Physical View on the Interactions Between Cancer Cells and the Endothelial Cell Lining During Cancer Cell Transmigration and Invasion

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There exist many reviews on the biological and biochemical interactions of cancer cells and endothelial cells during the transmigration and tissue invasion of cancer cells. For the malignant progression of cancer, the ability to metastasize is a prerequisite. In particular, this means that certain cancer cells possess the property to migrate through the endothelial lining into blood or lymph vessels, and are possibly able to transmigrate through the endothelial lining into the connective tissue and follow up their invasion path in the targeted tissue. On the molecular and biochemical level the transmigration and invasion steps are well-defined, but these signal transduction pathways are not yet clear and less understood in regards to the biophysical aspects of these processes.

To functionally characterize the malignant transformation of neoplasms and subsequently reveal the underlying pathway(s) and cellular properties, which help cancer cells to facilitate cancer progression, the biomechanical properties of cancer cells and their microenvironment come into focus in the physics-of-cancer driven view on the metastasis process of cancers. Hallmarks for cancer progression have been proposed, but they still lack the inclusion of specific biomechanical properties of cancer cells and interacting surrounding endothelial cells of blood or lymph vessels. As a cancer cell is embedded in a special environment, the mechanical properties of the extracellular matrix also cannot be neglected. Therefore, in this review it is proposed that a novel hallmark of cancer that is still elusive in classical tumor biological reviews should be included, dealing with the aspect of physics in cancer disease such as the natural selection of an aggressive (highly invasive) subtype of cancer cells displaying a certain adhesion or chemokine receptor on their cell surface.

Today, the physical aspects can be analyzed by using state-of-the-art biophysical methods. Thus, this review will present current cancer research in a different light from a physical point of view with respect to cancer cell mechanics and the special and unique role of the endothelium on cancer cell invasion.
The physical view on cancer disease may lead to novel insights into cancer disease and will help to overcome the classical views on cancer. In addition, in this review it will be discussed how physics of cancer can help to reveal and propose the functional mechanism which cancer cells use to invade connective tissue and transmigrate through the endothelium to finally metastasize.

Finally, in this review it will be demonstrated how biophysical measurements can be combined with classical analysis approaches of tumor biology. The insights into physical interactions between cancer cells, the endothelium and the microenvironment may help to answer some “old,” but still important questions in cancer disease progression.

**Keywords:** Contractile forces; focal adhesions; stiffness; cytoskeletal remodeling; integrins; viscoelasticity; RNAi; biomechanics.

**Abbreviations**

MLCK : myosin light chain kinase  
RLC : regulatory light chain  
EMT : epithelial-mesenchymal transition

**1. Introduction**

Interactions between cancer cells and endothelial cells play an important role during the malignant progression of cancer. Malignant cancer progression involves the process of metastasis and is the worst scenario in cancer disease as it is the main cause of cancer deaths. The process of metastasis includes many steps, which follow a linear propagation. The cascade of metastasis starts with the spreading of cancer cells from the primary tumor, which migrate into the local tumor microenvironment. Moreover, these cancer cells transmigrate into blood or lymph vessels (intravasation), get transported through the vessel flow, adhere to the endothelial cell lining, grow and form a secondary tumor either in the vessel or cancer cells possibly transmigrating through the endothelial vessel lining (extravasation) into the extracellular matrix of connective tissue. These cancer cells then migrate further deeply into the targeted tissue, grow and form a secondary tumor, which means that the tumor has metastasized (Fig. 1).

Many aspects of classical tumor biology research have been investigated and thus, eight hallmarks have been postulated such as sustained proliferative signaling, evading growth suppressors, avoiding immune destruction, activation of invasion and metastasis, enabling and promoting replicative immortality, induction of angiogenesis, resistance to cell death and deregulation of cellular energetics. However, these proposed hallmarks of cancer do not account for the physical aspects of cancer and their role in malignant cancer progression.

Among the molecules regulating cancer cell motility and invasion are cell-cell adhesion receptors such as E-cadherin-Notch signaling, cell-matrix adhesion receptors such as integrin receptors (α6β3, αvβ3, αvβ5, α5β1) and chemokine receptors such as CXCR2 and CXCR4. All these proteins may also play a role in cancer cell–endothelial cell interactions during cancer metastasis. Despite all these current findings and even the novel approaches based on genomics and proteomics, cancer
Invasive cancer cell without cell-cell adhesions
Non-invasive cancer cells with cell-cell adhesions
Basal membrane
Invasion
Spreading of cancer cells
Adhesion
Transport via blood or lymph vessels
Adhesion
Primary tumor
Invasive cancer cell without cell-cell adhesions
Primary tumor
Non-invasive cancer cells with cell-cell adhesions
Basal membrane
Invasion
Spreading of cancer cells
Adhesion
Transport via blood or lymph vessels
Adhesion
Secondary tumor
Secondary tumor
Extracellular matrix
Extravasation
Intravasation
Macrophage
Fig. 1. The consecutive steps of the process of the metastasis formation cascade. Certain selected cancer cells can weaken their cell-cell adhesions, cross the tumor boundary including the basement membrane around the primary tumor, disseminate from the primary tumor, invade into the 3D microenvironment such as the extracellular matrix of connective tissue, transmigrate through the basement membrane of blood or lymph vessels (intravasation) possibly with the help of tumor-associated macrophages. Once entered the vessels, cancer cells are transported through the whole body and possibly transmigrate through the endothelial vessel lining (extravasation) and the basement membrane to migrate into the extracellular matrix of connective tissue and form a secondary tumor in cancer-type specific targeted organs. Another possibility is that cancer cells stick in the vessels after they adhered to the endothelial cell wall, assemble and grow inside the vessel as a secondary tumor.

research does not fundamentally change the cancer death rates, but improves clinical diagnosis substantially in the field of cancer research regarding the classification and detailed staging of tumors, numerous marker proteins, and mapping of specific human cancer-types.

Despite these biological improvements, a main criticism remains: the expression levels of numerous genes and molecules, which are differently regulated during cancer progression, depend on the cancer disease stage. In particular, it is still not fully understood how they regulate cancer progression. A reason may be that these genomic and proteomic based methods do not account for the localization of molecules in special compartments such as lipid rafts,¹⁰ their activation or assembly state, their life-time, turn-over, modification and recycling rate.¹¹–¹⁵
As the whole complexity of malignant cancer progression seems not to be covered by the genomic or proteomic based methods, biophysical methods are promised to obtain more insights into malignant cancer disease progression. In more detail, classical physical approaches will be adopted to complex soft matter such as cancer cells and novel biophysical methods will be developed in order to apply them to cancer research. These novel physical approaches have changed the direction of recent cancer research and have broken down the classical view on cancer disease during the last decade.

