The integrin alphav beta3 increases cellular stiffness and cytoskeletal remodeling dynamics to facilitate cancer cell invasion

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The integrin alphav beta3 increases cellular stiffness and cytoskeletal remodeling dynamics to facilitate cancer cell invasion

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Abstract. The process of cancer cell invasion through the extracellular matrix (ECM) of connective tissue plays a prominent role in tumor progression and is based fundamentally on biomechanics. Cancer cell invasion usually requires cell adhesion to the ECM through the cell-matrix adhesion receptors integrins. The expression of the $\alpha v \beta 3$ integrin is increased in several tumor types and is consistently associated with increased metastasis formation in patients. The hypothesis was that the $\alpha v \beta 3$ integrin expression increases the invasiveness of cancer cells through increased cellular stiffness, and increased cytoskeletal remodeling dynamics. Here, the invasion of cancer cells with different $\alpha v \beta 3$ integrin expression levels into dense three-dimensional (3D) ECMs has been studied. Using a cell sorter, two subcell lines expressing either high or low amounts of $\alpha v \beta 3$ integrins ($\alpha v \beta 3^{\text{high}}$ or $\alpha v \beta 3^{\text{low}}$ cells, respectively) have been isolated from parental MDA-MB-231 breast cancer cells. $\alpha v \beta 3^{\text{high}}$ cells showed a threefold increased cell invasion compared to $\alpha v \beta 3^{\text{low}}$ cells. Similar results were obtained for A375 melanoma, 786-O kidney and T24 bladder carcinoma cells, and cells in which the $\beta 3$ integrin subunit was knocked down using specific siRNA. To investigate whether contractile forces are essential for $\alpha v \beta 3$ integrin-mediated increased cellular stiffness and subsequently enhanced...
cancer cell invasion, invasion assays were performed in the presence of myosin light chain kinase inhibitor ML-7 and Rho kinase inhibitor Y27632. Indeed, cancer cell invasiveness was reduced after addition of ML-7 and Y27632 in $\alpha\nu\beta^3_{\text{high}}$ cells but not in $\alpha\nu\beta^3_{\text{low}}$ cells. Moreover, after addition of the contractility enhancer calyculin A, an increase in pre-stress in $\alpha\nu\beta^3_{\text{low}}$ cells was observed, which enhanced cellular invasiveness. In addition, inhibition of the Src kinase, STAT3 or Rac1 strongly reduced the invasiveness of $\alpha\nu\beta^3_{\text{high}}$ cells, whereas the invasiveness of $\beta^3$ specific knock-down cells and $\alpha\nu\beta^3_{\text{low}}$ cells was not altered. In summary, these results suggest that the $\alpha\nu\beta^3$ integrin enhances cancer cell invasion through increased cellular stiffness and enhanced cytoskeletal remodeling dynamics, which enables the cells to generate and transmit contractile forces to overcome the steric hindrance of 3D ECMs.

Online supplementary data available from stacks.iop.org/NJP/15/015003/mmedia

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1. Introduction

In malignant tumor progression, the invasion of cancer cells through the extracellular matrix (ECM) of connective tissue plays a prominent role for metastasis formation [1–3]. This process of metastasis formation consists of multiple steps involving the detachment of invasive cells from the primary tumor mass, cancer cell migration into the surrounding tissue and transmigration (intravasation) through blood or lymph vessels possibly with the help of macrophages and their extravasation into the target organs [4–7].

The metastatic process requires dynamic biomechanical alterations of the homotypic intercellular interactions of cancer cells and heterotypic intercellular interactions of cancer cells and other cell types. Additionally, these interactions of cancer cells are modulated by components of the ECM [8]. Indeed, several matrix-cell surface adhesion receptors such as cadherins and integrins have been identified to act as either as negative or positive regulators of cancer cell invasion and metastasis [9–12].

Cell-surface expressed integrins couple the ECM to cytoskeletal actin microfilaments. This coupling is an initiation signal for focal adhesion proteins to cluster underneath the cell membrane to focal contacts and focal adhesions [13]. Thus, focal adhesion proteins such as vinculin and focal adhesion kinase are critical for the process of cellular motility into ECM [14–17]. In focal adhesions, cell surface transmembrane receptors of the integrin family promote the extracellular interaction with ECM ligands and couple the microenvironment with the cytoskeletal actin–microfilament system [18–19]. Indeed, focal adhesion sites, or rather their components, not only function to facilitate cell-matrix adhesion or cell motility, but also serve as a membrane anchor for the cytoskeleton and transduction of biochemical and biomechanical signals from the ECM to the cell and vice versa [18–22].

Moreover, the β3 integrin subunit is a component of the αvβ3 integrin heterodimer, which plays a prominent role in blood vessel formation/angiogenesis and hence promotes tumor growth [23–26].

Additionally, the β3 integrin subunit is required for the transendothelial migration of WM239 melanoma, PC-3 prostate and breast carcinoma cells [27–29]. In several malignancies, cancer cells express αvβ3 integrins which correlate with tumor progression in melanomas, gliomas, ovarian and breast cancer [30–34]. In particular, in breast cancer, αvβ3 characterizes the metastatic phenotype and hence, the expression of this integrin-type is increased in invasive tumors and found frequently in distant metastases [35–36].

As most of the previous studies on cancer cell migration examined only individual cell lines from specific tumor types, the results are still difficult to generalize. In particular, it is unknown whether molecular determinants of cancer cell migration exist which are common to different tumor cell types. In order to approach this problem the study analyzes cancer cell lines from breast, skin, kidney and bladder for their ability to invade into dense three-dimensional extracellular matrices (3D ECMs) in the absence and presence of high and low αvβ3 integrin expression in vitro.

The aim of this study was to analyze the role of the αvβ3 integrins for cancer cell invasion under controlled in vitro conditions, and to characterize the biomechanical invasion strategy that is activated by αvβ3 integrins. The 2.4 mg ml⁻¹ synthetic 3D ECMs with subcellular-sized pores were used for the invasion assays [37–39]. The invasiveness of cancer cells in such a system depends basically on a balanced regulation of biomechanical processes including cell adhesion and de-adhesion [40], cytoskeletal remodeling [41], protrusive force.
generation [40, 42] and matrix properties such as stiffness, pore size, ECM protein composition and enzymatic degradation [43].

In particular, it was investigated whether the expression of αvβ3 integrins facilitates 3D ECM invasion through enhanced cellular stiffness and increased cytoskeletal remodeling dynamics, as needed to overcome the steric hindrance of dense 3D ECMs. To address this issue, cancer cells with high and low endogenous αvβ3 integrin expression were isolated using flow cytometry and used for invasion and biomechanical analysis. αvβ3<sup>high</sup> cancer cells displayed increased invasiveness into 3D ECMs compared to αvβ3<sup>low</sup>. Consistently, knock-down of the β3 integrin subunit in αvβ3<sup>high</sup> cells decreased cancer cell invasion into 3D ECMs. The αvβ3 integrin specificity of the invasion-enhancing effect was analyzed systematically by measuring cell cytoskeletal remodeling and cellular stiffness. Moreover, it was explored whether αvβ3 integrin mediated invasiveness in 3D ECMs is ROCK, MLCK, Src, STAT3 and Rac1 signaling dependent. Taken together, this study reveals that αvβ3 integrins contribute substantially to the invasiveness of cancer cells by promoting the signaling for the transmission and generation of contractile forces and hence, increasing cellular stiffness.

