cells treated with antisense or mismatch-control ODN. Significant differences in adhesive properties to C I or C IV under these conditions were not observed if expression of FAK was reduced (not shown). If FRNK was hyperexpressed in Hep-G2 cells the adhesive behavior to collagen was not significantly altered. Similar adhesion rates to C I and C IV under static conditions were found compared to control transfected cells after 10 to 60 minutes. (Figure 3A) Some of these experiments were repeated using laminin or fibronectin as adhesive substrates and differences between the ECM components were not found (not shown). Furthermore, FRNK-transfected cells did not show cell spreading or adhesion-mediated hyperphosphorylation of FAK within 10 to 60 minutes (Figure 3B).

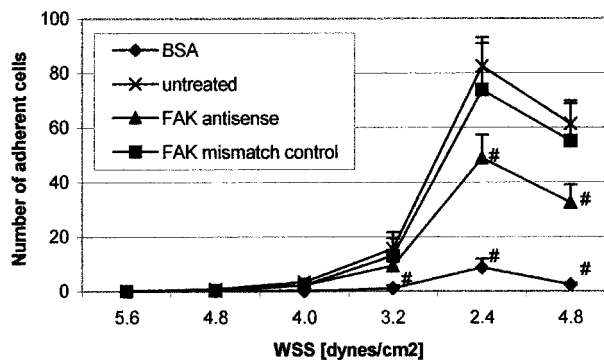
### *FRNK and Reduced Expression of FAK Decrease Dynamic Cell Adhesion*

For investigation of FAK-related alterations of dynamic adhesive properties, we examined adhesion of HT-29LMM cells where FAK expression was reduced or endogenously inhibited. Treatment of cells with FAK-AS2 or FAK-AS control did not affect initial cell-ECM interactions and rolling of cells under flow conditions. Short-term cell sticking without definitive adhesion was also found to be similar. The flow rates or WSS, respectively, where FAK-AS2 and FAK-AS control-treated cells began to adhere to C I- or C IV-coated surfaces were similar, and significant differences between WSAT values were not found. However, using lower fluid flow or reduced shear forces, which represented lower WSS, the numbers of adherent cells were significantly reduced if expression of FAK was inhibited (Figure 4). The rates of adherent cells at low wall shear stress WSS (dynamic adhesion rates DAR) but not



**Figure 3.** Static cell adhesion and spreading of FRNK-transfected cells. **A:** Hep-G2 cells transfected with EGFP-plasmid (//) or pcDNA3.1-plasmid (○) containing FRNK or in its genuine form (mock) were allowed to adhere to collagen IV in microtiter plate assays for 30 minutes. Significant differences in the numbers of adherent cells were not found between FRNK- and control-transfected cells. Values are shown as mean ± SD from quadruplicate experiments. Comparable results were obtained using collagen I or colon carcinoma cells (not shown). **B:** Hep-G2 cells adherent to collagen IV for 30 minutes were fluorescence-stained for FAK (epitope within FRNK-fragment, B1) and F-actin (phalloidin, B2) in a double-labeling technique. The cell expressing FRNK remained round without cell spreading and reorganization of the actin cytoskeleton. In addition, FRNK-transfected cells did not show adhesion-specific phosphorylation of FAK (B4), whereas mock-transfected cells demonstrated typical focal adhesions with phosphorylated Tyr397 residues of FAK (B3). This inhibition was confirmed by Western blot (not shown).

adhesion stabilization rates (ASR) demonstrated highly significant differences between FAK-AS2 and control cells (Table 1). Almost identical results were obtained if HT-29LMM were pretreated with the PTK-inhibitor Genistein using different concentrations that were able to inhibit adhesion-mediated Tyr-phosphorylation of FAK (Table 2). In contrast, other PTK inhibitors, such as the Erbstatin analog, Tyrphostin25 or Tyrphostin47, that were unable to modify FAK phosphorylation did not demonstrate any effect on dynamic adhesion of HT-29LMM cells (not shown).