In particular, a novel hallmark adding the aspect of physics to classical cancer research is that the primary tumor and the tumor microenvironment alter the survival conditions (such as cell-division) and cellular properties of a certain set of cancer cells, which subsequently lead to the selection of an aggressive (highly invasive) subtype of cancer cells. This aggressive subtype of cancer cells may be able to reduce cell-cell adhesions to neighboring cells, cross the tumor boundary of the primary tumor, invade into the extracellular matrix scaffold and transmigrate through the endothelial lining of vessels.

This review will focus mainly on our work of the selection of an aggressive subtype of cancer cells expressing high amounts of a certain integrin or chemokine receptor on their cell surface. In addition, it will highlight the impact of mechanical properties of invasive cancer cells, the mechanical properties of the cancer microenvironment, and their impact on the migration mode of cancer cells. Moreover, this review will focus on the dimensionality of cancer cell migration, on the novel invasion promoting role of the endothelial lining from vessels and on the mechanical properties of cancer cells and their possible modulation by the endothelium as well as on the alterations of the endothelium’s mechanical properties. Finally, our findings will be set into the broad range of knowledge on current results in the field of cancer research and their impact on further cancer research will be discussed. The next two chapters will discuss how the selection process of an aggressive cancer cell phenotype can take place.

2. Selection of Aggressive Cancer Cell Subtypes Expressing a Certain Cell-Matrix Receptor

The selection of an aggressive phenotype of cancer cells in a primary tumor seems to be the onset of the malignant cancer progression cascade. Although the process of metastasis occurs rarely among the numerous cancer cells within a primary tumor, once started the metastasis has a worse prognosis for the patient. In principle one single cancer cell may be sufficient to cause malignant cancer progression. However, a few of these cancer cells undergo a transition or selection process in order to be able to follow the step-by-step series of the metastatic progress. How this selection process works is still elusive. We suggest that alterations of the mechanical properties may play a pronounced role in selecting aggressive and highly invasive cancer
cells. The following gives an example of how such a selection process may work and when it may start during cancer disease progression.

The process of cancer metastasis is a complex process that includes sequential steps and is responsible for over 90% of cancer-related deaths. When focusing on the onset of metastasis, cancer cells spread from the primary tumor, cross the tumor boundaries such as the basement membrane and tissue compartments, migrate or flow through vastly different microenvironments, including the tumor stroma, the blood vessel endothelium, the vascular system and the tissue at a secondary target site, where the probability of metastasis occurring is increased compared to other non-targeted sites (Fig. 1). What role the mechanical properties of the home tissue of the primary tumor and the secondary target tissue does play is still under investigation and discussion. Currently, we suggest that similar mechanical properties of the two tissue types may enhance the ability of cancer cells to build a secondary tumor at this particular site. Until now, it is not known what determines the specificity of target tissues for cancer metastasis for a certain cancer type. Together with enhanced cellular invasiveness, specific cellular morphology, a certain cytoskeletal architecture and certain biomechanical properties, cancer cells can restructure and thus even adapt their microenvironment to promote tumor progression and finally to metastasize in targeted organs. These findings may suggest that cancer cells need a special microenvironment, where they are able to alter biochemical and mechanical properties. As we have analyzed the expression profile of highly invasive cancer cell types and non-invasive cancer cell types, when co-cultured with endothelial cells for 16 hours compared to monocultured cancer cell types, our findings are that the α5β1 integrin and the αvβ3 integrin expression are both increased in co-cultured highly invasive cancer cells compared to monocultured cancer cells, but not in co-cultured non-invasive cancer cells compared to monocultured cancer cells.

These results indicate that the afore-mentioned two integrins may play a role in cancer cell transendothelial migration and invasion. Therefore, we selected subcell lines from parental cell lines such as MDA-MB-231 (breast), T24 (bladder), 786-O (kidney) and A375 (skin) using a flow cytometer, which express high, intermediate and low amounts of α5β1 or αvβ3 integrins on their cell surface. Indeed, these subcell lines showed altered invasive properties due to integrin expression levels. In particular, the metastatic signal transduction cascade is supposed to be triggered by the proliferation of the primary tumor, genetic alterations of primary cancer cells, activation of signaling pathways and the selection of an aggressive cancer cell subtype. This aggressive cancer cell subtype should be able to weaken the cell-cell adhesions and be able to cross the tumor boundaries such as other cancer cells within the primary tumor and the basement membrane. These initial steps may further promote the invasiveness of this special subtype of cancer cells including their ability to form protrusions, intravasate into blood or lymph vessels and finally, to metastasize. Despite the fact that many steps are not understood in detail, however, how the detachment of a certain subtype of cancer cells from the primary tumor epithelium
occurs and how this subtype invades the underlying tumor stroma is able to be studied at the biomechanical, cellular and molecular levels. In summary, this process can be described by the well-known epithelial-mesenchymal transition (EMT), which was initially defined in the process of embryogenesis.\textsuperscript{23} The role of the EMT in cancer metastasis has been extensively studied\textsuperscript{24,25} and is still controversially discussed. Agreement has been reached about the key components of the EMT that are E-cadherin, Notch receptors (both cell-cell adhesion molecules), matrix-metalloproteinases (that degrade the extracellular matrix of connective tissue) and cytokeratins, which can lead to enormous changes in the physical and mechanical properties of cancer cells. In particular, the reduction of cell-cell adhesion between neighboring epithelial cancer cells and morphological alterations of the cell shape from cuboidal epithelial to fibroblastoid mesenchymal\textsuperscript{26} as well as in their aspect ratio.\textsuperscript{21} These alterations may cause the loss of intercellular adhesions, which initially hold the primary tumor together and may now serve as a selection pressure that is applied to select for an aggressive and highly invasive possibly mesenchymal cancer cell subtype, which can invade the tumor microenvironment.\textsuperscript{21,25}

However, it has still not been fully investigated what regulates the molecular and physical mechanisms that enhance the invasiveness of these special subtype of aggressive cancer cells and enable them to migrate into the tumor microenvironment. A question arises whether these subtypes of cancer cells possess specific stroma adhesion receptors in order to migrate through antigens such as CD44, which can bind to hyaluronic acid (increased in the surrounding tumor microenvironment).

