Role of integrin adhesions in cellular mechanotransduction

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Chapter 6

General Discussion

Cells and their surrounding extracellular matrix (ECM) form a continuous network that is in constant homeostasis. This dynamic equilibrium requires complex cellular feedback mechanisms including force feedback loops. The ability of cells to sense and respond to the mechanical cues from their environment and, vice versa, to apply forces onto their environment is called cellular mechanotransduction and has been implicated in both physiological and pathological conditions. The work in this thesis is aimed at elucidating the different cellular mechanisms that take role in cellular mechanotransduction. I mainly focused on integrin mediated cell-matrix adhesions that are known to play a critical role in this process [1] (chapter 1).

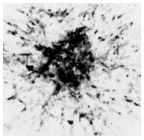
Studying cell matrix adhesions in different dimensionalities
In order to address the role of the cell-matrix adhesions in different contexts we made use of several techniques. 3D spheroid cultures allowed elucidation of the role of mechanotransduction at tissue level; whereas polyacrylamide gels with tunable stiffness and a cyclic cell stretcher allowed the study of the role of mechanical cues in cell function, and elastomeric PDMS micropillars with tunable stiffness allowed for measurements of cell traction forces.

In addition to developing state-of-the-art experimental techniques, extensive data (e.g. image) analysis was required. For this purpose,

scripts were written to recognize cells, nuclei, cell matrix adhesions, actin cables, collagen fibers, and determine aspects such as size and orientation. Additional computational analysis tools were developed for quantitative image analysis using information from PDMS micropillar displacements and dSTORM. Together these advances and implementations made it possible to perform this research and study the role of cell-matrix adhesions in cellular mechanotransduction.

How does the integrin composition of cell-matrix adhesions affect cellular mechanotransduction?
Alterations in expression levels of integrins have been previously related to activation of distinct cellular signaling mechanisms [2, 3] and (breast) cancer metastasis [4, 5]. It has also been shown that different integrins (e.g. those containing αv or β1 subunits) play distinct roles in generation of cellular traction forces [3, 6]. In chapter 3, we show that cells adhering to fibronectin either through integrin αvβ3 or α5β1 apply comparable levels of traction forces and are able to sense differences in environment stiffness. This appears in contrast to an earlier report showing that the complementary regulation of myosin II activity and mDia-mediated actin polymerization by these two integrins is required for rigidity sensing [3]. One way in which our work differs from this study is the fact that we expressed these integrin to levels supporting similar adhesion efficiency instead of equimolar levels. Moreover, all cells used in our model expressed some level of αv, providing a very different model system from the one used by Schiller et al [3].

We find that the integrin expression profile affects the orientation, rather than the amplitude of traction forces (chapter 3). This is accompanied by differential regulation of cytoskeletal architecture through the activity of Rho-ROCK signaling. Our findings demonstrate that cells, via altering integrin composition of their adhesions, are able to tune their inside-out force generation and outside-in force sensing possibly through Rho GTPase signaling pathways. We observe that expression of β1 integrins supports formation of long actin filaments resulting in higher centripetal orientation of forces, whereas expression of αvβ3 supports formation of shorter actin fibers, more random traction forces, and it allows cells to more robustly respond to external mechanical cues; e.g. more effective reorganization of actin cytoskeleton upon cyclic stretch and cell spreading and adhesion formation at softer substrates. Interestingly, αvβ3 frequently emerges with cancer invasion and tumor angiogenesis



[7]. The distinct properties of this integrin in regulation of mechanotransduction as identified by us, may contribute to these aspects of cancer progression.

