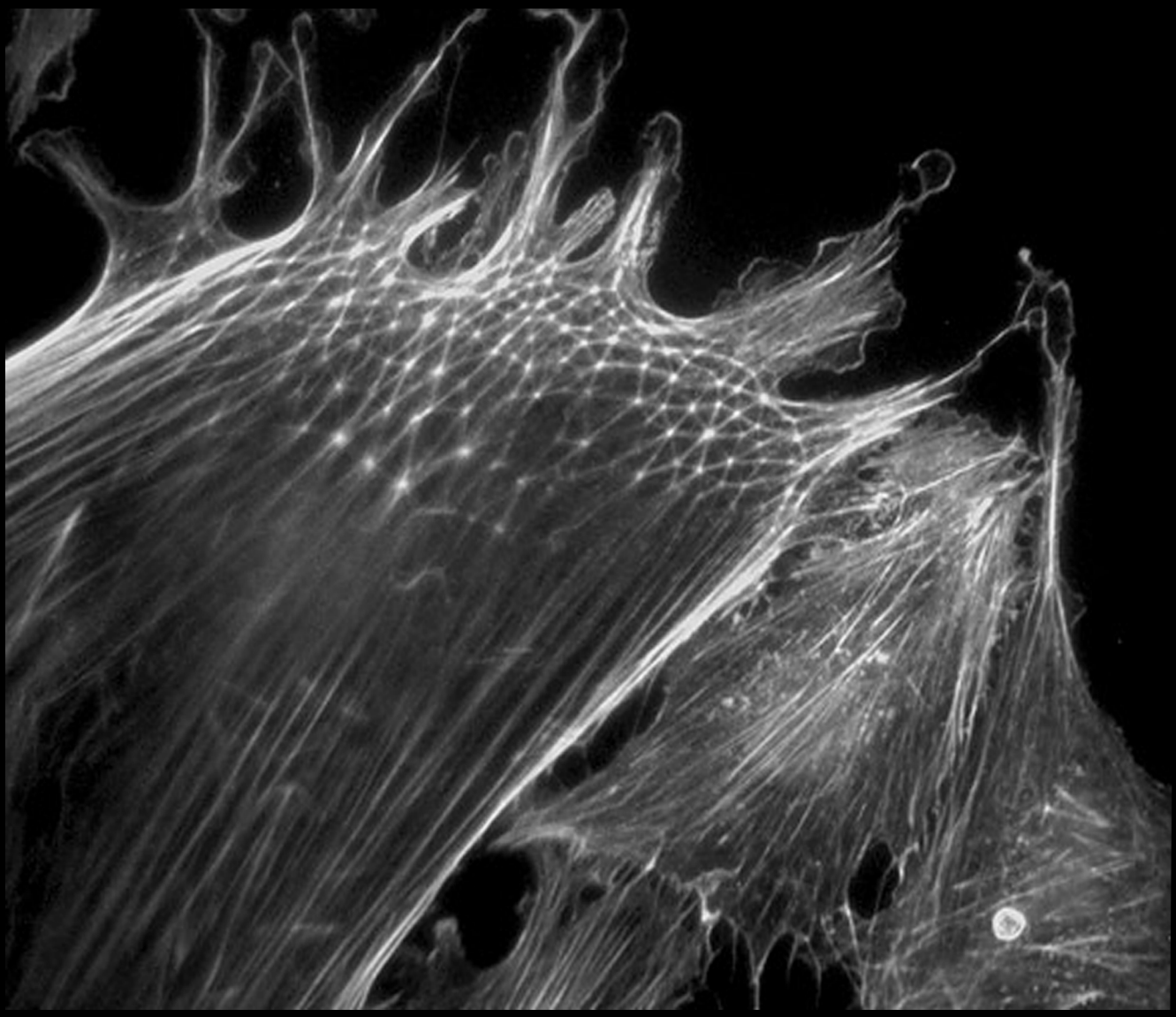
Mechanotransduction via Integrins

Functions in adhesion, cellular tension and cell fate



Tessa Gaarenstroom
Master Thesis - Cancer Genomics & Developmental Biology
Supervised by dr. C. Rabouille
Department of Cell Biology, UMC Utrecht