Not only can the stroma alter the embedded tumor by accumulation, binding or production of substances (by stroma cells), cancer cells may also alter their microenvironment drastically in order to migrate into it and invade. The movement of cancer cells in a 3D microenvironment also depends on the enzymatic digestion of the extracellular matrix or by sheddases cutting cell-matrix adhesion receptors on the cell surface of the cancer cells such as ADAM-10 and ADAM-17 (see below). This enzymatic mode of cell invasion may follow other constrictions compared to the non-enzyme-driven cell invasion.\textsuperscript{20}

However, the integrin-dependent mode functions in the presence and absence of enzymatic digestion. Recently, we found that $\alpha_{5}\beta_{1}$ integrin expression facilitates cancer cell invasion into the extracellular matrix and even enhances the transmigration through endothelial cell monolayers grown to confluent monolayers on top of 3D extracellular matrices.\textsuperscript{4,21} The latter finding was surprising and unexpected as the endothelium was always presented as a strong “passive” barrier for cancer cell invasion. In contrast, we reported for the first time that the endothelium can act as an enhancer or inducer of cancer cell invasiveness into 3D extracellular matrices.\textsuperscript{21}

In addition, also $\alpha_{v}\beta_{3}^{\text{high}}$ integrin expressing cancer cells showed increased invasiveness into 3D extracellular matrices compared to $\alpha_{v}\beta_{3}^{\text{low}}$ cells.\textsuperscript{22} What about other integrins and their effect on cancer cell invasion?

Finally, other integrins such as collagen binding integrins such as $\alpha_{1}, \alpha_{2}, \alpha_{10}$ or $\alpha_{11}$ have to be analyzed for their potential in regulating cancer cell invasion. Besides
the cell-matrix adhesion receptors such as integrins, other cell surface receptors such as chemokines may play a role in cellular invasiveness. Thus, another approach for the selection of a highly aggressive subtype of cancer cells is discussed in the next chapter below.

3. Selection of Aggressive Cancer Subtypes Expressing a Chemokine Receptor

In the chapter above we have discussed the selection of cancer cell lines expressing high and low amounts of integrins. Now we will discuss the selection process of cancer cells expressing high and low amounts of the chemokine receptor CXCR2 and their subsequent ability to invade and transmigrate. Simultaneously, as we determined the expression pattern of the highly invasive and non-invasive cancer cells in co-culture with endothelial cells and in monoculture, we also analyzed the endothelial cells in co-culture and monoculture with highly invasive and non-invasive cancer cells. We report that chemokines such as Gro-β (CXCL2) and interleukin-8 (CXCL8) are increasingly expressed when primary microvascular endothelial cells are co-cultured with highly invasive cancer cells compared to monocultured endothelial cells, but not when co-cultured with non-invasive cancer cells.\(^4\)

As the receptor for these two chemokines is CXCR2, which is increasingly expressed on highly invasive cancer cells compared to non-invasive cancer cells, we isolated subclones from parental breast cancer cells (MDA-MB-231) with high and low CXCR2 expressions on their cell surface (Fig. 2). These CXCR2\(^{\text{high}}\) subcell lines showed increased invasiveness compared to the CXCR2\(^{\text{low}}\) subcell lines in 3D extracellular matrix and even the transmigration through an endothelial cell monolayer was increased in terms of numbers and invasion depths. Moreover, these CXCR2\(^{\text{high}}\)

![Fig. 2](image-url)  
Fig. 2. Schematic drawing of the selection process of highly and weakly invasive cancer cells from a parental human cancer cell line. A cancer cell line shows weakly and highly expression of the CXCR2 chemokine receptor on its cell surface. Thus, the cells are selected as single clones showing either weakly or highly expression of the CXCR2 receptor. These cells grow to cell lines and are called subgroup cancer cell lines.
and CXCR2<sub>low</sub> subcell lines possess different cellular stiffness and altered contractile forces transmission and generation that may explain their different invasiveness. How mechanical properties of cancer cells account for increased invasiveness will be discussed in the following chapter.

4. Mechanical Properties of Cancer Cells Facilitate Invasion

It is still not known what the prerequisites for the cell motility in 2D and 3D systems are. However, the ability of cancer cells (of epithelial origin) to migrate into 3D connective tissue does not depend on a single parameter. Instead, it rather depends on certain mechanical parameters, which regulate the migration velocity of cancer cells through a dense 3D extracellular matrix (pore size around 2 µm). Among these parameters are certain properties such as: (a) cell adhesion and de-adhesion dynamics (turn-over of focal adhesions, adhesion strength), (b) cytoskeletal remodeling dynamics, cell-fluidity and cell-stiffness, (c) matrix remodeling by secretion of extracellular matrix proteins and digestion through matrix degrading enzymes and (d) the generation and transmission of protrusive or contractile forces.<sup>4</sup><sup>-7</sup><sup>-30</sup> Each of these parameters cannot be treated as a single one, they must be related to the others to see how strong the particular effect on cell invasion and transendothelial migration is (Fig. 3). Thus, the balance between these parameters is crucial for the efficiency of cancer cell invasion, the speed of invasion and invasion depths in 3D extracellular matrices.<sup>31</sup> These parameters can vary depending on the cancer cell type and shift towards a single parameter, but still these parameters are all important for determining cancer cell invasiveness and invasion efficiency except for cancer cell types which are not of epithelial origin. In this case, these parameters vary a lot and some may even play no role.