Regulation of the molecular composition of cell matrix adhesions with traction force application

The relationship between the molecular composition of cell matrix adhesions and force has not been unraveled. In chapter 4 of this thesis, localization analysis of super resolution images obtained with direct stochastic reconstruction microscopy (dSTORM) allowed us to quantify the number of talin, paxillin, vinculin and focal adhesion kinase (FAK) molecules in cell matrix adhesions. By combining this method with traction force microscopy we were able to obtain a quantitative relationship between the molecular composition of the adhesion and force application. We observed that there was a 1:2:2 relation with force induced recruitment of talin:paxillin:vinculin molecules on a relatively stiff substrate, whereas no relation was observed between force application and number of FAK molecules. Given the role of talin, paxillin, vinculin and FAK in transducing the force from integrins to the actin cytoskeleton [8] as well as their role in regulating the actin cytoskeleton by signaling through RhoGTPases [9], these findings indicate that changes in force levels alter adhesion mediated signaling and actin mediated force feedback control.

It has been shown that phosphorylation of paxillin and FAK as well as the interaction between and talin and vinculin are force dependent [10–12]. Combined with these findings, our data indicates that FAK phosphorylation rather than FAK recruitment is related to increased traction forces. Interestingly, lowering the stiffness of the substrate leads to dramatic change in the stoichiometry of the investigated proteins within adhesions and we show that this is mainly due to a reduced force associated with vinculin. This demonstrates that environmental stiffness modulates the relation between traction forces and the molecular composition of cell matrix adhesions. Others have demonstrated that vinculin can be recruited by paxillin without vinculin activation or by talin, leading to vinculin activation and binding to actin fibers [13]. Together with our findings, this suggests that soft substrates support force-induced vinculin-paxillin interaction leading to a pool of inactive vinculin molecules. Instead, more rigid substrates support force-induced talin-vinculin interaction leading to vinculin activation and coupling to the actin cytoskeleton and hence, a much higher force induction per re-

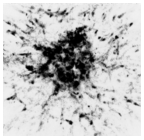
cruited vinculin (in chapter 1 force dependent vinculin-talin and vinculin-paxillin interactions are discussed in more detail).

We investigated a small subset of cell matrix adhesion proteins. Other molecular force sensors, including the integrins, are also present in cell-matrix adhesions as discussed in chapter 1 of this thesis. In order to fully understand the underlying molecular regulation of force-feedback control, force dependent abundance and activity of all these proteins as well as other possible candidates need to be addressed. Where antibodies are available, our dSTORM-based approach can be applied and results may be integrated with current ongoing proteomics analyses of cell matrix adhesions [14] in order to get an overview of cell matrix adhesion dynamics in relation to traction force. This is highly relevant as alterations in force feedback mechanisms can result in- and drive pathologies.

Quantification of molecules from dSTORM images: the next step in super resolution microscopy?

The method we used in chapter 4 to obtain the number of molecules from the dSTORM image can be readily applied to any super resolution image given that there is significant signal amplification (i.e. multiple localizations observed per protein). In chapter 4 we addressed any possible shortcoming or error in quantifying the number of molecules with our method apart from possible antibody under-labeling. Having used high concentrations of both primary and secondary antibodies, we believe that the numbers we reported are the best estimates possible with current technologies.

As dSTORM can be performed with commercially available antibodies no genome editing is necessary for application of our method. Therefore it does not suffer from risks associated with gene tagging such as alterations in protein localization, activity, and expression levels. Additionally, since the effect of labeling and photophysics on different localizations obtained during one acquisition cycle will be theoretically the same, and our method only relies on the positional information, it can readily be applied to the localization distributions without any prior knowledge of the setup used. Lastly, and uniquely to our approach, the fraction of a given protein in a given area (e.g. a cell matrix adhesion) undergoing certain post-translational modifications (e.g. protein phosphorylation and ubiquitination) can be addressed with this technique by using general and modification-specific antibodies against a protein of interest.



Consequences of cellular force application for cancer progression- remote collagen network orientation

The complex interplay between tumor cells, tumor stroma and the surrounding extracellular matrix (ECM) has been shown to play an essential role in tumor progression [15]. In chapter 2 we observe that tumor cells orient a collagen network through ROCK mediated contractility. Expression of β1 integrins and ROCK signaling has been implicated in tumor progression through matrix crosslinking [16]. Here, in agreement with earlier reports from others and us [4, 5], depletion of β1 integrins has very different effects depending on the cell type. Our findings suggest a direct relation between tumor expansion/cell migration and collagen reorganization that is not determined by the expression of β1 integrins. Other collagen receptors (e.g. syndecans and discoidin domain collagen receptors) or integrins binding to other ECM proteins might be important for this relation instead.

