

Resistance to the mTOR Inhibitor Temsirolimus Alters Adhesion and Migration Behavior of Renal Cell Carcinoma Cells through an Integrin $\alpha 5$ - and Integrin $\beta 3$ -Dependent Mechanism¹

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Abstract

Inhibitors of the mammalian target of rapamycin (mTOR) have improved the treatment of renal cell carcinoma (RCC). However, chronic drug exposure may trigger resistance, limiting the utility of these agents. The metastatic behavior of RCC cells, susceptible (RCC^{par}) or resistant (RCC^{res}) to the mTOR inhibitor temsirolimus, was investigated. Adhesion to vascular endothelium or immobilized collagen and fibronectin was quantified. Chemotactic motility was evaluated with a modified Boyden chamber assay. Integrin α and β subtype receptors were analyzed by flow cytometry and Western blot analysis. The physiological relevance of the integrins was then determined by blocking studies and small interfering RNA knockdown. Adhesion to endothelial cells and to fibronectin (not to collagen) and chemotaxis were enhanced in RCC^{res} compared to RCC^{par}. RCC^{res} detached from fibronectin and motile activity further increased under retreatment with low-dosed temsirolimus. $\alpha 5$ integrin was diminished inside the cell and at the cell surface, whereas the $\beta 3$ subtype was reduced intracellularly but elevated at the plasma membrane. In RCC^{par}, blocking $\alpha 5$ surface receptors enhanced RCC-collagen but reduced RCC-fibronectin interaction, whereas the opposite was true for RCC^{res}. Chemotaxis of RCC^{par} but not of RCC^{res} was strongly diminished by the $\alpha 5$ antibody. Blocking $\beta 3$ significantly lowered chemotaxis with stronger effects on RCC^{res}, compared to RCC^{par}. Importantly, $\beta 3$ knockdown reduced chemotaxis of RCC^{par} but upregulated the motile behavior of RCC^{res}. Temsirolimus resistance is characterized by quantitative alterations of integrin $\alpha 5$ and $\beta 3$ expression, coupled to functional changes of the integrin molecules, and forces a switch from RCC adhesion to RCC migration.

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Introduction

Renal cell carcinoma (RCC) is one of the most aggressive tumor types. Approximately one third of patients have already developed metastases at diagnosis, and up to 40% of patients undergoing surgical resection will have disease recurrence. Once metastasized, the 5-year survival rate is less than 5% [1].

Increasing knowledge about the molecular alterations driving a cell to become malignant has led to the development of novel compounds targeting those pathways, which are aberrantly activated in cancer. This is particularly true for the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling, which is dysregulated in RCC [2], and activation of this pathway has been suggested to correlate with aggressive behavior and poor prognosis in RCC tumors [3].

In the targeted treatment of RCC, mTOR inhibition plays a principal role. Temsirolimus has been approved for the first-line treatment of patients with RCC with poor prognosis, whereas the oral mTOR inhibitor everolimus has been recommended for patients with

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advanced progressive RCC or for patients with failed vascular endothelial growth factor–targeted therapy [1,4].

Though mTOR targeting offers significantly enhanced response rates, it is rarely curative [5]. The reason for the insufficient therapeutic response has not been fully elucidated. It is argued that chronic drug exposure may activate an undesired escape mechanism, leading to resistance development. It has recently been demonstrated that long-term mTOR blockade triggers undesired feedback loops in RCC cells [6], associated with drug nonresponsiveness and accelerated tumor growth [7]. Similar effects have been observed with resistant prostate cancer cells, evidenced by elevated cell cycle progression compared to those from drug-sensitive sublines [8].

When discussing the pros and cons of mTOR inhibitors, it must be kept in mind that invasion and metastasis are critical for malignant tumor progression. They are the main causes of treatment failure. How circulating RCC cells are transferred from blood vessels into the target tissue when resistance toward mTOR inhibitors develops is unclear. The object of the present study was to drive RCC cells into temsirolimus resistance and investigate altered adhesion and invasion dynamics. Because adhesion molecules of the integrin family are critically involved in the process of tumor transmigration and metastasis [9,10], modification of integrin α and β subtype expression was analyzed and correlated to the invasive behavior of the tumor cells.

Materials and Methods

Cell Culture

Kidney carcinoma Caki-1, KTCTL-26, and A498 cells were purchased from LGC Promochem (Wesel, Germany). The tumor cells were grown and subcultured in RPMI 1640 medium (Gibco/Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. The temsirolimus-resistant subline was cultivated for 12 months by exposing the parental cells to temsirolimus (Torisel; LC Laboratories, Woburn, MA), starting at 1 nM/ml and increasing stepwise to 1 μ M/ml. The resistant variants were termed Caki^{res}, KTC^{res}, and A498^{res}. The parental control cells were designated Caki^{par}, KTC^{par}, and A498^{par}.

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical veins and harvested by enzymatic treatment with dispase (Gibco/Invitrogen). Human endothelial cells were grown in Medium 199 (M199; Biozol, Munich, Germany), supplemented with 10% FCS, 10% pooled human serum, 20 mg/ml endothelial cell growth factor (Boehringer, Mannheim, Germany), 0.1% heparin, 100 ng/ml gentamycin, and 20 mM Hepes buffer (pH 7.4). Subcultures from passages 2 to 5 were employed.

Drug Treatment

Temsirolimus was dissolved in DMSO as a 10 mM stock solution and stored as aliquots at –20°C. Before experiments, temsirolimus was diluted in cell culture medium to the final concentration. Control cell cultures received cell culture medium alone. To exclude toxic effects of the compounds, cell viability was determined by trypan blue (Gibco/Invitrogen).

To analyze the influence of temsirolimus on adhesion and chemotactic movement of resistant compared to sensitive tumor cells, cell culture medium of Caki^{res}, KTC^{res}, or A498^{res} cells containing 1 μ M temsirolimus was replaced by temsirolimus-free medium to avoid unspecific effects. A medium change was also carried

out in the drug-sensitive cell culture system. After 3 days, 10 nM/ml temsirolimus was added to both resistant and sensitive cells (controls received fresh medium without temsirolimus), and adhesion and chemotactic movement were analyzed.