2. Results

2.1. High αvβ3 integrin expression leads to enhanced cell invasion

To investigate the effect of the αvβ3 integrin expression on invasion, two subcell lines were isolated from the parental breast cancer line MDA-MB-231 that expressed either high or low amounts of αvβ3 integrins on their cell surface (figure 1(A)). In the following, these subcell lines are referred to as αvβ3<sup>low</sup> and αvβ3<sup>high</sup> cells. Between these subcell lines, the difference in the expression of αvβ3 integrin was 46-fold, which is shown as mean fluorescence intensities (MFIs) (figure 1(A)). Using cytofluorometry, the αvβ3 integrin expression levels were confirmed to be stable during culture for more than 50 passages (data not shown). All experiments in this study were performed on cells derived from a single isolation that had been obtained by cell sorting with respect to αvβ3 integrin subunit expression. In independent experiments, the sorting of parental cells in total three times over the course of 2 years was repeated, and each time stable αvβ3<sup>low</sup> and αvβ3<sup>high</sup> phenotypes with similarly high differences in the invasion behavior were established (data not shown). This finding confirms that the αvβ3<sup>low</sup> and αvβ3<sup>high</sup> phenotypes can be obtained reproducibly. In addition, the expression profiles of other relevant integrins such as α1, α2, α5 and β1 integrin subunits were analyzed on the cell surface of αvβ3<sup>low</sup> and αvβ3<sup>high</sup> cells as well as parental MDA-MB-231 cells (figure S1, see the supplementary data, available from stacks.iop.org/NJP/15/015003/mmedia). Between αvβ3<sup>low</sup> and αvβ3<sup>high</sup> cells there was no difference in integrin cell surface expression observed in all other integrins tested, whereas the parental MDA-MB-231 showed significantly increased levels of α5 integrin subunit expression compared to both αvβ3<sup>low</sup> and αvβ3<sup>high</sup> cells (figure S1). The expression of αvβ3 integrin on the cell surface of parental MDA-MB-231 is between the two low and high αvβ3 integrin subtypes (figure S2).

The percentage of cells that were able to invade into a 3D ECM significantly was higher for αvβ3<sup>high</sup> than for αvβ3<sup>low</sup> cells (figure 1(B)). In addition, the invasion profile (cumulative probability) of the invasive cells showed that αvβ3<sup>high</sup> cells invaded deeper into the 3D ECM (figure 1(C)). To investigate whether the effect of the αvβ3 expression on invasiveness is cancer cell-type specific, αvβ3<sup>high</sup> and αvβ3<sup>low</sup> cells were isolated from A375 melanoma cells (17-fold
Figure 1. Effect of αvβ3 expression on cancer cells invasion. (A) αvβ3 integrin expression of αvβ3low and αvβ3high cells derived parental MDA-MB-231 breast cancer cells. In each histogram, left curves are isotype controls and filled gray curves show integrin expression. One representative experiment out of at least three is shown. The bar graphs contain MFI (mean ± SD) values (n = 3). ** p < 0.01. (B) A higher percentage of αvβ3high cells (black) invaded into 3D ECMs compared to αvβ3low cells (gray) after 3 days. *** p < 0.001. (C) Invasion profiles showed that αvβ3high cells migrated deeper into 3D collagen matrices compared to αvβ3low cells. (D) αvβ3 integrin expression of 375αvβ3low and 375αvβ3high cells derived parental A375 melanoma cells. In each histogram, left curves are isotype controls and filled gray curves show integrin expression. One representative experiment out of at least three is shown. The bar graphs contain MFI (mean ± SD) values (n = 3). ** p < 0.01. (E) A higher percentage of 375αvβ3low cells (black) invaded into 3D ECMs compared to 375αvβ3low cells (gray) after 3 days. *** p < 0.001. (F) Invasion profiles showed that 375αvβ3high cells (black) invaded into 3D ECMs compared to 375αvβ3low cells (gray) after 3 days. *** p < 0.001. (G) αvβ3 integrin expression of 786αvβ3low and 786αvβ3high cells derived parental 786-O kidney cancer cells. In each histogram, left curves are isotype controls and filled gray curves show integrin expression. One representative
difference between $\alpha\nu\beta^3_{\text{high}}$ and $\alpha\nu\beta^3_{\text{low}}$, figure 1(D)), 786-O human kidney carcinoma cells (8-fold difference between $\alpha\nu\beta^3_{\text{high}}$ and $\alpha\nu\beta^3_{\text{low}}$, figure 1(G)) as well as $\alpha\nu\beta^3_{\text{high}}$ and $\alpha\nu\beta^3_{\text{low}}$ cells from T24 bladder carcinoma cells (6-fold difference between $\alpha\nu\beta^3_{\text{high}}$ and $\alpha\nu\beta^3_{\text{low}}$, figure 1(J)). The cell invasiveness of $\alpha\nu\beta^3_{\text{high}}$ cells derived from A375, 786-O and T24 cells was higher than that of $\alpha\nu\beta^3_{\text{low}}$ cells indicated by increased numbers of invasive cells (figures 1(E), (H) and (K)) and the invasion profiles (figures 1(F), (J) and (L)) showing that the $\alpha\nu\beta^3_{\text{high}}$ cells invaded deeper into 3D ECMs. These results confirm that the cell invasiveness increases with integrin $\alpha\nu\beta^3$ expression levels in several cancer cell-types.

2.2. High $\alpha\nu\beta^3$ integrin expression leads to reduced cell motility in two dimensions (2D)

To investigate the motility of the $\alpha\nu\beta^3$ integrin expression on cell motility in a second alternative approach to cross-validate the invasion results in 3D and to compare the results with well-known two-dimensional (2D) migration assays, the movement of the subcell lines $\alpha\nu\beta^3_{\text{low}}$ and $\alpha\nu\beta^3_{\text{high}}$ cancer cells were analyzed after 8 h of cell adhesion on 50 $\mu$g ml$^{-1}$ fibronectin-coated glass slides for 2–6 h in a 2D microenvironment. The $\alpha\nu\beta^3_{\text{low}}$ cells with reduced $\alpha\nu\beta^3$ integrin expression showed significantly faster migration speeds (figure 2(A)) and reduced migration persistence of movement (figure 2(B)) compared to the $\alpha\nu\beta^3_{\text{high}}$ cells with high $\alpha\nu\beta^3$ integrin expression. As the results of 2D migration assays in this study and in other studies [44] have been reported to be contrary to the results obtained using a 3D microenvironment, the invasiveness of the subcell lines was analyzed in the following using a 3D ECM migration assay. The latter method of investigating cell motility in a tissue microenvironment seems to more reliable and mimics the tumor microenvironment better than a planar substrate, on which the surfaces of cells do not have contact with the substrate/microenvironment in all directions.