Abstract

Overview

I – Integrins and cellular tension
- I.1 *Integrins – extracellular domain*
- I.2 *Integrins – intracellular domain*
- I.3 *Tensegrity*
- I.4 *Cytoskeletal prestress*
- I.5 *Forces and tension*
  - Intracellular forces – Actin contractility
  - Extracellular forces – Extracellular matrix
- I.6 *Integrins as mechanotransducers*

II – Integrins as mechanotransducers
- II.1 *Mechanotransduction and global effects*
- II.2 *Local strengthening responses to applied forces*
- II.3 *Force and cell spreading*

III - Mechanotransduction and signalling – Focal adhesion dynamics
- III.1 *The role and regulation of Rho GTPases*
- III.2 *Integrin signalling and FA dynamics*
  - Rac activation and FA turnover
  - Adhesion strengthening
- III.4 *Movement and regulation of focal adhesions during migration*

IV - Mechanotransduction and signalling – Cell fate
- IV.1 *Cell adhesion and cytoskeletal tension dictate cell fate*
- IV.2 *Integrins and apoptosis*
- IV.3 *Mechanotransduction in cell cycle progression*
  - Integrin-associated signalling components
  - Cytoskeletal contractility and small GTPases
- IV.4 *Integrin crosstalk with growth factor signalling*
- IV.5 *Integrin influence on the gene level*
  - Transcriptional control
  - Translational control

V – Mechanotransduction during development and cancer
- V.1 *Mechanical control of tissue development and differentiation*
- V.2 *Extracellular forces and cancer progression*
  - Extracellular forces and proliferation
  - Metastasis
  - Breast cancer