Tumor Cell Adhesion

To analyze tumor cell adhesion, HUVECs were transferred to six-well multiplates (Falcon Primaria; BD Biosciences, Heidelberg, Germany) in complete HUVEC medium. When confluency was reached, RCC cells (resistant and sensitive) were detached from their culture flasks by Accutase treatment (PAA Laboratories, Cölbe, Germany). Cells (0.5×10^6) were then added to the HUVEC monolayer for 30, 60, or 120 minutes. Subsequently, nonadherent tumor cells were washed off using warmed (37°C) M199. The remaining cells were fixed with 1% glutaraldehyde. Adherent tumor cells were counted in five different fields of a defined size ($5 \times 0.25 \text{ mm}^2$) using a phase-contrast microscope, and the mean cellular adhesion rate was calculated.

Attachment to Extracellular Matrix Components

Six-well plates (Falcon Primaria) were coated with collagen G [extracted from calfskin, consisting of 90% collagen type I and 10% collagen type III; diluted to 400 μ g/ml in phosphate-buffered saline (PBS); Seromed, Berlin, Germany] or fibronectin (derived from human plasma, diluted to 50 μ g/ml in PBS; BD Biosciences) overnight. Unspecific cell binding was evaluated using culture plates treated with Poly-D-Lysine (Nunc, Wiesbaden, Germany). Plastic dishes served as the background control. Plates were washed with 1% BSA in PBS to block nonspecific cell adhesion. Tumor cells (0.5×10^6) were then added to each well for 30 minutes. Subsequently, nonadherent tumor cells were washed off, and the remaining adherent cells were fixed with 2% glutaraldehyde and counted under a microscope. The mean cellular adhesion rate, defined by $\text{adherent cells}_{\text{coated well}} - \text{adherent cells}_{\text{background}}$, was calculated from five different observation fields ($5 \times 0.25 \text{ mm}^2$).

Tumor Cell Motility (Chemotaxis)

Serum-induced chemotactic movement was investigated using six-well Transwell chambers (Greiner Bio-One, Frickenhausen, Germany) with 8- μ m pores. RCC cells (0.5×10^6) per milliliter were placed in the upper chamber in serum-free medium. The lower chamber contained 10% serum. After 20-hour incubation, the upper surface of the Transwell membrane was gently wiped with a cotton swab to remove nonmigrating cells. Cells, which had moved to the lower surface of the membrane, were stained using hematoxylin and counted under a microscope. The mean chemotaxis rate was calculated from five different observation fields ($5 \times 0.25 \text{ mm}^2$).

Integrin Surface Expression

RCC cells were detached from their culture flasks by Accutase (PAA Laboratories GmbH, Pasching, Austria) and washed in blocking solution (PBS, 0.5% BSA). The cells were then incubated for 60 minutes at 4°C with phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) directed against the following integrin subtypes: anti- α 1 (mouse IgG1, clone SR84), anti- α 2 (mouse IgG2a, clone 12 F1-H6), anti- α 3 (mouse IgG1, clone C3 II.1), anti- α 4 (mouse IgG1, clone 9 F10), anti- α 5 (mouse IgG1, clone IIA1), anti- α 6 (rat IgG2a, clone GoH3), anti- β 1 (mouse IgG1, clone MAR4), anti- β 3 (mouse IgG1, clone VI-PL2), or anti- β 4 (rat IgG2a; clone 439–9B; all: BD Biosciences). Tumor cell integrin expression was then measured using a FACScan (BD Biosciences; FL-2H (log

channel histogram analysis; 1×10^4 cells per scan) and expressed as mean fluorescence units. A mouse IgG1-PE (MOPC-21) or IgG2a-PE (G155–178; all: BD Biosciences) was used as an isotype control.

Western Blot Analysis

To investigate the integrin protein level in Caki^{res} and Caki^{par} cells, tumor cell lysates were applied to a 7% to 12% polyacrylamide gel and electrophoresed for 90 minutes at 100 V. The protein was then transferred to nitrocellulose membranes (1 hour, 100 V). After blocking with nonfat dry milk for 1 hour, the membranes were incubated overnight with mAbs directed against integrin $\alpha 3$ (rabbit, polyclonal, 1:1000; Chemicon/Millipore, Schwalbach, Germany), integrin $\alpha 5$ (mouse IgG2a, 1:5000, clone 1; BD Biosciences), and integrin $\beta 3$ (mouse IgG1, 1:2500, clone 1; BD Biosciences).

HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG (both: 1:5,000; Upstate Biotechnology, Lake Placid, NY) served as the secondary antibody. The membranes were briefly incubated with ECL detection reagent (ECL; Amersham/GE Healthcare, München, Germany) to visualize the proteins and then analyzed by the Fusion FX7 system (PepLab, Erlangen, Germany). β -Actin (1:1,000; clone AC-15; Sigma-Aldrich, Taufkirchen, Germany) served as the internal control.

Blocking and Knockdown Studies

RCC cells were incubated for 60 minutes with 10 μ g/ml function-blocking anti-integrin $\beta 3$ (clone B3A) or anti-integrin $\alpha 5$ (clone P1D6) mouse mAb (both: Millipore). Control cells were incubated with cell culture medium alone.

Additionally, tumor cells (3×10^5 per well) were transfected with small interfering RNA (siRNA) directed against integrin $\beta 3$ (2 μ M, HS_ITGB3_5 FlexiTube siRNA: NM_000212; Qiagen, Hilden, Germany) or integrin $\alpha 5$ (2 μ M, Hs_ITGA5_5 FlexiTube siRNA: NM_002205; Qiagen) with a siRNA/transfection reagent (HiPerFect Transfection Reagent; Qiagen) ratio of 1:6. Nontreated cells and cells treated with 5 nM control siRNA (AllStars Negative Control siRNA; Qiagen) served as controls. Subsequently, tumor cell adhesion to HUVEC, immobilized collagen, or fibronectin as well as RCC chemotaxis were analyzed as indicated above.

Statistics

All experiments were performed three to six times. Statistical significance was calculated with the Wilcoxon–Mann-Whitney U test. Differences were considered statistically significant at a *P* value less than .05. Inhibitory concentration of 50% (IC₅₀) values were calculated by CalcuSyn software (Biosoft, Cambridge, UK).