Fig. 3. Schematic image of a cancer cells invading a 3D matrix and the parameters regulating cancer cell invasion into dense 3D matrix scaffolds. The ability to invade through the extracellular matrix of connective tissue is regulated by a balance between at least four biomechanical processes: (i) contractile force generation and transmission as well as protrusive forces, (ii) transmission of contractile forces via cell-matrix adhesions (adhesion strength, de-adhesion), (iii) cytoskeletal (CSK) remodeling dynamics, cell fluidity, and stiffness), and (iv) enzymatic matrix degradation.
For cancer cells of epithelial origin, a disruption of the balance between these parameters leads to a drastic change of the invasion mode from epithelial to mesenchymal, or even to amoeboid with or without traction forces. Whether this transition is always full and holds true for all types of cancer cells needs to be further investigated. Taken together, the invasion strategy is determined by these biomechanical and biochemical parameters. How the balance of these parameters is regulated by cancer cells (of epithelial origin) and what role the tumor microenvironment regarding growth factors, cytokines, chemokines, matrix-protein composition, structure and concentration, and matrix mechanical stiffness plays is still not fully clear and thus under intensive investigation.\(^{17}\)

There are two different mechanisms that are currently presented and discussed: The first mechanism is the degradation of the dense 3D extracellular matrix through the secretion of matrix metalloproteinases (MMPs) in order to facilitate cancer cell invasion.\(^{20,32,33}\) The second mechanism is the cutting of cell-cell adhesion molecules such as NOTCH receptors, ephrins or E-cadherins from the cell surface of cancer cells by sheddases such as the secretases ADAM-10 and ADAM-17.\(^{34–40}\) This reduction of cell-cell adhesions may then facilitate signal transduction leading to nuclear translocation (together with a transcription factor) of cell-cell adhesion proteins such as $\beta$-catenin and induction of gene expression, and finally induction of cancer cell invasiveness. Furthermore, the sheddase ADAM-17 can also cleave pro-TNF-$\alpha$ exposed on the cell surface of cancer cells to release TNF-$\alpha$ into the microenvironment.\(^{41}\) This may then activate nearby endothelial cells, which are subsequently stimulated to facilitate cancer cell transmigration. Besides the enzymatic degradation of shedding of membrane receptors, another parameter regulating the invasion speed and the invasion depth of cancer cells in dense 3D extracellular matrices is the physical property of cancer cells to generate and transmit contractile forces.\(^{4,42}\) Recently, biophysical methods for measuring contractile forces in 3D collagen or fibrin matrices have been presented.\(^{43–46}\) In more detail, the invasion of cancer cells can be analyzed by taking z-stack images using a confocal scanning microscope. In some cases, matrix embedded beads may serve as markers as well as be part of the collagen fiber structure. The tracking of collagen fibers is more sophisticated, but also more reliable. Using collagen fibers as markers, the effect of marker (bead) phagocytosis is vanished as only a minor number of collagen fibers are digested and internalized compared to embedded beads in close neighborhood to invasive cancer cells. In addition, bead internalization affects the mechanical properties of cancer cells such as stiffness and subsequently, it reduces the invasiveness of highly invasive cancer cells into dense 3D extracellular matrices.\(^{47}\)

As integrins were up-regulated in invasive cancer cells, we focused on them and supposed that they were possibly key players for the regulation of cancer cell invasion into dense 3D matrices as well as their cytoplasmatically associated focal adhesion molecules such as vinculin and focal adhesion kinase (FAK). In particular, integrins are important for facilitating the invasiveness of cancer cells because they regulate cell-matrix adhesion and de-adhesion, adhesion strength, transmission and
generation of contractile forces through outside in (via ligand-binding) or inside-out (via internal signaling through growth factors etc.), stimulation of integrin-receptors (clustering) as well as cytoskeletal remodeling dynamics. The integrins are a family of cell-matrix adhesion receptors and consist of two non-covalently linked \( \alpha \) (18 different ones) and \( \beta \) (8 different ones) subunits. Taken together, integrins facilitate transmembrane connections between the actomyosin cytoskeleton of the cells and their microenvironment such as an extracellular matrix fiber scaffold. In more detail, the connection between the cell’s cytoskeleton and the microenvironment seems to be facilitated by the focal adhesion protein vinculin which can act as a mechano-coupling and mechano-regulating protein. The focal adhesions of cancer cells possess two main functions in cell invasiveness: Firstly, they transmit contractile forces to the microenvironment. Secondly, they mediate the connection of cancer cells to its external substrate in order to withstand de-adhesion and apoptosis. In addition, the composition of focal adhesions may vary due to the integrin type and the integrin activation state. Moreover, the signaling through \( \alpha \) integrin subunits is not uniform among the different \( \alpha \) integrin subunits: for example, the activation of \( \alpha 4 \) and \( \alpha 9 \) integrin subunits diminishes the cell spreading, whereas the activation of \( \alpha 1, \alpha 3, \alpha 5, \alpha 6, \alpha v \) and \( \alpha \text{IIb} \) integrin subunits increases the cell spreading. Due to the integrin type and integrin activation state, the functional role of vinculin in focal adhesions may also be altered. Moreover, integrins regulate the function of other integrins: the \( \alpha 5/\beta 1 \) integrin has been reported to regulate the facilitated adhesions as well as the motility on extracellular matrices. Thus, we also investigated the effect of \( \alpha v/\beta 3 \) highly and lowly expressing cells on cancer cell invasion into dense 3D matrices. Indeed, we found that the \( \alpha v/\beta 3^{\text{high}} \) cells invaded more numerously and deeply into the 3D matrices compared to \( \alpha v/\beta 3^{\text{low}} \) cells. In addition to the impact of the mechanical properties on cancer cell functions as such as cellular motility, it is explained and discussed how cancer cells alter their microenvironment in the next chapter.