**Figure 4.** Effect of reduced FAK expression on dynamic cell adhesion to ECM. HT-29 cells treated with FAK antisense or mismatch-control oligonucleotides were used for adhesion assays in a laminar flow chamber. BSA was used as nonspecific adhesion substrate. Values are shown as mean ± SD from five different experiments. Numbers of adherent cells were compared to dynamic adhesion of untreated cells to C I using Student's *t*-test (#,  $P < 0.05$ ). Similar results were obtained using C IV as adhesive substrate.

Comparable to the antisense-mediated down-regulation of FAK expression of FRNK resulted in a significantly reduced dynamic cell adhesion (Figure 5), although initial interactions were not affected (WSAT:  $3.87 \pm 0.6$  versus  $3.3 \pm 0.3$  dynes/cm<sup>2</sup>;  $P = 0.086$ ). The ability of cells to stabilize their adhesions to collagen, however, was significantly inhibited by FRNK expression (ASR:  $71 \pm 8\%$  versus  $55 \pm 15\%$ ;  $P < 0.05$ ), and crawling of cells along the coated surfaces was regularly seen in FRNK-transfected cells.

### FAK-Independent Cell Adhesion to Endothelial Cells

FRNK-transfected Hep-G2 cells were compared for their static cell adhesion to HUVEC-coated surfaces. After 15 to 45 minutes FRNK-expressing cells and EGFP-control transfected cells showed comparable adhesion rates, but FRNK cells were slightly more adherent to HUVEC after 30 and 45 minutes ( $P < 0.005$ ) (Figure 6A). Under hydrodynamic conditions Hep-G2 cells demonstrated cell adhesion only at low flow conditions representing the fact that selectin-mediated adhesive bonds are less stable than integrin-mediated contacts. Therefore, the low flow interval was used at WSS of 1.6 dynes/cm<sup>2</sup>. However, the adhesive behavior under these conditions was also comparable between FRNK-transfected and control cells and wall shear adhesion threshold WSAT, dynamic adhesion rates DAR or adhesion stabilization rates ASR did not show significant differences (Figure 6B).

### Regulation of Metastatic Cell Adhesion in Vivo by FAK

This results suggested that FAK is required for integrin-mediated cell adhesion under the influence of shear forces comparable to the conditions for metastatic cell adhesion during hetogenous formation of distant metastases. Since metastatic tumor cell adhesion *in vivo* can be mediated by integrin interactions with ECM components

**Table 1.** Reduced Expression of FAK Decreases Dynamic Adhesion Rate to Collagen

Substrate	Oligonucleotide	WSAT [dynes/ cm <sup>2</sup> ]	DAR [cells]	ASR [%]
BSA		2.9 ± 0.5*	9 ± 3*	18 ± 19*
C I		4.6 ± 0.4	83 ± 10	74 ± 4
C I	FAK-AS2	4.2 ± 0.4 <sup>†</sup>	49 ± 9 <sup>†</sup>	66 ± 3 <sup>†</sup>
C I	FAK-AS control	4.2 ± 0.4 <sup>†</sup>	74 ± 17 <sup>†</sup>	74 ± 6 <sup>†</sup>

Wall shear adhesion threshold (WSAT), dynamic adhesion rate (DAR) and adhesion stabilization rate (ASR) were calculated as described. Values are given as mean ± SD from five different experiments. Expression of FAK in HT-29 cells was reduced using antisense ODN (FAK-AS2) or mismatch control ODN (FAK-AS control). Statistical analysis (Student's *t*-test) was performed for BSA- versus C I-coated surfaces, and untreated versus treated cells. (\**P* < 0.001; <sup>†</sup>not significant; <sup>‡</sup>*P* < 0.05).