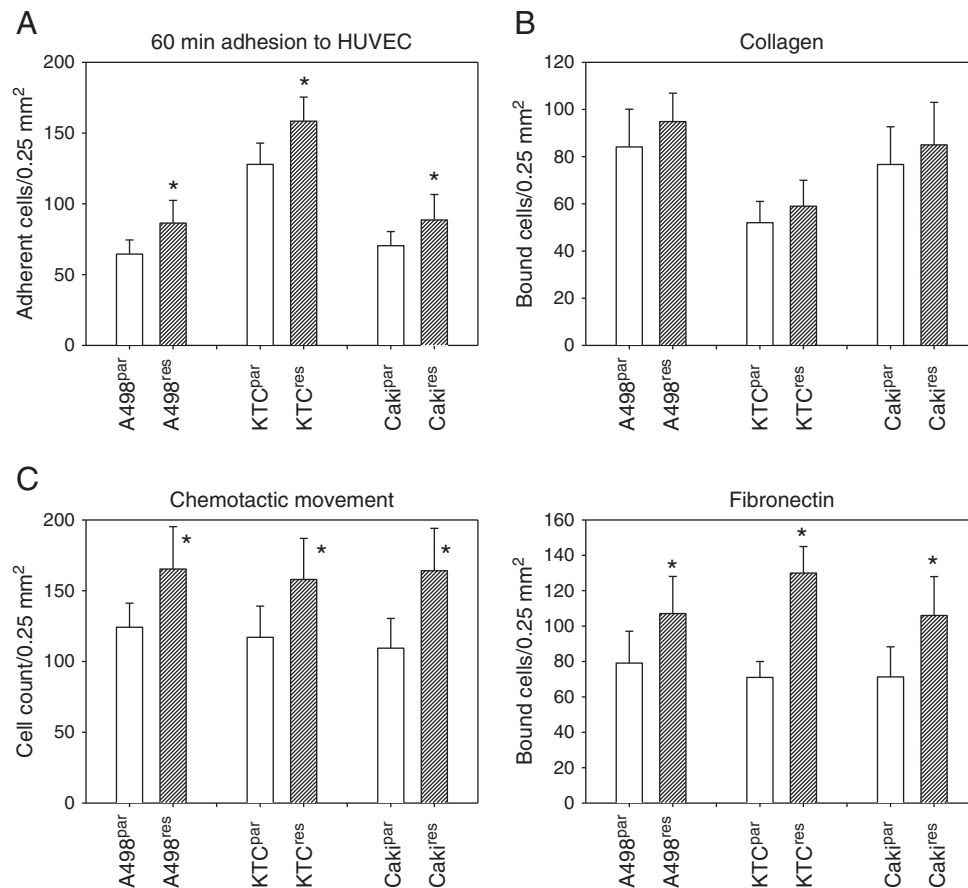


Figure 1. Adhesion and chemotactic behavior of temsirolimus-resistant (res) versus temsirolimus sensitive (par) RCC cells. (A) RCC adhesion to HUVEC after 60 minutes. (B) Adhesion to the extracellular matrix proteins collagen and fibronectin. Resistant (res) or sensitive (par) RCC cells were added to immobilized collagen or fibronectin for 60 minutes, and binding was measured. (C) Chemotactic movement was assessed in a Transwell chamber assay. Tumor cells were seeded in the upper chamber in serum-free medium, and 10% FCS, as the chemoattractant, was placed in the lower well. A to C show means calculated from five counts. Each diagram represents one of six experiments. * indicates significant difference between the resistant and the sensitive tumor cell subline.

Results

Tumor Cell Adhesion and Chemotaxis

IC₅₀ values were given as follows: A498^{par} = 3.32 ± 0.82; A498^{res} = 17.01 ± 0.32; KTC^{par} = 0.49 ± 0.18; KTC^{res} = 29.73 ± 8.76; Caki^{par} = 7.41 ± 3.24; and Caki^{res} = 160.53 ± 46.71 (each: nM/ml). Evaluation of tumor cell endothelial cell interaction revealed that more A498^{res}, KTC^{res}, or Caki^{res} cells adhered to HUVEC than did the respective parental cell lines (Figure 1A). Similar behavior was apparent for the matrix binding assay. Significantly more A498^{res}, KTC^{res}, or Caki^{res} cells bound to immobilized fibronectin (but not to collagen) compared to A498^{par}, KTC^{par}, or Caki^{par} (Figure 1B). Regarding chemotaxis, more A498^{res}, KTC^{res}, or Caki^{res} cells penetrated the Transwell membrane, compared to the parental cell lines (Figure 1C).

Tumor cells were retreated with a therapeutically relevant temsirolimus concentration (10 nM), and the response was analyzed. Drug treatment caused a significant reduction in the number of drug-sensitive cells adhering to HUVEC. This effect was not found in the resistant cell lines (Figure 2A, representative for KTCTL-26). Adhesion of A498^{par}, KTC^{par}, or Caki^{par} to collagen or fibronectin

increased with 10 nM temsirolimus. However, temsirolimus did not elevate A498^{res}, KTC^{res}, or Caki^{res} cell binding to collagen, and the number of bound cells was even diminished in the fibronectin-coated plates, compared to nontreated cells (Figure 2B). Inversely, chemotactic movement of A498^{par}, KTC^{par}, or Caki^{par} was diminished, whereas this was not true with respect to A498^{res}, KTC^{res}, or Caki^{res} cells. Chemotaxis of the resistant cell lines was increased, compared to the controls (Figure 2C).

Because all cell lines responded similarly to temsirolimus, subsequent experiments were limited to the cell line KTCTL-26.