2.3. $\beta^3$ integrin subunit knock-down decreases invasiveness into three-dimensional extracellular matrices (3D-ECMs)

To investigate the effect of $\beta^3$ integrin subunit on cell invasion, the $\beta^3$ integrin subunit was knocked-down in $\alpha\nu\beta^3_{\text{high}}$ cells derived from parental MDA-MB-231 breast cancer cells by

using specific β3 siRNA. Typically representative invasive cells of αvβ3high cells treated with control siRNA (left) or specific β3 siRNA (β3-1 (middle) and β3-2 (right)) are shown in figure 3(A). Specific knock-down of β3 in αvβ3high cells was over 98% with residual of 1.6% ± 0.29(n = 3) expression of the αvβ3 integrin for siβ3-1 and was over 95% with residual expression of 4.4% ± 0.33(n = 3) for siβ3-2 after 2 days (figure 3(B)). The percentage of cells that were able to invade into a 3D collagen matrix was higher for β3 expressing cells (control siRNA treated cells) compared to β3 knock-down cells (β3 siRNA treated cells; two different specific β3 siRNAs: siβ3-1 and siβ3-2) (figure 3(C)). In addition, the invasion profile (cumulative probability) of the invasive cells showed that control siRNA treated cells invaded deeper into the ECM compared to β3 knock-down cells (figure 3(D)). The invasion profiles of β3 knock-down cells reveal that these cells only invade less than 50 μm (figure 3(D)) and their average invasion depth was reduced from 173.56 ± 5.6 (n = 356) to 15.4 ± 1.4(n = 111) (figure 3(E)). These results indicate that β3 integrin subunit expression leads to enhanced cancer cell invasion in 3D collagen matrices. In addition, the parental MDA-MB-231 cells have also been treated with control siRNA and specific β3 integrin (siβ3-1) siRNA and analyzed for their invasiveness into 3D ECMs (figure S3(A), see the supplementary data, available from stacks.iop.org/NJP/15/015003/mmedia). The knock-down efficiency was at least over 90%(n = 4). Indeed, the percentage of invasive cells (figure S3(B)) and their invasion depths as shown in the invasion profiles is significantly reduced in β3 integrin knock-down cells compared to control siRNA treated wildtype cells (figure S3(C)). These results indicate that also parental MDA-MB-231 cells can migrate via an αvβ3 integrin facilitated pathway into dense 3D ECMs.

2.4. Effect of the αvβ3 expression on the stiffness of cancer cells

Several mechanical properties of cancer cells can determine the efficiency of cancer cell invasion into dense 3D ECMs and hence support metastasis formation. Among them are cellular stiffness, which may determine contractile force transmission and generation, as well as cytoskeletal remodeling dynamics, which reorganizes the cell’s acto-myosin cytoskeleton.
Figure 3. Knock-down of the β3 integrin subunit reduced the invasion-enhancing effect of the αvβ3 integrin into dense 3D-ECMs. (A) 183 μm deep invaded αvβ3high cell after treatment with control siRNA (left), 41 μm deep invaded αvβ3high cells after treatment with β3 integrin subunit specific siRNA (siβ3-1; middle) and 36 μm deep invaded αvβ3high cell after treatment with another β3 integrin subunit specific siRNA (siβ3-2; right). (B) αvβ3 integrin expression of αvβ3high cells treated with control (left) or two β3 specific siRNA siβ3-1 (middle) and siβ3-2 (right) for 2 days. In each histogram, left curves are isotype controls and filled gray curves show integrin expression. One representative experiment out of at least three is shown. The bar graphs contain MFI (mean ± SD) values (n = 3). ** p < 0.01. (C) A higher percentage (mean values ± SE) of control siRNA treated αvβ3high cells (black) invaded into 3D ECMs compared to siβ3-1 (light gray) and siβ3-2 (dark gray) treated αvβ3high cells after 3 days. *** p < 0.001. (D) Invasion profiles showed that control siRNA treated αvβ3high cells migrated deeper into 3D collagen matrices compared to siβ3-1 (light gray) and siβ3-2 (dark gray) treated αvβ3high 24αvβ3low cells (dark gray) after 3 days. (E) Invasion depth (mean values ± SE) is increased in control siRNA treated αvβ3high cells compared to specific b3 integrin siRNA siβ3-1 (light gray) and siβ3-2 (dark gray) treated αvβ3high cells.
including stress fibers and the turnover of focal adhesions connecting the external ECM to the cell’s cytoskeletal scaffold. Both mechanical properties may support $\alpha \beta 3$ integrin facilitated cancer cell invasiveness into 3D ECMs. In order to analyze whether the expression of the $\alpha \beta 3$ integrin affects cellular adhesion strength or mechanical stiffness, $\alpha \beta 3^{\text{high}}$ cells and $\alpha \beta 3^{\text{low}}$ cells were measured using magnetic tweezer microrheology (figure 4). External forces of up to 10 nN were applied to super-paramagnetic beads coated with fibronectin (also a component of the 3D ECMs, figure S4—available from stacks.iop.org/NJP/15/015003/mmedia). Cancer cells secrete fibronectin into the medium and it is also present in fetal calf serum (FCS; one batch used for all experiments; figure S4). Thus, the fibronectin beads were bound to $\alpha \beta 3^{\text{high}}$ cells (figure 4(A)) and $\alpha \beta 3^{\text{low}}$ cells (figure 4(B)). The displacement of the bound beads during a step-wise increased application of force (creep measurement) followed a power law [39].

The stiffness measurements (averaged over all forces from 0.5 to 10 nN) of the $\alpha \beta 3^{\text{high}}$ cells and $\alpha \beta 3^{\text{low}}$ cells showed that $\alpha \beta 3^{\text{high}}$ cells have higher stiffness values expressed as mean values (figure 4(C)). These results indicate that the $\alpha \beta 3$ integrin alters cell stiffness and stress-stiffening of cancer cells. To analyze whether there are differences independent of alterations between the two cell lines, we used a $\beta 3$-specific siRNA approach. The $\beta 3$-specific siRNA reduced the MFI on $\alpha \beta 3^{\text{high}}$ cells after 2 days (figure 4(E)). These stiffness results showed a pronounced reduction in $\alpha \beta 3^{\text{high}}$ cells that had been treated with the $\beta 3$-specific siRNA si$\beta 3$-1 for 2 days as well as si$\beta 3$-2 compared to control siRNA treated cells (figure 4(F)). The stiffness results for the $\alpha \beta 3^{\text{high}}$ cells and $\alpha \beta 3^{\text{low}}$ cells and knock-down experiments suggest that contractile forces may play a role in $\alpha \beta 3$ facilitated cell invasion. To investigate whether the difference in stiffness is a result of fibronectin engagement with the $\alpha \beta 3$ integrin or also a result of collagen engagement with the $\alpha \beta 3$ integrin, collagen type I coated beads were used to measure cancer cell stiffness (figures 4(H) and (I)). The difference in stiffness (figure 4(H)) was also significantly present when using collagen coated beads instead of fibronectin-coated beads (figure 4(C)). This indicates that the difference in stiffness between $\alpha \beta 3^{\text{high}}$ and $\alpha \beta 3^{\text{low}}$ cells is a result of fibronectin/collagen engagement with the $\alpha \beta 3$ integrin. These findings may indicate that the $\alpha \beta 3$ integrin facilitated invasiveness is stiffness dependent.