Previously, isolated cells have been shown to sense the presence of other cells up to ∼100 µm in collagen environments [17]. This was attributed to the alignment of fibrous collagen matrix induced by the cells. In our system we observed distant orientation of the collagen up to 2.5 mm. This distant orientation of collagen cannot be explained by local cellular secretion or degradation of collagen as the length scale over which we observe collagen orientation is way beyond tumor expansion areas, up to 5 times the tumor radius. This indicates that propagation of forces applied by the multicellular tumor spheroids over long distances through the fibrous collagen environment drives remote collagen alignment.

Consequences of cellular force application for cancer progression- a role for remote collagen network orientation in tumor angiogenesis?

Chemical signaling, mainly vasculature endothelial growth factor (VEGF) signaling, and its crosstalk with physical signaling have been studied in angiogenesis. It has been shown that physical cell-ECM interaction affects cellular response to VEGF [18, 19] as well as regulating VEGF expression [20, 21]. Additionally the physical properties of the matrix can alter the sprouting response to VEGF stimulation [22]. Our findings in chapter 2 further indicate that the physical signaling from the tumor cells can promote long distance directional tumor-angiogenesis. How this physical signaling compliments or controls VEGF chemical signaling remains unknown. Blocking VEGF receptor signaling or inducing

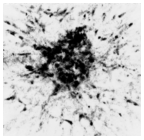
VEGF gradients in combination with sequential printing of tumor and endothelial cells in ECM scaffold can be used to address this question. Still, in chapter 2 we show that disruption of the mechanical connection between the primary tumor and the vasculature cells is sufficient to impair the directionality of vasculature cells. *In vivo*, such physical signaling by the tumor may help guide angiogenesis and thereby promote cancer progression. Therefore, interfering with mechanical tumor-ECM communication is a promising candidate approach to interfere with multiple aspects of progression including cancer growth, invasion, as well as tumor-angiogenesis.

Common signaling pathways regulating cell migration, adhesion size and cellular traction forces

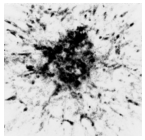
In chapter 5, using 2D screens we identified 11 candidate adhesome genes that regulate cell migration and adhesion dynamics. Performing time-lapse force measurements on 4 of these candidates has shown that in addition to causing impaired cell migration and larger cell-matrix adhesions, knockdown of these genes resulted in a general trend for increased traction forces and slower force turnovers. The relation of force to adhesion size and migration has been also studied previously [23, 24]. The family of Rho GTPases and downstream ROCK signaling might play an essential role in this relation as the orchestrators of actin cytoskeleton. In chapter 2 of this thesis we have shown that high ROCK activity, in addition to formation of longer actin fibers and centripetal force generation in cells discussed in chapter 3, supports tumor expansion and tumor induced collagen reorganization. In chapter 5 we further show that high traction forces and slow force turnover, which suggest high ROCK activity, is observed in combination with impaired cell migration. Our findings together indicate that cell migration, adhesion formation and force generation are interrelated and ROCK signaling has an important role in this relation.

Concluding remarks

It follows from Newton's law of motion that force generation is essential for cell migration. Even in assays where anchorage independent migration of cells was characterized, the force generation mechanism has been shown to be necessary for cell motility [25]. Hence it is not surprising to see the altered adhesion structures, cell migration and force application being related. Rho GTPases can regulate all three of these cellular mechanisms and focusing on the cell protrusions shows how Rho GTPases can



be dynamically mediated in a molecular level [26]. This regulation, together with other signaling pathways, controls mechanosensing at the molecular and multicellular level and plays an important role in both physiological and pathological conditions. With the work presented in this thesis, further understanding of molecular signaling in control of cellular mechanosensing as well as the role of mechanosensing in cancer has been achieved.



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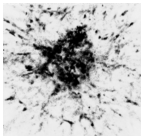

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