5. Cancer Cells Alter Their Microenvironment

As we have discussed above, the mechanical properties of cancer cells have a pronounced impact on their invasiveness. It has been supposed that the mechanical properties of cancer cells are determined or regulated by their microenvironment. Besides the mechanical properties of cancer cells, the microenvironmental mechanics can also be altered by transmigrating cancer cells. In particular, the invasive cancer cells can express MMPs on their surface such as MT1-MMP, which promote the digestion of the laminin- and collagen IV-rich basement membrane and subsequently, the digestion of the extracellular matrix tissue microenvironment leads to major restructuring of the 3D extracellular matrix, either local and global by secreting additional matrix components in the 3D matrix scaffold (Fig. 3). When invasive cancer cells pass the tumor stroma, they may sense the complex composition of the connective tissue scaffold consisting of collagen type I and fibronectin
through their particular cell-matrix receptors. For example, in close neighborhood to the primary tumor site the connective tissue scaffold has been reported to be stiffer compared to the matrix scaffold of normal healthy connective tissue due to elevated levels of collagen and additionally, due to elevated crosslinking of collagen fibers by lysyl-oxidase through cancer associated fibroblasts. This also enhances the stability of collagen fiber networks and increases their resistance to matrix degradation. In more detail, the crosslinking of collagen fibers triggers the outside-in signaling of the cell-matrix receptors, the integrins, as well as the binding of certain ligand to these receptors. Moreover these dramatic alterations of the extracellular matrix’s structure and mechanical properties may induce and enhance the proliferation of cancer cells, increase cancer cell invasion and represent possibly a positive feedback-loop. It is still elusive whether the effect of extracellular matrix stiffening of mammary tumors is transferable to other solid epithelial originated tumors. In more detail, how the stiffening process of the extracellular matrix is regulated and evoked is still not yet clear, in particular, what regulates the mechanical stiffening on the molecular level is under investigation. The knowledge of these molecular and biochemical regulation processes may help to understand how the whole process of cancer cell invasion starts and hence how it could be inhibited. Recently, we reported that the α5β1 integrin facilitates cancer cell invasion into the extracellular matrix, which needs to have a certain mechanical stiffness. Moreover, the α5β1 integrin facilitates cancer cell invasion even through endothelial cell monolayers grown to confluent layers on top of 3D extracellular matrices without disrupting, destroying or remodeling of the endothelial cell monolayer. However, it is still not yet shown what role sheddases such as ADAM-8, ADAM-10 or ADAM-17 play and whether they enhance or decrease cancer cell invasion and transendothelial migration. Taken together, this is still elusive and has to be determined in future studies. Not only do cancer cells alter the physical properties and the structure of their microenvironment, this interaction between cancer cells and their microenvironment is not restricted to cancer cells acting on the microenvironment, thus in addition the microenvironment has a broad impact on the cancer cell’s properties and functions as described and discussed in the next chapter.

6. The Microenvironment Alters the Biochemical and Mechanical Properties of Cancer Cells

As the microenvironments around primary tumors are altered by the primary tumor itself and by invasive cancer cells, the mechanical properties of cancer cells may also be altered, caused by their microenvironment. The mechanical properties of tumor microenvironments are altered compared to normal healthy tissue. Thus, the tumor microenvironments seem to be a key element for the regulation of cancer cell motility through 3D connective tissue and transendothelial migration through blood or lymph vessels. In line with this, we and others have reported that the microenvironment of tumors is no passive compartment. Rather, it regulates the
progression of cancer disease by behaving as an active element, which is important in regulating its mechanical properties in order to provide malignant tumor progression. In particular, the tumor microenvironment has been regarded as highly critical for all steps of the cancer metastasis process. In particular, we have demonstrated for the first time that the endothelial microenvironment of a tumor is an active element for enhancing and initiating the invasion of certain cancer cells into dense 3D extracellular matrices, where the pore size is smaller than the cancer cell’s diameter. In addition, external physical properties of the tumor microenvironment can be of geometric origin such as pore size, crosslinks, concentration and composition between collagen fibers of the 3D extracellular matrix or mechanical origin such as the matrix’s stiffness, which may affect the ability of cancer cells to invade into the matrices. These parameters cannot be seen as independent or unrelated, they are indeed connected and cannot always be varied independently. In more detail, the structure of the tumor microenvironment is determined by matrix stiffness, pore size, connection points or crosslinking proteins, extracellular matrix fiber network composition and concentration, fiber thickness, bending and orientation of the fibers. These parameters are not separately modulated and are not independent of each other, instead, they are rather related. In particular, for example the gel’s elastic module depends on the density of crosslinks and the rigidity of the polymer chains or fibers (depending on the persistence length). Moreover, even the matrix’s stiffness and the pore size are affected if the composition of the collagen types is varied. If collagen V is taken, the pore-size and the fibril diameter are supposed to be the same as if collagen type I is taken, but the matrix stiffness can be altered by varying the ratio between collagen types I and V. The cancer cells and the primary tumor can alter all these parameters of the surrounding tumor microenvironment. In addition, tumor-associated cells such as endothelial cells, macrophages or fibroblasts are able to alter or adjust these properties of matrices in order to adapt the external matrix to an optimal substrate for a single or collective invasion of cancer cells.

When cancer cells are too stiff or too soft, however, they can probably not alter highly crosslinked collagen fibers or bundles of the extracellular matrix scaffolding through applying a force in order to enlarge the pores or bend the fibers and thus increase their migration efficiently. Moreover, even the ability of cancer cells to degrade the extracellular matrix by MMPs may be altered and hence, is not sufficient to provide cancer cell invasion in certain circumstances. In summary, the mechanical properties of a primary tumor’s local microenvironment seems to play an important role in mediating the invasive and aggressive properties of cancer cells. Our understanding of the mechanical properties has been increased, but still many important questions remain. For example, does the matrix stiffness of the local tumor microenvironment facilitate the selection of an aggressive and highly invasive cancer cell sub-type? In particular, does the selection of this invasive cancer cell subtype underlie similar principles as for the differentiation of mesenchymal...
stem cells into distinct lineages? The latter question is partly answered as the differentiation of stem cells has been shown to be consistent with differences in tissue compliance. What role does the mechanical properties of the matrices play in facilitating cancer cell invasion and transmigration? Moreover do the invasive cancer cells form podosomes on 2D matrices or invadosomes in a 3D microenvironment? How many cancer cell types form podosomes or invadosomes? Do cancer cells use protrusive force-dependent or blebbing surface tension-dependent invasion modes? How is the formation of these structures regulated by the proteolytic digestion of the extracellular matrix?

Taken together, the physical and mechanical interactions between a cancer cell and its extracellular matrix (a collagen-rich scaffold on which the primary tumor grows) plays a key role in allowing cancer cells to migrate from a tumor to nearby tissues by crossing the tumor boundary as well as compartment boundaries, and if the primary tumor is strongly malign, cancer cells interact with tumor blood or lymph vessels. During the steps of intravasation and extravasation, invasive cancer cells may undergo large elastic deformations to penetrate endothelial cell-cell contacts or even to migrate through a living endothelial cell lining vessel walls, which then still stays intact after the transmigration step and reassembles to a closed endothelial monolayer after the transmigration event of a cancer cell. How this is regulated is still not yet known, but it is currently under investigation. Once having entered the vascular system, a cancer cell has to deal with the vessel flow (applying shear stress on the circulating and adhering cancer cells), which may impact the cancer cell’s migration velocity and adhesion strength to endothelial vessel walls. All this can also influence the binding efficiency of the cancer cells (within the vessels) to endothelial cells and may in particular determine the translocation of sites where a secondary tumor will be formed and finally grow.