2.5. Effect of the $\alpha \beta 3$ expression on cytoskeletal remodeling dynamics

Cancer cell invasion and metastasis involves dynamic filamentous actin cytoskeletal remodeling and assembly/disassembly of focal adhesion sites. In more detail, cytoskeletal remodeling means activation of ERK1/2, Src and focal adhesion kinase signaling pathways. These alterations include formation and dissolution of stress fibers, dynamic actin accumulation at the cellular periphery and formation of lamellipodia and filopodia. Many biomechanical parameters such as adhesion/de-adhesion, contractile forces, cellular stiffness, cytoskeletal remodeling dynamics (cellular fluidity) and matrix degradation through secreted enzymes determine the migration speed and the invasiveness of cancer cells. Indeed, cellular stiffness and cytoskeletal remodeling dynamics are related, but it is still unclear whether they are positively or negatively correlated. During cell invasion into a dense 3D ECM with pore sizes smaller than the cell’s diameter, the invasive cell might change its shape and restructure its cytoskeleton to move forward in these dense 3D ECMs. The dynamics of cytoskeletal remodeling processes can also be measured with magnetic tweezer microrheology. The power-law exponent $b$ characterizes the visco-elastic response of cancer cells and assumes typical values between 0 for elastic solid materials and 1 for viscous fluid materials. The $b$-values were significantly increased in $\alpha \beta 3^{\text{high}}$
Figure 4. Stiffness and cytoskeletal remodeling dynamics are increased in αvβ3 integrin expressing cancer cells. A fibronectin coated bead is connected to αvβ3<sup>high</sup> cells (A) and αvβ3<sup>low</sup> cells (B) as shown as scanning electron microscopic images. (C) The stiffness (mean values ± SE) of αvβ3<sup>high</sup> cells and αvβ3<sup>low</sup> cells as well as (F) of αvβ3<sup>high</sup> cells transfected with control siRNA (control) and two β3 integrin subunit specific (siβ3-1 and siβ3-2) siRNAs was measured after force application to fibronectin-coated beads using magnetic tweezers. (E) Flow cytometric analysis (MFI as mean ± SD, n = 3) of αvβ3 expression on the cell surface of αvβ3<sup>high</sup> cells transfected with control siRNA (right histogram) or two β3 integrin subunit specific (siβ3-1 and siβ3-2; left and middle histogram, respectively) siRNAs was measured, respectively. (D) Creep exponent b (cell fluidity and cytoskeletal remodeling dynamics; mean values ± SE) of αvβ3<sup>high</sup> cells and αvβ3<sup>low</sup> cells as well as (G) αvβ3<sup>high</sup> cells transfected with control siRNA (control) and two β3 integrin subunit specific (siβ3-1 and siβ3-2) siRNAs was also determined after force application to fibronectin-coated beads using magnetic tweezers. The values are expressed as mean ± SE. 87–105 cells were measured for each condition. (H) The stiffness (mean values ± SE) of αvβ3<sup>high</sup> cells and αvβ3<sup>low</sup> cells was measured after force application to collagen type I (collagen)-coated beads using magnetic tweezers. (I) Creep exponent b (mean values ± SE) of αvβ3<sup>high</sup> cells and αvβ3<sup>low</sup> was also determined after force application to collagen-coated beads using magnetic tweezers. ***p < 0.001.
cells at all external forces applied to fibronectin-coated bound beads compared to $\alpha v_3^{\text{low}}$ cells, indicating that these cells were more fluid-like and that the cytoskeletal remodeling dynamics was increased in $\alpha v_3^{\text{high}}$ cells (figure 4(D)). These results were confirmed by $\alpha v_3^{\text{high}}$ cells that had been treated with two $\beta^3$-specific siRNAs (si$\beta$3-1 and si$\beta$3-2) for 2 days, as these cells displayed significantly increased cellular fluidity and hence, increased cytoskeletal remodeling dynamics compared to control siRNA treated cells (figure 4(G)). To analyze whether the collagen engagement of $\alpha v_3$ integrins lead to similar results of cellular fluidity and cytoskeletal remodeling dynamics, the $b$-value measurements were performed with collagen-coated beads. The $b$-values were significantly increased in $\alpha v_3^{\text{high}}$ cells at all external forces applied to collagen-coated bound beads compared to $\alpha v_3^{\text{low}}$ cells, indicating that these cells were more fluid-like (figure 4(I)) and that the cytoskeletal remodeling dynamics was increased in $\alpha v_3^{\text{high}}$ cells. Taken together, the fibronectin/collagen engagement of the $\alpha v_3$ integrin is critical for determining cytoskeletal remodeling dynamics (cellular fluidity). These findings suggest that the $\alpha v_3$ integrin facilitated invasiveness possibly depends on the cytoskeletal remodeling dynamics.

2.6. The $\alpha v_3$ facilitated cell invasion depends on the transmission and generation of contractile forces

To investigate whether contractile forces are essential for $\alpha v_3$ integrin-mediated increased cellular stiffness and subsequently enhanced cancer cell invasion, invasion assays were performed in the presence of myosin light chain kinase inhibitor ML-7 and Rho kinase inhibitor Y27632. Indeed, cancer cell invasiveness was reduced after addition of ML-7 and Y27632 in $\alpha v_3^{\text{high}}$ cells, but not in $\alpha v_3^{\text{low}}$ cells (figures 5(A)–(D)). In particular the number of invasive cells of $\alpha v_3^{\text{high}}$ cells was significantly reduced as well as their invasion depths after addition of ML-7 and Y27632 (figures 5(A) and (C)). Moreover, after addition of the contractility inducer calyculin A (Cal A), an increase in pre-stress in $\alpha v_3^{\text{low}}$ cells was observed, which enhanced cellular invasiveness (figures 5(B) and (D)), whereas no significant change occurred in $\alpha v_3^{\text{high}}$ cells regarding numbers of invasive cells or invasion depths (figures 5(A) and (C)). In addition, the effect of the contractile force inhibitors and the inducer of contractile forces on cellular stiffness were analyzed by adding these drugs to $\alpha v_3^{\text{high}}$ and $\alpha v_3^{\text{low}}$ cells using magnetic tweezer method. Indeed, ML-7 and Y27632, which both reduced contractile forces in $\alpha v_3^{\text{high}}$ cells, also decreased the cellular stiffness, whereas the inducer of contractile forces Cal A had no further effect on the cellular stiffness of $\alpha v_3^{\text{high}}$ cells (figure 5(E)). In contrast, both inhibitors, ML-7 and Y27632, could not further reduce the stiffness of $\alpha v_3^{\text{low}}$ cells, whereas the inducer of contractile forces Cal A was able to increase cellular stiffness of $\alpha v_3^{\text{low}}$ cells (figure 5(F)). These results suggest that the cellular stiffness regulates the transmission or generation of contractile forces, which are needed to overcome the hindrances of dense 3D ECMs. These findings may indicate that the $\alpha v_3$ integrin facilitated invasiveness depends on cellular stiffness which subsequently enables the cells to transmit and generate contractile forces.