that are directly accessible for circulating tumor cells in the space of "Disse", we used our *in vivo* model to evaluate the role of FAK for cell arrest in the hepatic microcirculation under physiological conditions. Considering the transfection rates of Hep-G2 cells expressing EGFP, adhesive interactions of these cells within the liver microcirculation was slightly reduced compared to untreated cells. However, the localization of cell adhesions, remaining vessel lumen of affected sinusoids and the time course of increasing numbers of adherent cells during the first ~15 minutes and early migration into the hepatic parenchyma starting within 5 minutes after cell injection were not influenced by EGFP transfection. In contrast, if cells expressed FRNK we observed a dramatic reduction in their ability to arrest within the sinusoids. The numbers of adherent and total numbers of arrested cells were highly significantly lower than EGFP-transfected cells (*P* < 0.0001). In addition, cell migration into the parenchyma was completely impaired by FRNK-expression (*P* < 0.001) (Figure 7).

### Discussion

Various studies have shown that integrin-mediated interactions between tumor cells and ECM components within host organs appear to be crucial for the organ-specific formation of distant metastases.<sup>6,50,51</sup> For example, integrin expression can profoundly influence metastatic tumor progression in various tumor entities.<sup>52-55</sup> The mechanisms by which integrins take part in various steps of these processes *in vivo* during blood-borne metastasis formation are only partially understood and appear to involve changes in tumor cell binding affinities and signal transduction into the cells.<sup>56,57</sup> Intravital microscopy technologies have been recently used to investigate this

metastatic tumor cell adhesion within host organ microcirculation, such as in liver and lung, and their invasion into the host organ parenchyma.<sup>58-62</sup> In these studies contradictory results were reported regarding the type of entrapment (mechanical entrapment versus active cell adhesion) and the requirement of invasion into host organ parenchyma (invasion versus intravascular proliferation). Using colon carcinoma cells our group observed a lack of mechanical entrapment in rat liver sinusoids and a rapid extravasation into the liver parenchyma,<sup>48</sup> suggesting that active regulation of adhesion molecules is involved in organ-specific colonization of host organs. As described above circulating tumor cells appear to be able to directly bind to ECM components in the space of "Disse" via different types of integrins (laminin- and fibronectin-binding integrins), whereas other integrin heterodimers seem to be mainly involved in the migration into host organ parenchyma (collagen-binding integrins). These observations suggested that the regulation of integrin function, their affinity and/or avidity, might have an important role in the organ-specific colonization of distant organs by metastasizing tumor cells. FAK is a very important regulator of "outside-in" integrin-mediated cell signaling and may also regulate these "inside-out" integrin functions.

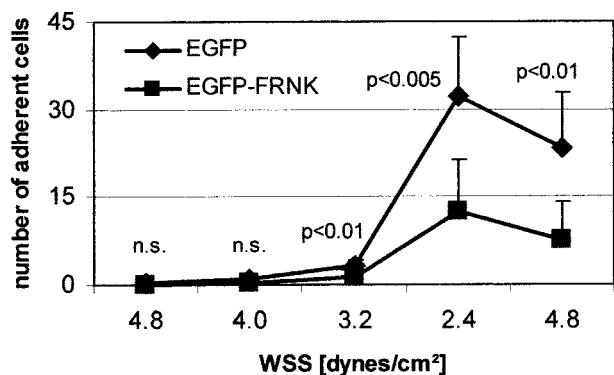
In our study we provide *in vitro* and *in vivo* evidence that FAK is required for integrin-mediated direct tumor cell adhesion within the liver, a major metastatic target organ. Since down-regulation of FAK in different tumor cell entities did not inhibit adhesive interactions between these tumor cells and EC, the loss of cell adhesions in FAK-inhibited cells appears to support our previous findings that direct integrin-mediated tumor cell adhesions with ECM components in the space of "Disse" can occur that are regulated by integrin-mediated signaling. We have previously found that adhesive interactions of colon car-