Integrins Are Modified in KTC^{res} Cells

Surface levels of integrin α and β adhesion receptors were analyzed by a FACScan. The integrin subtypes α 3 and β 1 were strongly expressed, and α 1, α 2, α 5, and β 3 were moderately expressed, whereas α 6 and β 4 were only minimally detectable on KTC^{par} cells (Figure 3A). Comparative analysis between KTC^{par} and KTC^{res} cells revealed distinct differences of the integrin expression pattern. The α 3 integrin subtype was slightly elevated, and the β 3 subtype member was strongly enhanced, whereas integrin α 5 was dramatically downregulated on the KTC^{res} cell membrane, compared to KTC^{par}

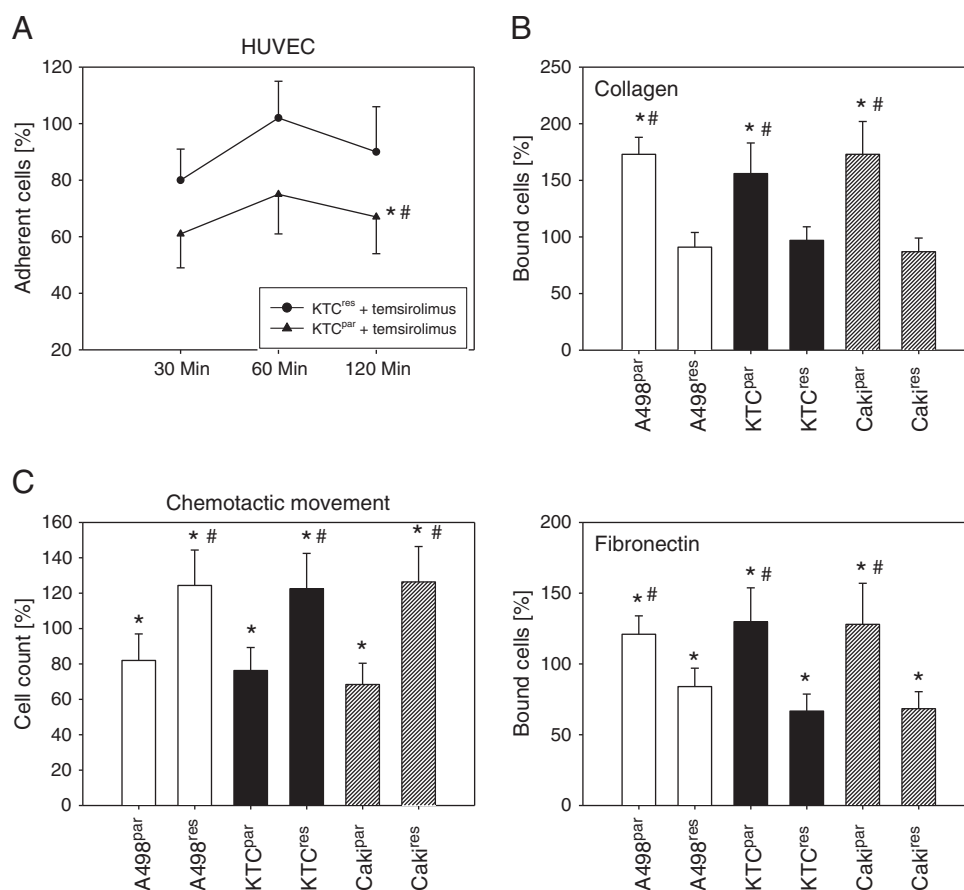


Figure 2. Short-term treatment with low-dosed temsirolimus differentially alters adhesion and migration of resistant and nonresistant RCC cells. The resistant tumor cells were treated with fresh medium (without temsirolimus) for 3 days and then exposed to 10 nM temsirolimus. Medium change followed by 10 nM temsirolimus treatment was also carried out with the drug-sensitive cell lines. A shows time-dependent RCC adhesion to HUVEC (representative for KTC^{par} and KTC^{res}), B shows the collagen and fibronectin binding assay, and C demonstrates chemotactic behavior of the tumor cell sublines evaluated by the Transwell chamber assay (60-minute values). Percentage is related to controls not treated with 10 nM temsirolimus, set to 100%. Each diagram represents one of six experiments. * indicates significant difference to the temsirolimus-free control. # indicates significant difference between the resistant and the sensitive RCC cell line.

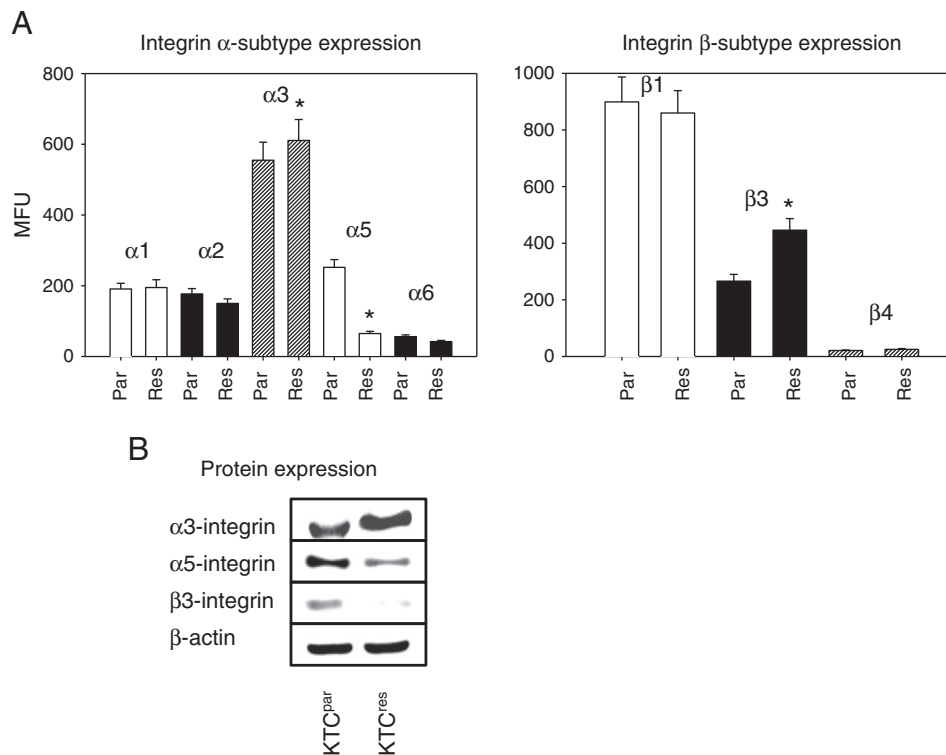


Figure 3. Integrin α and β expression in KTC^{par} and KTC^{res} cells. A depicts the FACS results given as mean fluorescence units. Tumor cells were washed in blocking solution and then stained with specific mAbs as listed in [Materials and Methods](#) section. To evaluate background staining of PE-conjugated antibodies, goat anti-mouse IgG1-PE or IgG2a-PE was used. Fluorescence was measured using a FACScan flow cytometer. * indicates significant difference between the resistant and the sensitive tumor subline. (B) Modification of intracellular integrin protein level. Tumor cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted on the membrane incubated with appropriate mAbs. β -Actin served as the internal control. The figure shows one representative from three separate experiments.