2.7. The $\alpha v_3$ facilitated cell invasion is inhibited by the Src, STAT3 and RAC1 inhibitors

The addition of a Src kinase inhibitor (Src inh) to $\alpha v_3^{\text{high}}$ cells (figure 6(A)) and $\alpha v_3^{\text{high}}$ cells treated with control siRNA (figure 6(F)) reduced significantly the percentage of invasive cells into 3D ECMs, whereas it has no effect on the invasiveness of $\alpha v_3^{\text{low}}$ cells (figure 6(C))
Figure 5. Regulation of contractile forces. (A) Inhibition of contractile forces using myosin light chain kinase inhibitor (ML-7; 15 µM) or Rho kinase inhibitor (Y27632; 100 µM) reduced cancer cell invasion of \(\alpha\nu\beta^3\text{high}\) cells (A), whereas the invasiveness of \(\alpha\nu\beta^3\text{low}\) cells (B) was not affected. *** \(p < 0.001\). (C) Invasion profiles of \(\alpha\nu\beta^3\text{high}\) cells showed that the invasion depth was reduced by ML-7 and Y27632, but the invasion depths of \(\alpha\nu\beta^3\text{low}\) cells treated with these inhibitors was not affected. Induction of contractile forces by the calyculin A inhibitor (Cal A inh; 1 nM), which inhibits myosin light chain phosphatase, increased the invasiveness of \(\alpha\nu\beta^3\text{low}\) cells ((B), (D)), whereas the invasiveness of \(\alpha\nu\beta^3\text{high}\) cells treated with Cal A inh even slightly reduced ((A), (C)). (E) Addition of the inhibitors ML-7 and Y27632 to \(\alpha\nu\beta^3\text{high}\) cells reduced their stiffness compared to control (DMSO) treated cells, whereas the stiffness of \(\alpha\nu\beta^3\text{low}\) cells was not affected by ML-7 or Y27632 (F). The addition of the inducer of cellular contractility Cal A had no effect on the stiffness of \(\alpha\nu\beta^3\text{high}\) cells (E), whereas the stiffness of \(\alpha\nu\beta^3\text{low}\) cells was significantly increased (F). *** \(p < 0.001\).

or \(\alpha\nu\beta^3\text{high}\) cells treated with specific \(\beta3\) siRNA (figure 6(H)). Furthermore, the addition of a STAT3 inhibitor (STAT3 inh) or Rac1 inhibitor (Rac1 inh) to \(\alpha\nu\beta^3\text{high}\) cells and \(\alpha\nu\beta^3\text{high}\) cells treated with control siRNA reduced significantly the percentage of invasive cells into 3D.
Figure 6. Inhibition of Src kinase (Src inh), STAT3 (STAT3 inh) or Rac1 (Rac1 inh) of αvβ3-mediated cell invasion. (A), (B) Percentage of invasive cells (mean ± SE) of αvβ3\textsuperscript{high} cells (A), αvβ3\textsuperscript{low} cells ((C), (D)) determined after 3 days in the presence of a Src inhibitory peptide (Src inh 30 μM), STAT3 inhibitor (STAT3 inh 30 μM), or Rac1 inhibitor (Rac1 inh, 100 μM) or DMSO as control. Invasion profiles of αvβ3\textsuperscript{high} cells (B) and αvβ3\textsuperscript{low} cells (D) treated with DMSO-control, Src inh, STAT3 inh or Rac1 inh revealed that the addition of these inhibitors to αvβ3\textsuperscript{high} cells reduced their invasion depths, whereas they had no effect on αvβ3\textsuperscript{low} cells. (E) αvβ3 integrin expression of αvβ3\textsuperscript{high} cells treated with control or β3 specific siRNA (siβ3-1) for 2 days. In each histogram, left curves are isotype controls and filled gray curves show integrin expression. One representative experiment out of at least three is shown. The bar graphs contain MFI (mean ± SD) values (n = 3). (F) A higher percentage of control siRNA treated αvβ3\textsuperscript{high} cells invaded into 3D ECMs compared with control siRNA treated αvβ3\textsuperscript{high} cells in the presence of Src inh, STAT3 inh or Rac1 inh. (G) Invasion profiles showed that
Figure 6. (Continued) control siRNA treated $\alpha\nu\beta^3_{\text{high}}$ cells (DMSO control; black) migrated deeper into 3D collagen matrices compared with control siRNA treated $\alpha\nu\beta^3_{\text{high}}$ cells after addition of Src inh, STAT3 inh and Rac1 inh. (H) A similar percentage of specific $\beta^3$ integrin (si$\beta^3_{-1}$) siRNA treated $\alpha\nu\beta^3_{\text{high}}$ cells in the presence of DMSO (control; black) invaded into 3D ECMs compared with specific $\beta^3$ integrin (si$\beta^3_{-1}$) siRNA treated $\alpha\nu\beta^3_{\text{high}}$ cells in the presence of Src inh, STAT3 inh or Rac1 inh. (I) Invasion profiles showed that specific $\beta^3$ integrin (si$\beta^3_{-1}$) siRNA treated $\alpha\nu\beta^3_{\text{high}}$ cells (DMSO control; black) showed no difference in invasion depths in 3D ECMs compared with control siRNA treated $\alpha\nu\beta^3_{\text{high}}$ cells after addition of Src inh, STAT3 inh and Rac1 inh. (J) Addition of the inhibitors Src inh, STAT3 inh and Rac1 inh $\alpha\nu\beta^3_{\text{high}}$ cells reduced their stiffness significantly compared to control (DMSO) treated cells, whereas the stiffness of $\alpha\nu\beta^3_{\text{low}}$ cells was not affected (K). $*** p < 0.001$.

ECMs, whereas it has no effect on the invasiveness of $\alpha\nu\beta^3_{\text{low}}$ cells (figure 6(C)) or $\alpha\nu\beta^3_{\text{high}}$ cells treated with specific $\beta^3$ siRNA (si$\beta^3_{-1}$) (figure 6(H)). Expression of the $\alpha\nu\beta^3$ integrin on $\alpha\nu\beta^3_{\text{high}}$ cells treated with control siRNA or $\beta^3$ integrin specific siRNA (figure 6(E)). Additionally, the invasion profiles show that the invasion depth is also reduced after addition of the Src, STAT3 and Rac1 inhibitors in $\alpha\nu\beta^3_{\text{high}}$ cells (figure 6(B)) and $\alpha\nu\beta^3_{\text{high}}$ cells treated with control siRNA (figure 6(G)), but not in $\alpha\nu\beta^3_{\text{low}}$ cells (figure 6(D)) or $\alpha\nu\beta^3_{\text{high}}$ cells treated with specific $\beta^3$ siRNA (figure 6(I)). Taken together, inhibition of the src kinase, STAT3 or Rac1 strongly reduced the invasiveness of $\alpha\nu\beta^3_{\text{high}}$ cells (figures 6(A) and (B)), whereas the invasiveness of $\beta^3$ integrin subunit knock-down cells (figures 6(H) and (I)) and $\alpha\nu\beta^3_{\text{low}}$ cells were not altered (figures 6(C) and (D)). These results suggest that the increased invasiveness may be facilitated by a Src kinase, STAT3 and Rac1 pathway. In addition, the effect of these inhibitors on cellular stiffness was analyzed in $\alpha\nu\beta^3_{\text{high}}$ and $\alpha\nu\beta^3_{\text{low}}$ cells using magnetic tweezers. The Src, STAT3 and Rac1 inhibitors reduced the cellular stiffness in $\alpha\nu\beta^3_{\text{high}}$ cells (figure 6(J)), but had no effect on the stiffness of $\alpha\nu\beta^3_{\text{low}}$ cells (figure 6(K)). These results demonstrate that the $\alpha\nu\beta^3$ integrin-facilitated invasiveness of cancer cells depends on the cellular stiffness.