**Table 2.** Genistein-Mediated Reduced Dynamic Adhesion Rate to Collagen

Substrate	Inhibitor	WSAT [dynes/ cm <sup>2</sup> ]	DAR [cells]	ASR [%]
BSA		3.4 ± 0.7*	20 ± 13*	35 ± 25*
C I		4.5 ± 0.7	148 ± 43	73 ± 12
C I	Genistein 10µM	4.3 ± 0.7 <sup>†</sup>	63 ± 14*	79 ± 9 <sup>†</sup>
C I	Genistein100µM	4.1 ± 0.8 <sup>†</sup>	68 ± 39 <sup>†</sup>	72 ± 15 <sup>†</sup>

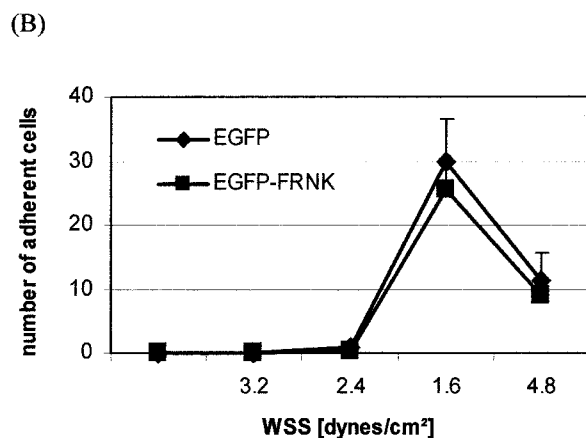
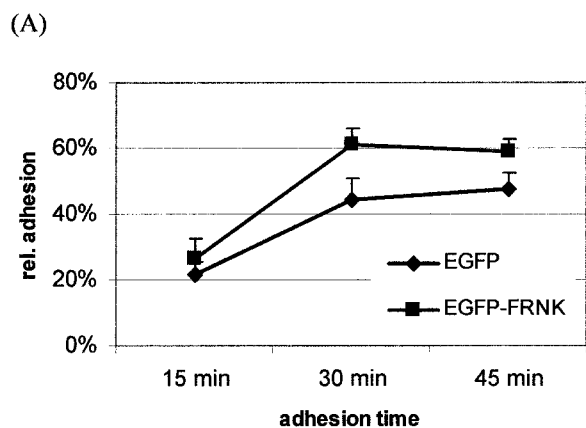
Wall shear adhesion threshold (WSAT), dynamic adhesion rate (DAR) and adhesion stabilization rate (ASR) were calculated as described. Values are given as mean ± SD from five different experiments. Cells were pretreated using different concentrations of Genistein. Statistical analysis (Student's *t*-test) was performed for BSA- versus C I-coated surfaces, and untreated versus treated cells. (\**P* < 0.001; <sup>†</sup>not significant, <sup>‡</sup>*P* < 0.05).



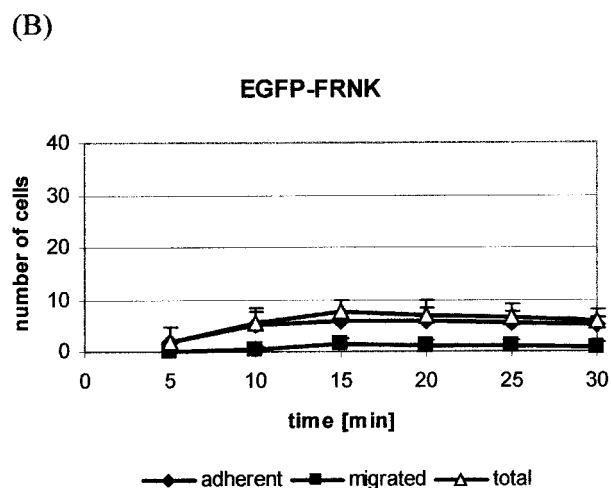
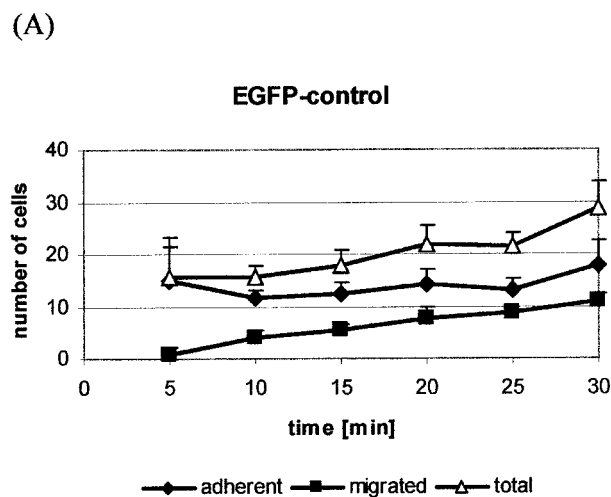