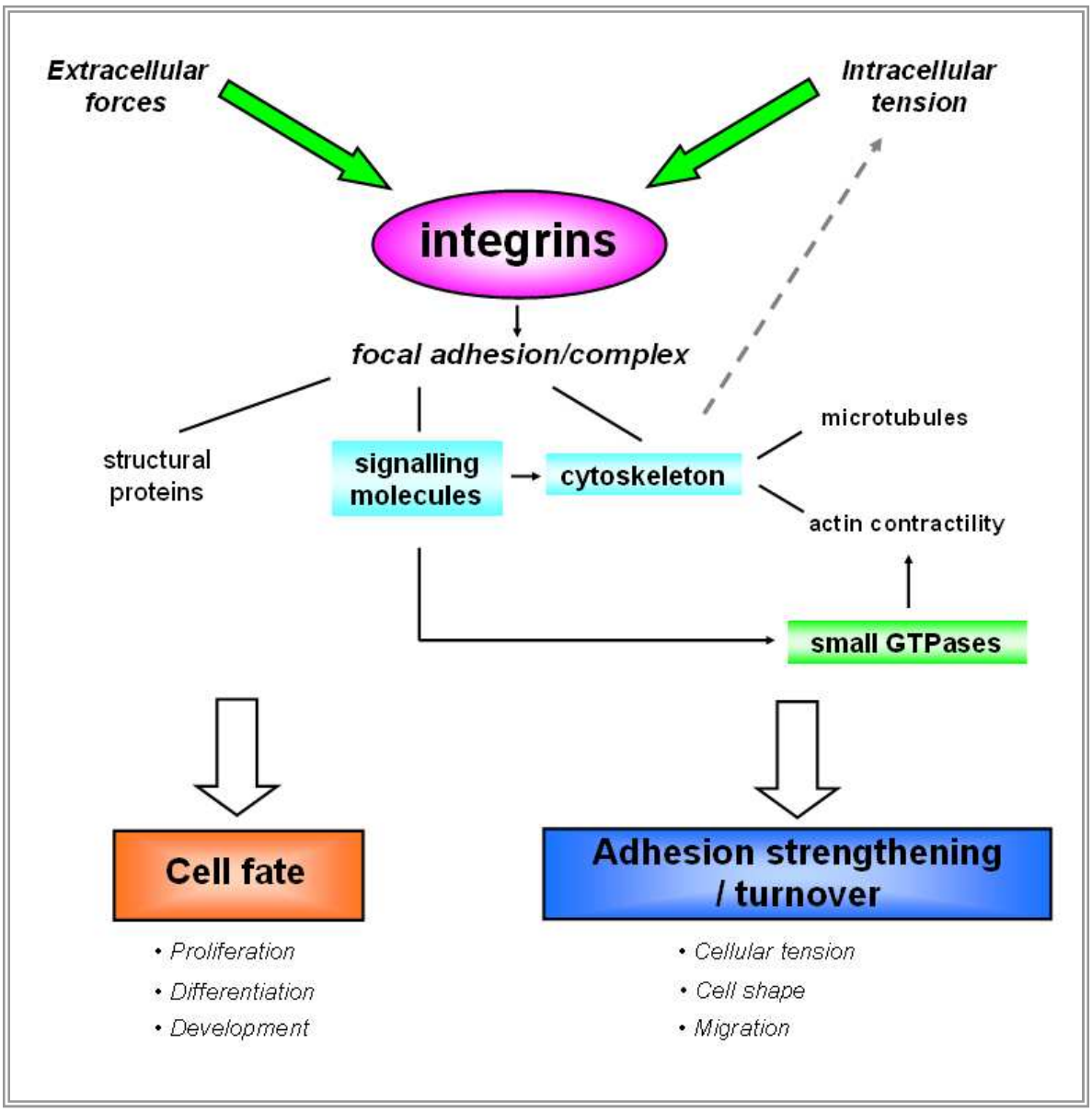
Conclusions

References

Abstract

During life and development, tissues and individual cells within an organism are under several types of mechanical stress. Forces are being exerted upon cell-matrix adhesions both from the extracellular environment and from within the cell, as the cytoskeleton actively generates tension. These mechanical cues are efficiently transduced via integrins, receptors for extracellular matrix components which are found in adhesive sites and are connected to the actin cytoskeleton. Through modulating the strength of these focal complexes and focal adhesions, as well as remodelling the cytoskeleton, forces from within the cell are balanced by those encountered on the outside, thereby balancing the tensional state of the cell. It appears that increased forces upon integrins correlate with greater intracellular tension via actomyosin contractility, as well as greater cell spreading. This occurs via the actions of small GTPases which balance actin polymerization and contractility, leading to adhesion modulation, while microtubules act as compressional struts to balance the generated tension. In addition to tensional and adhesive remodelling in response to applied forces, mechanotransduction via integrins also influences cell fate. In this work we look at the evidence that integrins efficiently transduce mechanical stresses and aid in the tensionally balanced state of the cell. Furthermore, we outline some general signalling mechanisms via which forces upon integrins affect processes as widespread as migration, proliferation, differentiation and apoptosis. It appears that downstream signal transduction impinges on most general growth and survival pathways, and that adhesion-mediated signalling is essential for a physiological tissue state. This therefore emphasizes the importance of mechanical tension sensing via cell-matrix adhesions in all cells, and perturbations in either integrins or their associated signalling and cytoskeletal machinery, as well as disturbed extracellular forces, can lead to pathological situations and malignancy.

Overview



Integrins receive mechanical forces, both from outside and inside the cell (green arrows). These transmembrane receptors are present in adhesive complexes termed focal adhesions or focal complexes, which contain signalling molecules and structural proteins that connect the integrins to the cytoskeleton. The cytoskeleton, which plays an important role in the generation of intracellular forces, is regulated by small GTPases, modulating contractile abilities. These in turn are receptive to modulation by integrin-associated signalling molecules, and thus forces transmitted through integrins feed back to modulate the cytoskeleton and intracellular tension. In addition, integrins can signal in response to mechanical forces to adjust focal adhesions, for instance during cell migration. Finally, mechanosensation via integrins cooperates with growth factor signalling pathways, thus influencing cell cycle progression and cell fate.