3. Discussion

Previously, the integrin $\alpha\nu\beta^3$ has been reported to be involved in the malignant progression of neoplasms involving tumor growth and metastasis formation [29–34]. Consistent with these studies we showed that high $\alpha\nu\beta^3$ integrin expression increased MDA-MB-231 breast carcinoma cell invasiveness into 3D ECMs. In contrast, using 2D migration assays it has been reported that the $\alpha\nu\beta^3$ integrin reduces the motility on ECM-protein ligand coated substrates [44]. Indeed, we confirmed that the motility of $\alpha\nu\beta^3_{\text{high}}$ and $\alpha\nu\beta^3_{\text{low}}$ cells was altered in terms of migration speed or persistence of migration. In particular, the $\alpha\nu\beta^3_{\text{low}}$ cells had a significantly higher migration speed, but migrated less persistently compared to $\alpha\nu\beta^3_{\text{high}}$ cells. These results demonstrate that the $\alpha\nu\beta^3$ integrin decreased the invasiveness on 2D planar substrates. Hence, these findings indicate that the effect of the $\alpha\nu\beta^3$ integrin
on cancer cell motility depends highly on the invasion microenvironment, in particular on 2D or 3D microenvironment. In particular, the dimensionality of the migration assay performed clearly affects the cellular motility and one should consider before a migration assay is used how this may influence the migrations results and mimic the natural cellular microenvironment. Additionally, we showed that high endogenous αvβ3 integrin expressing A375 (melanoma), T24 (bladder) and 786-O kidney cancer cell subclones increased the invasiveness into 3D ECMs compared to low endogenous αvβ3 integrin expressing subclones. These results suggest that the αvβ3 integrin facilitated invasiveness is not restricted to one cancer cell type, as it is transferable to other cancer types.

The invasion of cancer cells into their microenvironment such as connective tissue is a multistep event and depends on mechanical and biochemical properties of the microenvironment including ECM proteins [37, 43–45] and embedded cells [46] as well as cancer cells [47]. Here, we demonstrate that the high expression of the αvβ3 integrin enhances invasiveness of cancer cells into 3D ECMs through increased cellular stiffness, the transmission and generation of higher contractile forces pathway and increased cytoskeletal remodeling dynamics. The focus of this study was on the mechanism that leads to higher invasiveness of cancer cells with high expression of the αvβ3 integrin.

The functional mechanisms promoting the invasion of cancer cells are only fragmentarily investigated, but there is a common agreement that biomechanical factors may determine the speed of cell migration in dense 3D ECMs [40–41, 43, 46]. Among these factors are adhesion forces, degradation processes of the ECM through secretion of matrix-degrading enzymes, remodeling dynamics of the cytoskeleton, cellular stiffness and fluidity, and contractile force transmission and generation [39, 43, 48–50]. To investigate which of these biomechanical factors contribute to the higher invasiveness of cells with high αvβ3 integrin expression that were isolated from transfected cell lines with high and low αvβ3 integrin expression from MDA-MB-231 breast carcinoma cells. In each case, cells with high αvβ3 integrin expression were highly invasive. The knock-down of the β3 integrin subunit using β3 integrin subunit specific siRNA dramatically decreased their invasiveness into 3D ECMs.

Integrins are known to be involved in cell adhesion, transmigration and invasion processes and in coupling of the actomyosin cell cytoskeleton to the microenvironment. The activation of integrin receptors could be through conformational changes after ligand binding and possibly through biomechanical stimulation [17, 50]. The activation of integrins may be regulated through either increased affinity to ligands by enhancing the number of activated integrins and total integrins on the cell’s surface or integrin translocation into lipid rafts, whereas this has only been reported for β1 integrin subunits [50]. However, the αvβ3 integrin has been reported to be associated with receptor tyrosine kinases such as the VEGF-2 receptor to promote cell motility [52]. The latter was not addressed in this study, but the first was preliminarily reported not to be crucial because alternations in the expression levels of the β3 integrin were observed in aggressive, malignant tumors, but not in benign [34, 51]. Consistently, the knock-down of the β3 integrin subunit in αvβ3high cells reduced their invasiveness into 3D ECMs and impaired the mechanical alternations indicating that the αvβ3 integrin active function increases the invasiveness of cancer cells into connective tissue. Taken together, these results indicate that the αvβ3 integrin is a key player in facilitating cancer cell invasion into 3D ECMs by regulating the biomechanical properties of invasive cancer cells.

The signal transduction pathways that connect integrin adhesion events with cellular stiffness, cytoskeletal remodeling dynamics, traction force generation and cell invasion are
still elusive, although important components of other integrin receptors have been studied in
detail, such as the activation of α5β1 integrins by ECM ligands [50, 53], the formation of
focal adhesions following biochemical integrin activation [54], the connection between focal
adhesion assembly and contractile forces [50, 55–56], and between contractile forces and
3D cell invasion [39, 57]. Here, this article demonstrates that increased expression of αvβ3
integrins leads to increased cellular stiffness and cytoskeletal remodeling dynamics that enable
aggressive cancer cells to overcome the steric hindrance of dense 3D ECMs. These findings are
consistent with our previous studies that showed increased cancer cell stiffness and/or increased
contractile forces of highly invasive cancer cells expressing high amounts of α5β1 integrins
or CXCR2 receptors compared to the weakly invasive cancer cells expressing low amounts of
α5β1 integrins or CXCR2 receptors [8, 39]. The results of this study are in line with the other
studies [8, 39] and lead to the suggestion that several biochemical alterations such as integrins or
chemokine receptors on the cellular surface of cancer cells may affect biomechanical properties
in a similar way.