**Figure 5.** FRNK negatively regulates dynamic cell adhesion to collagen. Hep-G2 cells transfected with genuine EGFP-vector or EGFP-FRNK were used for dynamic adhesion assays on C I. Values are shown as mean  $\pm$  SD from six different experiments. WSAT was not significantly affected ( $4.7 \pm 0.6$  dyn/cm<sup>2</sup> versus  $4.1 \pm 0.3$  dyn/cm<sup>2</sup>), but DAR ( $7.8 \pm 6.8$  cells;  $P < 0.005$ ) and ASR ( $71 \pm 7\%$  versus  $55 \pm 15\%$ ;  $0.05$ ) were significantly reduced in FRNK-transfected cells. Similar results were obtained using C IV, but total numbers of cells were smaller, if this ECM component was used (not shown).

cinoma cells with ECM-coated surfaces under laminar flow conditions proceed in discrete steps: cell rolling, cell sticking or initial adhesion, and stabilization of cell adhesion.<sup>36</sup> Initial steps seem to be severely influenced by



**Figure 6.** FAK is not required for tumor cell adhesion to endothelial cells. Hep-G2 cells transfected with genuine EGFP-vector or EGFP-FRNK were used for (A) static or (B) dynamic adhesion assays on HUVEC *in vitro*. Values are shown as mean  $\pm$  SD from five different experiments. FRNK did not affect dynamic adhesion, but cell adhesion under static conditions was significantly higher in FRNK-transfected cells ( $P < 0.005$ ).



**Figure 7.** *In vivo* cell adhesion within the hepatic microcirculation. Hep-G2 cells transfected with (A) genuine EGFP-vector or (B) EGFP-FRNK were used for the intravital observation of circulating tumor cells within the liver sinusoids. Numbers of adherent cells, migrated cells and the total number of arrested cells (adherent + migrated) are shown as mean  $\pm$  SD from seven animals in each group. Expression of FRNK almost completely inhibited adhesive interactions of circulating Hep-G2 cells within the liver.

biophysical factors, and modulation of cell deformability and rigidity can determine the effectiveness of tumor cell arrest within host organ microcirculation.<sup>7</sup> Later events showed increasing receptor specificity and were apparently mediated, at least in part, by the same integrins as demonstrated in static adhesion assays.<sup>5</sup>

The functional status of integrins, which are known for their activation or affinity to bind to ECM components, is regulated by complex interactions with a number of cytosolic, cytoskeletal, and membrane-bound proteins.<sup>63</sup> Cells can modulate integrin-binding affinity and kinetics of interactions between integrin receptors and their adhesive ligands.<sup>9</sup> Using different integrin chimeras it was shown that this “activation” of integrins is related to only a limited number of intracellular factors, such as FAK,<sup>9</sup> but signal transduction is apparently required both for the adhesion itself and for further steps during tumor cell host organ colonization.<sup>64</sup> Surprisingly, reduced expression of FAK or its competitive inhibition by FRNK did not influ-