In the past, it has been found that the mechanical phenotype of cancer cells growing on soft or rigid substrates was altered. Moreover, the ability to build up a secondary tumor in the relatively soft tissue lung could be explained by the ability of these cancer cells to grow on soft matrices. Thus, mechanical properties of the extracellular matrix play a role in regulating the invasive properties of cancer cells. During the malignant progression of cancer the rigidity of the extracellular matrix material increases, which leads then to the accumulation of dense and crosslinked collagen fiber matrices around the primary tumor. This altered cancer microenvironment may serve as a signal for cancer cells of the primary tumor to increase their invasive properties in order to be able to migrate out of the primary tumor directly into the tumor microenvironment. Which invasion or migration mode will these cancer cells use? How do the physical properties of invasive cancer cells and their surrounding microenvironment affect and determine the migration mode is discussed in the following chapter.
7. Do Physical Properties Determine the Migration Mode?

The answer to this question is properly that physical properties can determine the migration mode together with biochemical properties that can also alter the invasion mode. An example is the inhibition of matrix degrading enzymes such as matrix-metalloproteinases in carcinoma and fibrosarcoma cells using a special cocktail of inhibitory enzymes that lead to a switch of the migration mode from a predominantly integrin-based mesenchymal motility to a faster amoeboid migration mode.\textsuperscript{33,86} Another example for a regulation of the invasion mode in the opposite direction is that an increase in contractility by using calyculin A converts a former more amoeboid invasion mode to a slightly mesenchymal invasion mode as shown for cancer cells expressing low amounts of the $\alpha_5\beta_1$ integrin.\textsuperscript{21} The motility of cancer cells in uncrosslinked (pepsin extracted collagen type I gels) can be amoeboid with and without contractile forces,\textsuperscript{18,42} whereas in crosslinked collagen gels the motility of cancer cells still requires MMPs such as MT1-MMP (MMP14).\textsuperscript{63,87,88} Taken together, if the MMP function directly depends on the collagen matrix microstructure such as the collagen concentration and crosslinking density, the MMP inhibition would solely be effective in diminishing cancer cell motility in highly crosslinked and highly concentrated regions of the extracellular matrix, whereas MMPs would be totally ineffective for less crosslinked and low density regions of the matrix. In addition, the external application of mechanical forces can enhance the MT1-MMP-driven proteolysis of the extracellular matrix.\textsuperscript{89} This finding leads to the hypothesis that there is a signal transduction-mediated connection between the forces sensing and the secretion of proteolytic enzymes. Finally, to prove this hypothesis, additional experiments are necessary. In particular, there is evidence that the physical properties of the tumor microenvironment are crucial in tumor initiation, progression and metastasis through a functional connection between physical forces and biochemical signal processes. However, many classical migration assays such as wound healing assay ignored the effect of the dimensionality on cell motility and that a 3D microenvironment includes more constraints compared to a 2D microenvironment. Therefore, this aspect is discussed in more detail in the following section.

8. Cancer Cell Motility in Three Dimensions

Previously, many physical and molecular mechanisms determining the motility speed of normal and cancer cells have been studied \textit{in vitro} assays using two-dimensional (2D) substrates.\textsuperscript{83–85} These assays have been called wound healing assays, where a scratch between the monolayer of a cell population re-induces their migration into the free space (gap) or inserts are used to culture cells in a monolayer until they reach the borders of these inserts; by removing the inserts, a gap is left where the cell can move in. The latter assay may be more suitable for the cells, as they are not hurt by the cut through the monolayer compared to the original classic
Despite these developments, we have reported that the dimensionality of the cell-culture system used to study cell invasion is crucial for the mode of cellular migration.\textsuperscript{21,52} The 3D microenvironment of the extracellular matrix \textit{in vivo} is characterized by certain features such as the pore size, connection points and fiber or bundle orientation, all of which cannot be found in extracellular matrix protein-coated 2D substrates.\textsuperscript{87} However, the underlying principle of 2D and 3D migration may be different. In more detail, several features seem to be crucial for 2D motility such as focal adhesions, stress fibers, broad lamellipodia, filopodial protrusions at the leading edge and apical polarization (Fig. 4). These 2D motility features are pronouncedly reduced in size and play no role for invasive cancer cells migrating through a 3D extracellular matrix.\textsuperscript{31,90–93}

Recently, we have shown that the motility on 2D and 3D substrates could be quite different. In particular, the expression of the mechano-coupling and mechano-regulatory protein vinculin is important for the regulation of cellular motility in 3D, but plays no role in 2D cell motility as vinculin knock-out cells migrate faster on a 2D substrate compared to vinculin-expressing (wild-type) cells.\textsuperscript{52} Additionally, recently it has been suggested that focal adhesions, composed of clustered integrins which structurally and dynamically couple the cellular actin-myosin cytoskeleton to the extracellular matrix proteins on 2D substrates, are altered when these cells are inside a 3D extracellular matrix.\textsuperscript{90} Our hypothesis is that other focal adhesion proteins may behave similar to vinculin. However, there could also be differences to focal adhesion proteins that do not act as mechano-coupling proteins.