(Figure 3A). No significant differences were seen with respect to $\alpha 1$, $\alpha 2$, $\alpha 6$, $\beta 1$, and $\beta 4$ integrins.

According to the flow cytometry data, integrin $\alpha 3$ protein was elevated, and $\alpha 5$ protein diminished in KTC^{res} compared to KTC^{par} cells. The $\beta 3$ integrin protein content was lowered in the drug-resistant tumor cells, compared to the drug-sensitive cells (Figure 3B). This finding contrasts with the FACS data demonstrating $\beta 3$ up-regulation under resistance.

Blocking Studies

Blocking studies were carried out to investigate the functionality of $\alpha 5$ and $\beta 3$ integrins, which were most strongly altered in KTC^{res} compared to KTC^{par} cells. Blockade of $\alpha 5$ on the cell surface by a mAb led to significant enhancement of tumor cell adhesion to HUVEC (Figure 4A). The effect was more pronounced in KTC^{res} compared to KTC^{par} cells. Integrin $\alpha 5$ blockade correlated with an increased binding of KTC^{par} but with a decreased binding of KTC^{res} cells to collagen. Inversely, $\alpha 5$ blocking reduced KTC^{par} binding but elevated KTC^{res} binding to fibronectin (Figure 4B). The motile behavior of KTC^{par} and KTC^{res} cells was influenced by $\alpha 5$, in as much as receptor blockade triggered a distinct (KTC^{par}) or moderate (KTC^{res}) loss of chemotactic activity (Figure 4C).

Knocking down the intracellular integrin $\alpha 5$ content by siRNA (Western blot controls are shown in Figure 5A) diminished the interaction of both KTC^{par} and KTC^{res} cells with endothelium as well as with the matrix proteins collagen and fibronectin, compared to

untreated controls (Figure 5B). Chemotaxis was not influenced (Figure 5C) with no differences between KTC^{par} and KTC^{res} cells.

Experiments were repeated using a $\beta 3$ integrin–blocking antibody. In doing so, tumor cell adhesion to HUVEC was significantly lowered, compared to the untreated controls (Figure 6A), whereby no quantitative differences were seen between KTC^{par} compared to KTC^{res} cells. A similar phenomenon was induced in the collagen and fibronectin binding assay (Figure 6B). Integrin $\beta 3$ blockade also prevented integrin migration through the transmembrane pores. However, the number of migrating KTC^{res} cells was reduced to a higher extent than for KTC^{par} cells (Figure 6C).

Chemotaxis of the KTCTL-26 tumor cells whose $\beta 3$ integrin had been knocked down was additionally investigated. This led to a significant reduction of KTC^{par} migration, whereas motility of KTC^{res} cells was upregulated (Figure 7).

Discussion

Evidence is presented here showing that temsirolimus resistance is coupled to enhanced RCC cell adhesion to vascular endothelium and to extracellular matrix components, accompanied by increased chemotactic activity. Transendothelial migration and motile spreading are critical steps in tumor dissemination and progression [11]. With this in mind, it is concluded that long-term exposure to temsirolimus may alter the invasive behavior, creating highly aggressive RCC cells. Interaction of the drug-resistant tumor cells with fibronectin, but not with collagen, was distinctly escalated. This

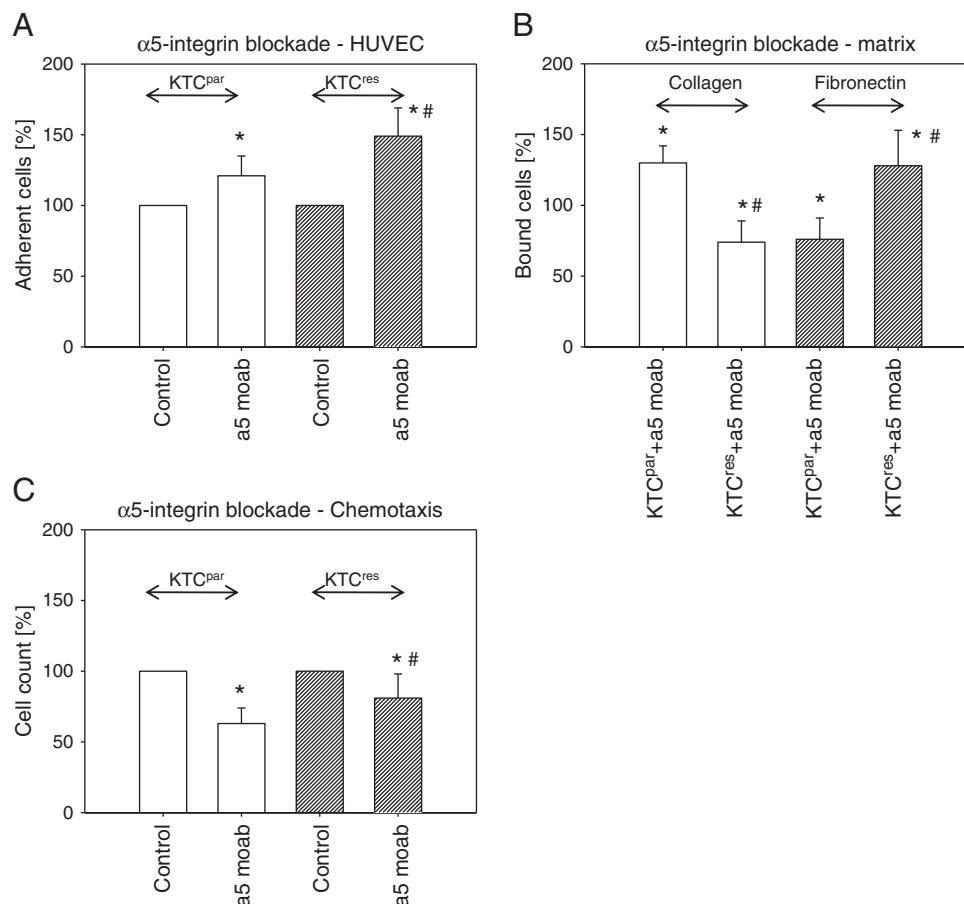


Figure 4. Influence of integrin $\alpha 5$ blockade on tumor cell adhesion to HUVEC (A), binding to immobilized collagen or fibronectin (B) and on chemotaxis (C). KTC^{par} or KTC^{res} cells were preincubated for 60 minutes with a function-blocking anti-integrin $\alpha 5$ mAb. Controls were untreated. Values are percentage difference to the 100% control. * indicates significant difference between the RCC control subline and the RCC subline treated with the function-blocking antibody. # indicates significant difference between KTC^{par} and KTC^{res} cells whose integrin subtype was blocked.