ence adhesive properties under static conditions. However, similar to the *in vivo* results with the almost complete loss of metastatic cell adhesions the numbers of adherent cells and adhesion stabilization were significantly reduced under hydrodynamic fluid flow *in vitro*, whereas initial cell binding was not different. Fewer cells were able to withstand shear forces and form definite cell attachments, even if the WSS was in the lower range of microcirculatory fluid flow ( $<3$  dynes/cm<sup>2</sup>). These results were confirmed by inhibition of FAK kinase activity using Genistein in our experiments and by others,<sup>65</sup> whereas other PTK inhibitors did not change adhesive properties.

In contrast to static cell adhesion, biophysical factors can influence adhesion processes under flow conditions as they occur within the microcirculation of host organs.<sup>66</sup> First, flow velocity determines available time for cell adhesion. The initial areas of cell contacts to the vessel walls are very small because of the more or less spherical shape of flowing tumor cells and the fact that initial cell contacts may occur via cell protrusions or filopodia. Alterations in cell shape and morphology (flattening of cells) are important for increasing the areas of cell-cell contacts. Additionally, cell flattening enables adherent cells to avoid high shear forces closer to the center of the parabolic curve of fluid flow. These factors as well as the character of flow are responsible for the strength of the shear forces acting against cell adhesion. Flow rate and viscosity of the perfusate are the main factors determining strength of cell shear stress.<sup>67</sup> In addition to these biophysical factors, shear stress and tension alone can induce functional reactions in circulating cells, such as leukocytes and platelets, or EC.<sup>30,68</sup>

In accordance with these reports in hematological cells, our results suggest that hydrodynamic fluid flow comparable to the microcirculation can induce changes in cell signaling in adherent epithelial tumor cells. The focal adhesion protein FAK showed increased Tyr-phosphorylation after a relatively short exposure to low shear forces. Similar results were previously found for EC and other circulating blood cells, but most studies used longer application times or higher shear forces up to 100 dynes/cm<sup>2</sup>. For example, shear stress can directly activate different signaling cascades in EC.<sup>69–71</sup> Using bovine aortic EC, Li et al<sup>72</sup> provided evidence that this phosphorylation of FAK appears to have an important role in shear-mediated signaling. Moreover, the organization and structure of cytoskeletal components, such as actin, can also be modified by shear stress.<sup>73</sup> Other studies demonstrated differences in adhesive properties of platelets between static and dynamic conditions.<sup>74</sup> In a previous study we have also shown similar differences for tumor cells adhesion to ECM components.<sup>5</sup> Our results suggest that comparable to cells physiologically exposed to shear forces of blood flow, circulating tumor cells can also respond to wall shear stress under hydrodynamic conditions by changes in Tyr-phosphorylation of FAK which mainly occur at the Tyr397-residue.<sup>7</sup> This responsiveness of FAK to external forces pointed to a specific role of this kinase for early steps of metastasis formation. FAK may therefore combine detection of external forces from the environment<sup>75</sup> in metastasizing tumor cells with their cellular response for effective adhesion stabilization providing the basis for further steps of metastasis for-

mation. Comparable to its role for cell motility and cell migration where FAK is an important regulator of mechanotransduction and focal adhesion turnover, this kinase also appears to be required for adhesion stability in circulating tumor cells.

In this study we presented the first evidence that FAK seems to be directly involved in early events of integrin-mediated metastatic tumor cell adhesion under dynamic conditions of fluid flow *in vitro* and *in vivo*. Our experiments suggested that FAK might be an important PTK that is not only involved in adhesion-mediated down-stream signaling and focal adhesion disassembly, but also responsible for the establishment of stabilized adhesive interactions. These interactions enable circulating tumor cells to resist shear forces within the microcirculation of metastatic target organs that is likely a prerequisite for successful formation of distant metastases.

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