Fig. 4. Schematic drawing of the different migration/invasion modes of cancer cells due to the dimensionality of the substrate for epithelial derived cancer cell types.
Another explanation for the differences in cellular motility on 2D substrates and in 3D matrices could give the appearance of collagen fibers in 3D extracellular matrices. Thus, the extracellular matrix may support the dynamically clustering of integrins, with sizes in the order a few nanometers and lifetimes of a few seconds, that seem to be necessary for cell invasion through a 3D microenvironment (Fig. 4). In particular, cells in vivo can initiate the bundling of collagen fibers by the generation of contractile forces of cellular protrusions such as filopodia and invadopodia.\textsuperscript{94} Moreover, the collagen bundles increase the surface area available for the cells that in turn induces the assembly of even larger focal adhesions.\textsuperscript{95} It still remains elusive whether cancer cells are able to from similar focal adhesions and build up stress fiber in a 3D extracellular matrix. How does the mechanical properties of the 3D extracellular matrix modulate the focal adhesion assembly and the actin stress fiber formation? Stress fibers, containing bundled actin filaments, play a prominent role in 2D cell motility systems, where they transmit the contractile forces required for the regulation of de-adhesion of the rear of a cell from the 2D substratum and the establishment of the actin flow at the leading edge of the migrating cell.\textsuperscript{82,96} In contrast to 2D systems, cells possess less stress fibers inside a 3D extracellular matrix compared to an extracellular matrix protein coated 2D surface. However, a quantitative analysis is still missing. In more detail, these 3D stress fibers are either localized to the cell cortex (called cortical action network) or radiate from the nucleus towards the plasma membrane to form pseudopodial protrusions.\textsuperscript{43} In particular, inhibition experiments blocking the actomyosin contractility are often less effective in 3D cell motility systems compared to 2D cell motility systems,\textsuperscript{43} which raises the question whether the stress fibers are dependent on dimensionality of the motility system.\textsuperscript{96,97} In contrast, we have recently reported that the 3D cell invasion of certain highly invasive cancer cells lines could be inhibited by using inhibitors reducing the actomyosin-dependent contractility such as the myosin light chain kinase inhibitor (ML-7), the Rho-kinase inhibitor (Y27632), or latrunculin A (actin-polymerizing inhibitor).\textsuperscript{19,21,30} In addition, it has been shown that also the apical polarization of the cells in 2D culture plays a role in cell migration, because when the polarization is reduced, the number of focal adhesions and stress fibers is significantly decreased, the functional role of focal adhesion proteins such as vinculin or FAK is fundamentally altered and certain proteins such as $\alpha$-actinin or myosin-II are enriched in stress fibers.\textsuperscript{98} Traction force microscopy results lead to the suggestion that in a 2D migration system, a lamellipodium actively pulls the rest of a cell through nascent focal adhesions newly established at the leading edge of the lamellipodium.\textsuperscript{99} However, 3D traction force microscopy reveals that cells inside a 3D extracellular matrix pull on nearby fibers of the matrix scaffold.\textsuperscript{43,46} In particular, pronounced matrix tractions occur close to active pseudopodial protrusions\textsuperscript{43} that pull with nearly equal forces at the leading and trailing edges of the migrating cell. As the release of the pseudopodia towards matrix collagen fibers can be asymmetric, this may then lead to a structural defect within the fiber scaffold normally at the rear of a migrating cell. Due to these results, it seems to be likely
that pseudopodial protrusions at the trailing edge of migrating cells are released first and thus, pull the rear of the cell forwards through the 3D extracellular fiber matrix scaffold\cite{100, 101} leading to persistent guided motion similar to the migration through a tunnel.

In contrast, the motion of cells in 2D is less persistent as this migration does not need to form a tunnel through which the cells walk through\cite{102}, the migrating cells secrete proteins or even whole cell parts to mark their migration path and maybe to chemically attract following migrating cells. As pseudopodia such as filopodia play a probing role in 3D extracellular matrices, they have no function on 2D substrates, where the extracellular environment is more uniform (Fig. 4). The pseudopodial protrusion activity in 3D extracellular matrices is regulated by focal adhesion components. For example, the migration speeds of p130CAS knock-out cells and zyxin knock-out cells can be correlated with the number of protrusions such as filopodia generated per time in 3D extracellular matrices\cite{90}. In more detail, the p130CAS knock-out cells move more slowly and zyxin knock-out cells more rapidly compared to their control wild-type cells in 3D extracellular matrices, whereas these knock-out cells exhibit the opposite motility phenotypes on 2D planar substrates (Fig. 4). The behavior of the vinculin knock-out cells has been shown to be similar to the migratory behavior of p130CAS knock-out cells depending on the dimensionality of the migration system\cite{50–52, 103}. Thus, the role of focal adhesion proteins in 2D motility systems cannot serve as a predictive model of their migratory role in the more physiologically relevant 3D migration systems. In particular, even the rate of filopodial protrusions formation does not seem to correlate with migration speed in 2D systems, whereas the rate of pseudopodial protrusions seems to be correlated with invasion speed in 3D migratory systems\cite{90}. These results lead to the following questions: does protrusion dynamics play a role in 2D migratory systems? Does protrusion dynamics play solely a role in 3D migratory systems?

Recently, another invasion mode has been reported for cancer cells that possess a relatively soft cytoskeleton. This invasion mode seems to be different from the well-known mesenchymal and amoeboid migration modes. In more detail, these soft cells use a pulsating migration mode in which slow and random migration appears for a long time and is suddenly transformed to short-lived pulses of fast and directed migration which interrupt the slow migration mode\cite{74}. Maybe this mode can be explained by the lobopodial invasion mode (Fig. 4), which has to be determined. These findings raise the question whether the EMT transition is still suitable for describing the migration of cancer cells in 3D matrices. Additionally, the soft cancer cells are surrounded by relatively stiff normal cells that migrate slow and a limited, little distance\cite{74}. How this interaction between the soft cancer cells and the stiff normal cells may appear is suggested, but still under investigation. The fast migration periods of these special cancer cell migrations can be induced by myosin-II-dependent deformation of their soft nucleus evoked by the transient crowding of the neighboring normal cells with stiff nuclei\cite{74}. Moreover, these neighboring stiffer normal cells can migrate due to cadherin-facilitated mismatch adhesions between
normal cells and cancer cells (less cadherins), but their movement is limited by the residual $\alpha$-catenin-mediated cell-cell adhesions such as homotypic E-cadherin adhesions between neighboring normal cells. These findings may explain the pulsating mode of cancer cell migration as these cancer cells cannot bind to normal cells and possess other mechanical properties.\(^7\)

In addition, these mechanical properties of cancer cells do not solely affect their own functions such as cell motility, however, they also regulate the motility of normal cells such as fibroblasts, which are then no longer hindered in their migration through strong cell-cell adhesion bonds. However, while this precise regulatory mechanism is still under investigation, its recovery may shed light on the initial process of the cancer cell spreading from the primary tumor site. As the initial spreading of cancer cells from the primary tumor involves the crossing of the basement membrane of the tumor, this may be a similar process compared to the crossing of blood or lymph vessels where cancer cells have to overcome the basement membrane of the vessels first in order to transmigrate (intrastate) into the particular vessel by overcoming or even by breaking down the endothelial cell lining barrier of the vessels. This is discussed in more detail in the next section.