Blocking of the myosin contraction through the MLCK inhibitor ML-7 or the ROCK
inhibitor Y27632 diminished the invasiveness of αvβ3^high^ cells indicating that the αvβ3-
facilitated increased invasiveness is contractile force dependent. In addition, both contractility
inhibitors reduced the stiffness of αvβ3^high^ cells. These findings indicate that the αvβ3
integrin facilitated invasiveness into 3D microenvironments depends on the cellular stiffness
and subsequently on the transmission and generation of contractile forces. However, it
remains elusive whether increased stiffness caused increased contractile forces or vice versa.
What is known is that cytoskeletal remodeling affects cellular stiffness and contractile force
transmission and generation. In αvβ3^low^ cells neither the inhibitor Y27632 nor the ML-7
inhibitor reduced the invasiveness or stiffness, whereas in αvβ3^high^ cells the invasiveness
and stiffness were significantly reduced indicating that αvβ3^high^ cells can employ invasion
strategies that rely on the cellular stiffness and the generation of contractile forces. Nonetheless,
by increasing the contractility of αvβ3^low^ cells through myosin light chain phosphorylation
using Cal A, they showed increased invasion. These results indicate that contractile forces
are needed for increased cell invasion. Furthermore, the inhibition of the Src kinase and
its downstream targets STAT3 and Rac1 reduced significantly the invasiveness of αvβ3^high^
cells, but had no effect on the invasiveness of αvβ3^low^ cells. These results suggest that Src
kinase/STAT3/Rac1 signaling pathways may be involved in the αvβ3 facilitated invasiveness
of cancer cells. Mutational alteration of the Src-binding motif of p130Cas has reported to
abolish the interaction between Src and its substrate p130Cas [58–59] and significantly reduce
levels of p130Cas phosphorylation at this site [60–61]. The ability of Src to phosphorylate
p130Cas is enhanced by mechanical extension of the interaction site, implying that p130Cas acts
as a mechanosensor [62] or as indicated in this study STAT3 or RAC1 act as a mechano-
regulatory proteins. In addition, p130Cas phosphorylation has been detected predominantly in
nascent integrin adhesion sites [60, 63], which are necessary for cell invasion into 3D ECMs
and contractile force transmission and generation. All three inhibitors (Src, STAT3 and RAC1
inhibitors) reduced the stiffness of αvβ3^high^ cells, but not of αvβ3^low^ cells indicating that the
αvβ3 integrin facilitated cancer cell invasiveness in a 3D microenvironment such as a 3D ECM
is stiffness and hence, contractile force dependent.

Finally, it can be concluded that the cellular stiffness and subsequently the transmission
and generation of contractile forces together with enhanced cytoskeletal remodeling dynamics
are the driving factors for increased the αvβ3 integrin-facilitated cell invasion into 3D ECMs,
and proposed that the measurement of biomechanical properties may be a novel factor in determining and explaining the malignancy of tumors.

4. Experimental procedures

4.1. Cells and cell culture

MDA-MB-231 (breast), T24 (bladder), 786-O (kidney) carcinoma cells and A375 (skin) melanoma cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, 1 g l⁻¹ glucose) containing 10% FCS (low endotoxin, < 0.1 EU ml⁻¹), 2 mM L-glutamine and 100 U ml⁻¹ penicillin–streptomycin (Biochrom, Berlin, Germany). Eighty per cent of confluent cancer cells were used in passages 5–30. Accutase was used for cell harvesting (less than 1% dead cells). Cells in every experiment were tested for mycoplasma contamination using a Mycoplasma detection kit (Roche, Mannheim, Germany). All other chemicals used were purchased from Sigma (Taufkirchen, Germany).

4.2. Isolation of subcell lines

The human breast cancer cell line MDA-MB-231, which expresses endogenous αvβ3 integrins, was used for the generation of subcell lines with high (αvβ3high cells) and low endogenous expression of αvβ3 integrins (αvβ3low cells). To isolate high and low expressing subcell lines MDA-MB-231 cells were stained with a mouse antibody against the human αvβ3 integrin (clone LM609, Millipore) for 30 min at 4 °C and then, with a secondary R-PE-labeled goat anti-mouse-IgG (F(ab)₂-fragment; Dianova) antibody for 30 min at 4 °C. Then, single cells of both subgroups (high and low) were sorted in ten 96 well plates using a cell sorter and grown to colonies and finally cultured to subcell lines at 95% humidity, 37 °C and 5% CO₂ in an incubator. Subcell lines high and low endogenous αvβ3 integrin expression were analyzed at least three times for each clone and remained stable over the whole investigation time (data not shown). Additionally, αvβ3 integrin subcell lines were isolated from T24, A375 and 786-O cancer cells with endogenous αvβ3 integrin expression [8, 29].

4.3. Flow cytometry

Cancer cells of 80%-confluency were harvested and resuspended in HEPES-buffer (20 mM Hepes, 125 mM NaCl, 45 mM glucose, 5 mM KCl, 0.1% albumin, pH 7.4). Cancer cells were incubated with mouse antibodies against human α1 (FB12), α2 (P1E6), α5 (Biozol), β1 (Biozol) and αvβ3 (LM609), all purchased from Millipore (Temecula, CA) unless otherwise stated. In all experiments, isotype-matched antibodies were used as controls (Caltag, Burlingame, CA). After 30 min per 4 °C, cells were washed and stained with a R-PE-labeled goat anti-mouse-IgG (F(ab)₂-fragment; Dianova). Flow cytometry measurements were performed using a FACSCalibur System (BectonDickinson, Heidelberg, Germany). Data are expressed as MFI values.

4.4. 3D ECM invasion assay

A 3D-collagen-invasion assay was used to study invasiveness of cancer cells. In particular, for a six well plate 3.5 ml collagen R (Serva, Heidelberg, Germany) and 3.5 ml collagen G (Biochrom,
Berlin, Germany) were mixed, 0.8 ml of 278 mM sodium bicarbonate (end concentration 26.5 mM) and 0.8 ml 10 × DMEM (Biochrom) was added. Air bubbles were avoided during the 3D-ECM preparation process [8]. The polymerization of the 3D ECMs started after the stirred solution was neutralized with 1 N sodium hydroxide and incubated at 37 °C, 95% humidity and 5% CO2. Immediately, 1.2 ml of the collagen solution was pipetted into each well of a six-well plate, and collagen mixtures were polymerized. Polymerized 3D ECMs were normally 500 µm thick. The 3D collagen-matrices were incubated overnight with 2 ml DMEM [39]. After removal of the incubation medium, 100 000 cancer cells were seeded on top of the 3D ECMs and cultured for 72 h at 37 °C, 5% CO2 and 95% humidity in DMEM containing 10% FCS. At this time period, differences in the invasiveness of cells were clearly visible. For serum-free cell invasion, cancer cells were cultured 24 h before and during the invasion assay in EX-cell293 medium (SAFCBiosciences, Lenexa, KS) with 100 U ml−1 penicillin–streptomycin. After 3 days, cancer cells cultured on and inside the collagen matrices were fixed with 2.5% glutaraldehyde solution in PBS-buffer, the fraction of cancer cells that invaded 3D ECMs and their invasion depth were measured by optical sectioning in 12 randomly selected fields of view. After this time, differences in the invasiveness of cell lines were clearly visible. Non-invasive cells can be readily identified by their nuclei (stained with 1 µg ml−1 Hoechst 33342 dye) located in one layer that coincides with the location of the topmost collagen fibers. A cell was counted as invasive when its nucleus is located below the layer formed by the non-invasive cells. Because of the depth of field of a 40 × 0.6 NA objective, the uncertainty of this method is ∼5 µm. The invasion depth was determined by focusing the microscope to the center of the nucleus; the value was read from the motorized z-drive of the microscope and was corrected for the refractive index of water (1.33). The z-focus at the gel surface served as the reference. To determine the percentage of invasive cancer cells (cells inside the 3D ECMs), the adherent cells on top of the 3D collagen matrices were also counted [8]. The invasion profile plots only contain the invasive cancer cells. The percentage of cancer cells that invaded is given in the bar graphs.