is clinically important because Knowles et al. recently demonstrated that fibronectin is the dominant factor promoting lung metastasis of RCC [9]. In good accordance, comparative analysis of primary and metastatic RCC cells displayed an increased capacity of the metastatic subtype to strongly attach to fibronectin, whereas cross talk with collagen was only of minor relevance [12]. Therefore, long-term use of temsirolimus may change the RCC phenotype, driving the fibronectin-dependent invasion process forward. This hypothesis is supported by the present investigation, whereby the tumor cells exposed to a therapeutically relevant temsirolimus dosage exhibit altered binding of the resistant RCC cells only to fibronectin. In contrast, both collagen and fibronectin binding to temsirolimus-sensitive RCC cells was altered, with collagen-dependent adhesion being more modified than fibronectin-dependent adhesion.

In drug-resistant prostate cancer cells, an inverse correlation between adhesion and migration properties has been reported [10]. Although the complex scenario of metastatic colonization is not fully understood, there is no doubt that loosening tumor-matrix contact is a necessary prerequisite to allow motile crawling into the surrounding tissue [11,13]. It is therefore not surprising that the basal attachment rate of the drug-resistant RCC to fibronectin was higher than the one of the drug-sensitive cells but was then diminished under short-term retreatment with low-dosed temsirolimus. At the same time, the

resistant tumor increased its motile activity, indicating a behavioral switch from being adhesive to becoming invasive. Such a two-step process could play a role during resistance acquisition. The first step may involve facilitating fibronectin instead of collagen-dependent tumor-matrix interaction, and the second step may involve a conversion from an adhesive to an invasive phenotype. Isogai et al. have defined a critical role of fibronectin in providing a cellular switch between stationary and migratory cell phases [14], which would support this hypothesis.

The mechanism responsible for increased motile behavior indicates modification of the integrin expression pattern. The $\alpha 5$ integrin subtype was drastically downregulated on the surface membrane as well as within the cytoplasm of drug-resistant RCC cells. Detailed information on the role of integrin $\alpha 5$ is sparse. Studies on A498 cells have revealed that $\alpha 5$ regulates tumor binding to fibronectin [15] and controls chemotaxis [16]. This corroborates the present data demonstrating diminished contact of KTC^{par} cells to fibronectin and reduced migratory potential once $\alpha 5$ surface expression has been blocked. Nevertheless, the situation appears more complex than initially thought because KTC^{res} behaved differently under $\alpha 5$ blockade, compared to the KTC^{par} cells. The pronounced effect of $\alpha 5$ on KTC^{par} chemotaxis was not seen with KTC^{res}. Most notably, attachment of KTC^{res} to collagen was inhibited, and attachment to