9. Transmigrating Invasive Cancer Cells Regulate the Biomechanical Properties of the Endothelial Cell Lining

The role of the endothelial cell lining of blood or lymph vessels on the regulation of cancer cell invasiveness into a 3D extracellular matrix is still elusive. The regulation of cancer cell transmigration is a complex scenario that is not yet fully characterized. In numerous previous studies, the endothelium has been reported to act as a passive barrier against the invasion of cancer cells.\(^104,105\) In more detail, the endothelium has been found to decrease pronouncedly the invasion of cancer cells and hence, finally, cancer metastasis.\(^106\) In contrast to these numerous reports, several recent reports propose a novel paradigm in which endothelial cells actively regulate the invasiveness of certain cancer cells by increasing their dissemination through vessels\(^107\) or by enhancing the invasiveness of cancer cells into 3D matrices.\(^4\) In particular, although several adhesion molecules have been identified to play a role in tumor-endothelial cell interactions and hence they even promote metastasis formation, however, the role of endothelial cell’s mechanical properties during cancer cell transmigration and invasion are still elusive. It has been suggested that altered mechanical properties of endothelial cells may support one of its two main functions in cancer metastasis: they act either as a passive barrier or they serve as an active enhancer for cancer cell invasion. As a main biochemical pathway of the tumor-endothelial interaction, it has been reported that the involvement of cell adhesion receptors and integrins such as platelet endothelial cell adhesion molecule-1 (PECAM-1) and $\alpha v/\beta 3$ integrins play a role, respectively.\(^108\) As integrins are known to connect the extracellular matrix and the actomyosin cytoskeleton,\(^109-111\) the linkage between the adhesion receptor and the actin cytoskeleton is facilitated through
the mechano-coupling focal adhesion and cytoskeletal adaptor protein vinculin\textsuperscript{51} and additionally determines the amount of cellular counter-forces that maintain the shape of the cells, their morphology and stiffness.\textsuperscript{112} In particular, a broad biophysical approach to investigating the endothelial barrier break-down in the presence of co-cultured invasive cancer cells is still elusive. As microrheologic measurements such as magnetic tweezer rheology are well suited for the precise analysis of the endothelial cell’s mechanical properties such as cellular stiffness during the co-culture with invasive or non invasive cancer cells compared to mono-cultured endothelial cells, endothelial stiffness is found to be influenced by co-cultured cancer cells.\textsuperscript{69} In particular, highly-invasive breast cancer cells can influence the cellular mechanical properties of co-cultured human microvascular endothelial cells by reducing the stiffness of endothelial cells pronouncedly, whereas non-invasive cancer cells were not able to affect endothelial cell stiffness.\textsuperscript{69} In addition, nanoscale particle tracking method diffusion measurements of actomyosin cytoskeletal-bound fibronectin-coated beads being markers for structural changes of the intercellular cytoskeletal scaffold can be used to measure the actomyosin-driven cytoskeletal remodeling dynamics. Thus, we find that cytoskeletal remodeling dynamics of endothelial cells are enhanced in co-culture with highly-invasive cancer cells, whereas they are even not altered in endothelial cells co-cultured with non-invasive cancer cells.\textsuperscript{69} Finally, these findings indicate that highly-invasive breast cancer cells can alter actively the mechanical properties of co-cultured endothelial cells compared to monocultured endothelial cells, whereas non-invasive cancer cells were not able to alter the mechanical properties of endothelial cells. Thus, our results have provided for the first time an explanation for the breakdown of the endothelial barrier function of vessel wall monolayers and supported the special role of the neighboring endothelial cells surrounding primary tumors. Taken together, we have discussed how cancer cells can alter the mechanical properties in order to transmigrate through the endothelial cell lining of blood or lymph vessels. The next chapter raises the question or suggestion that endothelial cells may alter the mechanical properties of cancer cells are either reversible or non-reversible during their transmigration.

10. Do Endothelial Cells Alter the Mechanical Properties of Certain Invasive Cancer Cells?

Preliminary data of our group suggest that endothelial cells are indeed able to alter the mechanical properties of cancer cells. How long these alterations last have not yet been investigated. We hypothesize that these cancer cells, which transmigrated through the endothelial cell lining underwent massive morphological shape change followed by induction of signal transduction events after adhesion and transmigration through the endothelium. These may include the expression of genes that alter the mechanical properties of cancer cells. On the side of the endothelium, we have seen that these mechanical and biochemical alterations are there and seem to
last longer than the duration of the whole transmigration process of cancer cells. Moreover, these alterations may also broadly affect the endothelial lining through a mechanically-driven signaling process across the endothelial monolayer.\textsuperscript{113,114} On the side of the transmigrating cancer cell, the mechanical alterations of the endothelium may direct the cancer cell to the side of the transmigration process and may dictate the transmigration mode such as paracellular transmigration or transcellular transmigration. Whether this turns out to be true has to be further investigated in more detail.

11. Conclusions and Future Directions

The biomechanical interactions of cancer cells with their local microenvironment during the process of metastasis seems to be a key point in understanding the spreading of cancer cells from primary tumor sites and may also help to predict the overall survival rate of the patient more accurate. In particular, the physical and material properties of cancer cells regulate their migratory behavior and their transport through the human body after entering the blood or lymph vessels and hence, support or inhibit metastasis. Mechanical forces from the microenvironment may additionally regulate cancer cell motility (of epithelial origin) in the structurally complex extracellular matrix scaffold during invasion, intravasation and extravasation of cancer cells in and of the vascular system. Hence, insights into the role of physical and mechanical processes regulating metastasis can be a prerequisite for the development of new approaches for cancer diagnosis and treatment. Taken together, besides providing effective prognostic and diagnostic tools for therapies inhibiting metastasis, the knowledge of the role of biomechanics in cell motility may also inspire inverse strategies to promote wound healing in terms of connective tissue regeneration after injuries. The effects of key mechanical properties of the tumor microenvironment such as mechanical forces, stiffness, pore sizes and steric hindrances on cancer progression as well as the mechanical properties of stromal cells and endothelial cells on cancer cell invasion in general and after usage of therapeutic drugs have to be explored systematically. However, cutting-edge genetic or biochemical approaches need to be combined with novel and state-of-the-art biophysical measurements of cancer cell mechanics and the mechanical properties of the tissue microenvironment.

The effective combination of physics, molecular biology and biochemistry may provide the strength to reduce divergent effects of potential cancer drugs on cellular or organ responses in animal cancer disease models and cancer patients, and subsequently, may lead to more appropriate and efficient cancer treatments. The novel field of “physics of cancer” is currently rooted in biological physics and soft matter physics. The biophysics research is certainly more than simply serving as a sink for providing novel techniques for oncologists. Rather, it reveals novel aspects important for the understanding of cancer progression and helps to refine the functional pathways involved in cancer disease progression.
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