4.5. Modulation of cell invasion

To inhibit or modulate cell invasion, 15 µM myosin light chain kinase inhibitor ML-7 (Calbiochem), 100 µM Rho kinase inhibitor Y27632 (Sigma) or 1 nM Cal A (inhibits serine/threonine phosphatases and induces contraction, Calbiochem) were added to the 3D ECM invasion assay prior to cell seeding. Moreover, to analyze the αvβ3-facilitated signaling, cell invasion was inhibited or modulated by addition of 30 µM Src tyrosine kinase inhibitor (Cat. No. 567805, Calbiochem), or 30 µM of the STAT3 inhibitor peptide (Cat. No. 573096, Calbiochem), which blocks the STAT3 activation and suppresses constitutive STAT3-dependent Src transformation, or 100 µM Rac1 inhibitor (Calbiochem).

4.6. Migration speed in 2D

10 000 αvβ3low or αvβ3high cells were seeded on FN or collagen type I coated glass slides. Cell movements were computed from phase-contrast images recorded with 10× magnification using a Fourier-based difference-with-interpolation algorithm [64]. The MSD of cell movements with time t was described with a power-law relationship MSD = D(t/t0)β [65–67], where t0 is the time interval of the image recordings (1 min), D is the apparent diffusion coefficient and the
power-law exponent $\beta$ is a measure of persistence, with $\beta \sim 1$ for randomly and $\beta \sim 2$ for ballistically migrating cells [64]. The average migration speed over any time period can be obtained from the square root of the MSD and is shown in the figure. The MSD of cancer cells reveals that the migration process is not a Brownian random walk but is superdiffusive due to directional persistence and temporal fluctuations in migration speed.

4.7. Enzyme-linked immunosorbent assay

Supernatant from 100 000 cells of each subcell line was collected after 3 days of cell invasion and the FCS was measured. Fibronectin concentrations were determined using a Fibronectin-Elisa Kit according to the manufacturer’s instructions (R&D systems).

4.8. siRNA transfection

200 000 80%-confluent cells were seeded in Ø 3.5 cm dishes and cultured in 2 ml DMEM complete medium. Five microliters of a 20 $\mu$M $\beta$3 integrin subunit siRNA (target sequences for $\beta$3: si$\beta$3-1 CCGCTTCAATGAGGAAGTGAA or Allstar-control RNAi-solution (control-siRNA), 12 $\mu$l HiPerFect-Reagent (Qiagen) and 100 $\mu$l DMEM were mixed [39]. RNAi-mediated $\beta$3 knock-downs were confirmed by flow cytometry using anti-αvβ3-integrin and Cy2-labeled anti-mouse-IgG antibodies (Dianova). The transfection efficiency was determined by flow cytometry to be >99% using 20 $\mu$M Alexafluor546-labeled siRNA.

4.9. Magnetic tweezer

Magnetic tweezers were used to apply a staircase-like sequence of step-forces ranging from 0.5 to 10 nN to superparamagnetic epoxylated 4.5 $\mu$m beads, coated with 100 $\mu$g ml$^{-1}$ fibronectin from human plasma (Roche Diagnostics, Mannheim, Germany, Cat. No. 11080938001) or 100 $\mu$g ml$^{-1}$ collagen type I (Biochrom) [39, 49]. Fibronectin was present in 3D-ECMs, because it was embedded into the matrices from the 10% FCS in the cell culture medium as well as from cultured cancer cells, which secrete fibronectin. 2 $\times$ 10$^5$ fibronectin coated beads were sonicated, added to 10$^5$ cells, and incubated for 30 min at 37 $^\circ$C and 5% CO$_2$. All measurements were performed at 37 $^\circ$C with an inverted microscope (DMI-Leica) after at least 30 min of bead binding, which is sufficient to connect the coated beads to the cytoskeleton of cancer cells. Once the coated beads are firmly connected to the cytoskeleton of cancer cells, the molecular details of the connection matter little and have no impact on the motion of coated beads [8]. The creep response $J(t)$ of cells during force application followed a power law in time, $J(t) = a(t/t_0)^b$, where the pre-factor $a$ and the power-law-exponent $b$ were force-dependent, and the reference time $t_0$ was set to 1 s. The bead displacement in response to a staircase-like force pattern followed a superposition of power laws from which the force dependence of $a$ and $b$ was determined by a least-squares fit [49, 68]. The parameter $a$ (units of $\mu$m nN$^{-1}$) characterizes the elastic cell properties and corresponds to a compliance (the inverse of stiffness) [49]. The force–distance relationship in units of nN $\mu$m$^{-1}$ is related to cellular stiffness in units of Pa by a geometric factor that depends on the contact area between the coated bead and the cancer cell (or the degree of bead internalization), and the cell height. If those parameters are known, the geometric factor can be estimated from a finite element analysis [69]. Without knowledge of the cell height and coated bead internalization, the typical strain $\varepsilon$ can be estimated as the bead displacement $d$ divided by the bead radius $r$, and the typical stress $\sigma$ as the applied
force $F$ divided by the bead cross-sectional area $\pi r^2$ such that the cell stiffness corresponds to: $G = \sigma / \varepsilon = r \cdot dF / (\pi r^2) [70]$. For 4.5 $\mu$m coated beads as used in this study, the geometric factor is 0.14 $\mu$m$^{-1}$, and a cell with an apparent stiffness of 1 nN $\mu$m$^{-1}$ would have a ‘proper’ stiffness of 140 Pa.

The power-law-exponent $b$ reflects the stability of force-bearing membrane and cytoskeletal structures connected to coated beads. Values of $b = 1$ and 0 indicate Newtonian-viscous and elastic behavior, respectively [68]. A non-zero power-law exponent indicates that an amount of the deformation energy during magnetic force application is not elastically stored in the cytoskeleton, instead, it is dissipated as heat due to the remodeling of cytoskeletal structures to which the bead is bound [68]. Thus, the dissipation is directly coupled to the rate at which the elastic bonds within the cytoskeletal network break up and finally turn over. The turnover of acto-myosin bonds contributes to the dissipative properties [71], and is not considered as a remodeling event, but it enables contractility-driven morphological shape changes of the cytoskeleton. In cancer cells, the power-law exponent $b$ usually falls in the range between 0.1 and 0.5, whereby higher $b$-values have been linked to a higher turn-over rate of cytoskeletal structures [72]. The $a$- and $b$-values are averaged over all applied forces and all coated beads measured (bound to cells) and are expressed as mean ± SE.

### 4.10. Statistical analysis

The flow cytometry data, 2D migration data and enzyme-linked immunosorbent assay data were expressed as mean values ± SD. All other data were expressed as mean values ± SE. Statistical analyses were performed using the two-tailed Student’s $t$-test. A value of $p < 0.05$ was considered to be statistically significant.

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