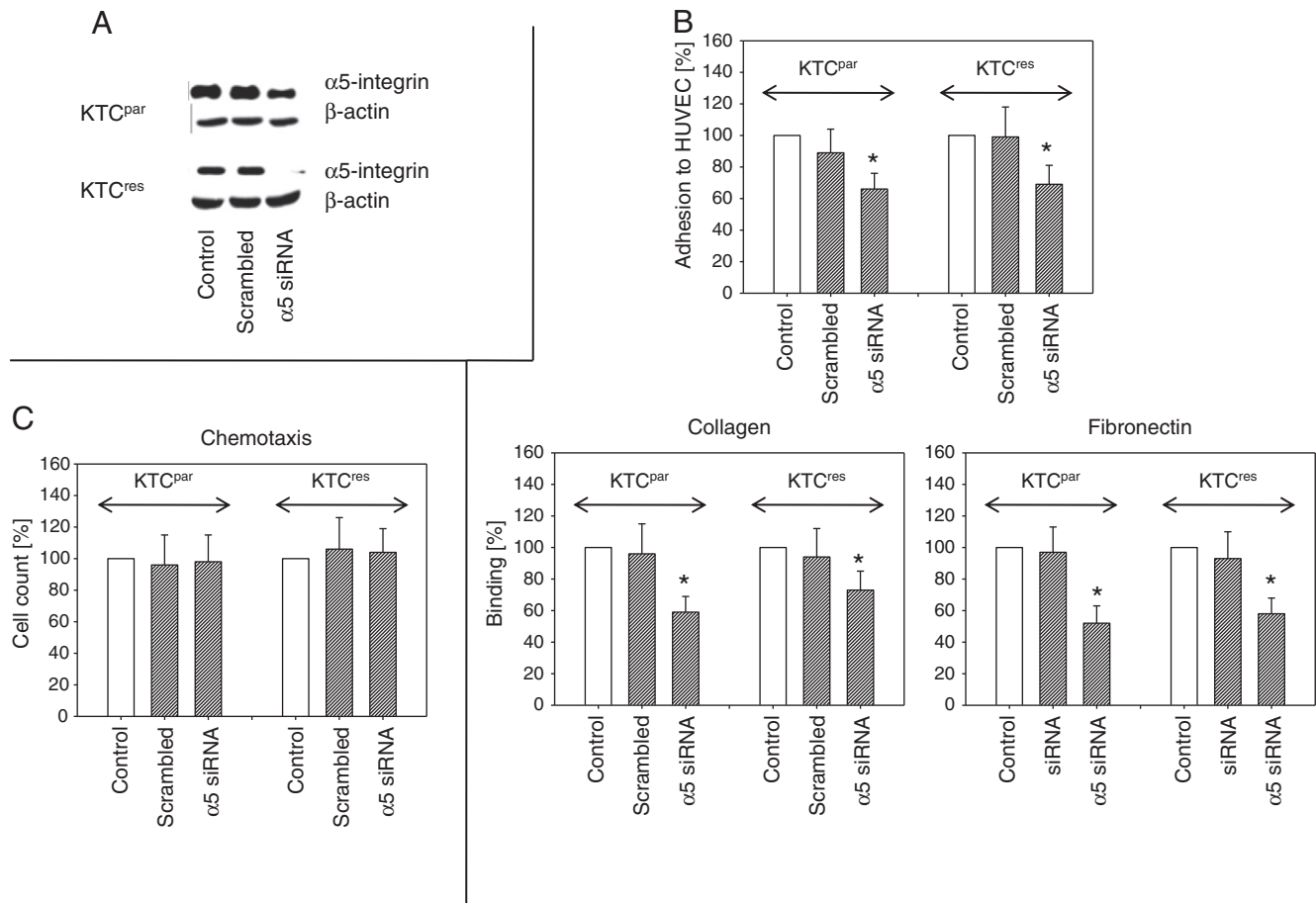


Figure 5. Influence of integrin $\alpha 5$ knockdown on tumor cell adhesion and motility. KTC^{par} and KTC^{res} cells were transfected with $\alpha 5$ siRNA or scrambled siRNA, and knockdown was controlled by Western blot analysis (A). Cells were then subjected to the HUVEC, collagen, and fibronectin adhesion assay (B) or to the chemotaxis assay (C). Values are shown as percentage difference to controls, set to 100%. Each diagram represents one of six experiments. * indicates significant difference to the siRNA-free control.

fibronectin was enhanced, whereas KTC^{par} responded to $\alpha 5$ blockade in the opposite way. Obviously, the relevance of the $\alpha 5$ receptor for KTC^{par} is not transferable to the KTC^{res} cells. On the basis of the present investigation, a functional switch of the $\alpha 5$ integrin during resistance development is proposed, in as much as this integrin subtype may no longer control the tumor cell's motility but rather shifts the tumor cell's binding affinity from collagen to fibronectin.

Change of the integrin function seems also to be reflected in the endothelial cell binding assay, because blocking $\alpha 5$ distinctly enhanced KTC^{res} but only slightly elevated KTC^{par} adhesion to HUVEC. Apart from hypothesizing differences in linking $\alpha 5$ to a (still unknown) endothelial cell receptor, HUVECs are predestined to deposit collagen and fibronectin on their surface [17]. Given that matrix proteins serve as the specific integrin ligands [18], $\alpha 5$ may promote KTC^{res} accumulation along the endothelial fibronectin fibers. However, involvement of $\alpha 5$ in KTC^{par} adhesion includes both collagen and fibronectin with a reciprocal relationship. Consequently, only mild alterations of KTC^{par} binding to HUVEC in the presence of the $\alpha 5$ antibody can be expected.

The different effects of $\alpha 5$ on temsirolimus-responsive compared to temsirolimus-nonresponsive RCC cells were not inducible by knocking down the $\alpha 5$ protein content. Therefore, it seems likely that the $\alpha 5$ surface receptor is the relevant factor responsible for modifying

tumor cell adhesion. Loss of $\alpha 5$ together with a functional switch has recently been observed in everolimus-resistant prostate cancer [10]. Presumably, the role of $\alpha 5$ seen in drug-resistant RCC is not restricted to this tumor entity. Nevertheless, further experiments on different tumor types are required to investigate whether the role of the $\alpha 5$ integrin in mTOR inhibitor-based regimen can be generalized.

Blocking the $\beta 3$ integrin surface molecule diminished RCC chemotaxis with KTC^{res} being more influenced than KTC^{par} cells. Considering the strong elevation of this receptor on the KTC^{res} membrane, it seems likely that membranous $\beta 3$ is, at least partially, responsible for the enhanced migratory activity seen in the resistant RCC tumor cells. Because the $\beta 3$ level inversely correlates with the KTC^{res}-binding activity, receptor enhancement might also be responsible for fibronectin detachment occurring during temsirolimus retreatment. Although no data from others are available regarding this issue, $\beta 3$ integrin expression correlated well with the invasive potential of lung [19], breast [20], and colorectal [21] carcinomas as well as of melanoma cells [22]. Classification of 45 human tumor cell lines derived from various tissues has revealed cell surface localization of $\beta 3$ integrin receptors exclusively in cell lines crossing an endothelial cell barrier [23]. Hence, upregulating $\beta 3$ along the RCC cell surface under chronic temsirolimus treatment might

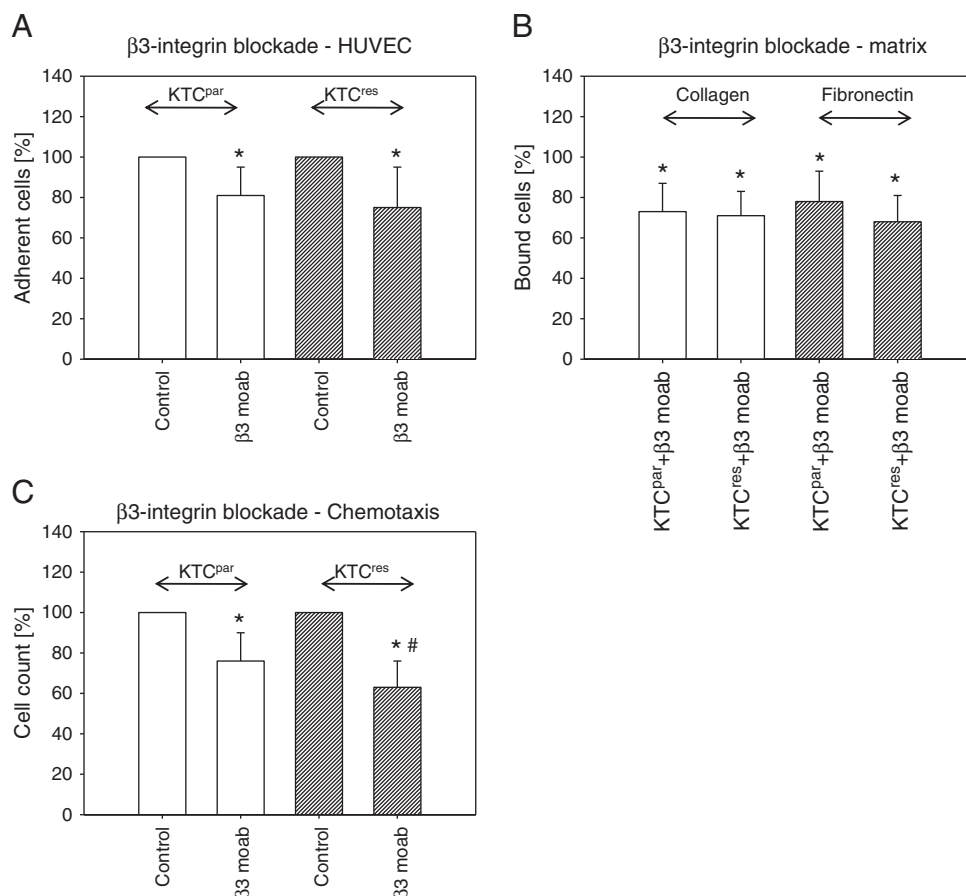


Figure 6. Integrin $\beta 3$ blocking studies. KTC^{par} or KTC^{res} cells were preincubated for 60 minutes with a function-blocking anti-integrin $\beta 3$ mAb, and tumor cell adhesion to HUVEC (A), tumor cell binding to immobilized collagen or fibronectin (B), and chemotactic motility (C) were evaluated. Controls remained untreated. Each experiment was repeated five times. Mean values from one representative test are shown as percentage difference to the 100% control. * indicates significant difference between the control RCC and RCC cells treated with the function-blocking antibody. # indicates significant difference between KTC^{par} and KTC^{res} cells whose integrin subtype was blocked.

entail the severe risk of accelerating metastatic tumor spreading. The development of undesired countermechanisms caused by an mTOR inhibitor regimen should therefore be carefully controlled. Whether

the evaluation of the $\beta 3$ expression level in patients with cancer might be an innovative tool to monitor drug response is the subject of ongoing studies.

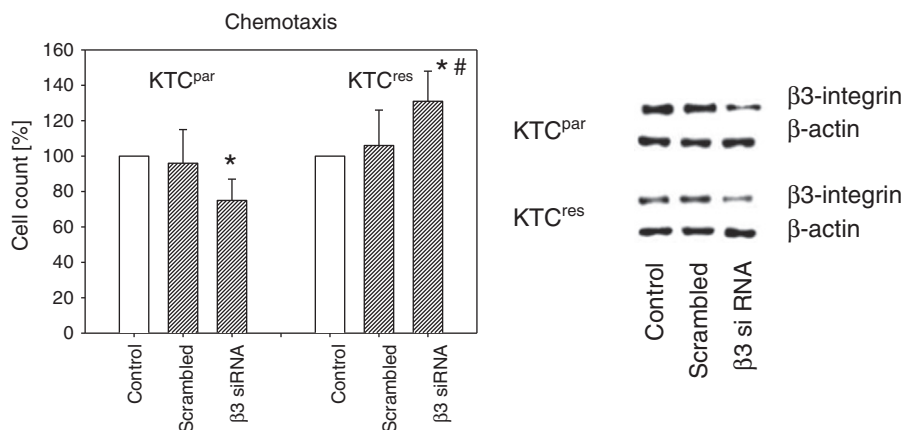


Figure 7. Knockdown of integrin $\beta 3$ differentially alters chemotaxis of KTC^{par} and KTC^{res} cells. Tumor cells were transfected with $\beta 3$ siRNA or scrambled siRNA. Controls remained untreated. Efficacy of receptor knockdown was evaluated by Western blot analysis (right panel). Subsequently, cells were subjected to the chemotaxis assay. Values are shown as percentage difference to the 100% control. * indicates significant difference to the untreated control. # indicates significant difference between KTC^{par} and KTC^{res} cells whose integrin subtype was knocked down.

Diminishing the cytoplasmic integrin $\beta 3$ pool by siRNA knock-down differentially altered the chemotactic activity of KTC^{PAR}, compared to KTC^{RES} cells. Indeed, loss of this protein significantly lowered KTC^{PAR} but increased KTC^{RES} chemotaxis. Reduction of the intracellular $\beta 3$ content, becoming overt during resistance acquisition, is therefore a signal that RCC cells undergo conversion toward a highly motile phenotype. Because loss of cytoplasmic $\beta 3$ is paralleled by enrichment of this receptor on the cell membrane, it may be assumed that $\beta 3$ is translocated from the intracellular space to the outer cell surface. The same reciprocal distribution of $\beta 3$ has been observed in Caki^{RES} and A498^{RES}, indicating a common mechanism of redistribution (data not shown). Indeed, trafficking integrins has been documented to play an important role in regulating invasive migration [24]. Because both reduced intracellular $\beta 3$ as well as enhanced $\beta 3$ surface expressions separately promote RCC migration, dynamic receptor trafficking may further encourage metastatic dissemination.

The different chemotactic response of sensitive and resistant tumor cells in the presence of $\beta 3$ siRNA points to a functional switch of the $\beta 3$ integrin, as has already been postulated with the $\alpha 5$ molecule. The $\alpha 5$ subtype forces fibronectin-RCC interaction, possibly as a prerequisite for initiating invasion, whereas $\beta 3$ drives the invasion process forward. The molecular background underlying the functional switch of $\alpha 5$ and $\beta 3$ is still a matter of debate. Flevaris et al. indicate that the $\beta 3$ integrin may inhibit the RhoA signaling pathway, subsequently inducing the conversion from adhesion to migration [25]. This is important because everolimus has recently been demonstrated to prevent migration of drug-sensitive cells by RhoA activation [26] and, consequently, activates the motile machinery by diminishing RhoA. Although how RhoA contributes to the conflicting processes of stable adhesion and motile spreading is not well understood, it is plausible to assume modification of the $\beta 3$ -RhoA cross-communication in RCC cells during resistance development. Whether this speculation is transferable to the $\alpha 5$ integrin is not yet clear. However, a link from $\alpha 5$ to RhoA has recently been observed in melanoma cells [27], making the existence of an $\alpha 5$ -RhoA axis in RCC cells likely.

This study shows that temsirolimus resistance drives RCC cancer cells to become highly motile. The process is accompanied by two different processes: 1) quantitative alteration of the integrin $\alpha 5$ and $\beta 3$ expression and 2) functional change of the integrin molecules, forcing the switch from adhesion to migration. Analysis of the integrin-driven alterations of the intracellular signaling machine is the subject of ongoing experiments.

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