Chapter I - Integrins and Cellular Tension

Tissue integrity is essential for the survival of every multicellular organism, as is the controlled growth and differentiation of individual cells within these tissues. Cells need to maintain controlled cell shape and often adhere to both neighbouring cells and to the extracellular matrix (ECM). In this manner, they provide shape and function to the tissue they are part of. Via these cell-cell and cell-ECM adhesions, cells are able to withstand forces exerted onto them.

Individual molecules, cells and tissues are exposed to several kinds of mechanical forces and stress. Examples of load-bearing events on a greater scale are muscles tensed over bones or shear stress exerted by blood flow against the blood vessel wall. Also, morphogenesis events during development can occur along changes in tissue shape or position by exerting mechanical forces upon them [7]. On a cellular level, forces from the ECM act upon the cell, and conversely the cell can also exert forces upon the environment. These mechanical cues must be converted into biochemical signals, to ensure a correct cellular response to changes in tension [5].

The contacts between the ECM and cells are very dynamic, as they can be robust and strong, or can consist of transient and dispersed interactions, facilitating either tight adherence or migration of cells. The main transmembrane molecules involved in cell-matrix interactions are integrins, which with their extracellular domain bind various ligands in the extracellular environment. Integrins are clustered together in focal complexes or focal adhesions, and are linked to both the cytoskeleton as well as various signalling components. In this way, they are able to transduce mechanical signals from the environment to the intracellular machinery, inducing cytoskeletal and signalling responses.

I.1 *Integrins – extracellular domain*

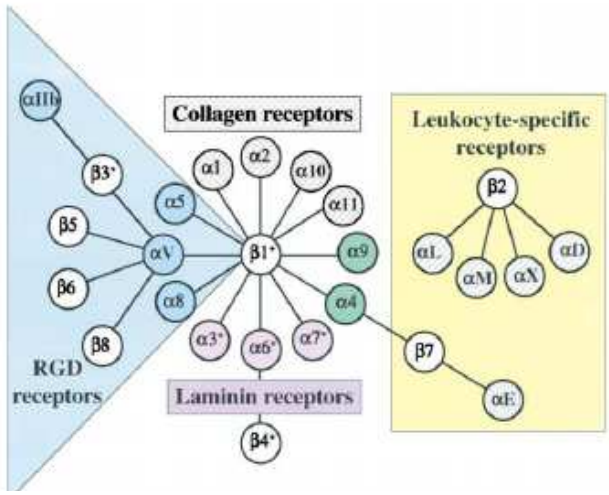


Figure 1 *– Schematic overview of the interaction of the different α and β chains to form functional integrin heterodimers. These can then be subdivided in different classes, based on the substrate they recognize.[1]*

Integrins are transmembrane proteins which form heterodimers, composed of an α and β chain. Different classes of these 18 α and 8 β chains can associate together and form several possible combinations, dictating ligand specificity or cell-type specific expression. For instance, certain integrins are expressed by leukocytes, while others play a role in wound healing, and yet others are necessary for epithelial adhesion to the basement membrane. The α and β subunits interact and bind to their ligands in the extracellular matrix via their extracellular domains, which contain 'head' regions which can be folded in several states of activity [8]. A schematic overview of the different integrin heterodimers and their ECM ligands are shown in **figure 1.**

Most integrins recognize laminin or

the RGD peptide sequence present in several matrix molecules, such as fibronectin and vitronectin, but various other sequences in ligands are bound by specific subsets of integrins [1]. Leukocyte integrins can also bind cell surface molecules of the Ig superfamily, and integrins involved in wound healing, when activated, bind to proteins such as Von Willebrand factor or thrombospondin. Cells *in vitro* are often cultured on vitronectin, which is abundant in serum, or on glass coated with fibronectin, and the major integrin present in the adhesions to the substrate is thus $\alpha 5\beta 1$ [8].

As integrins are involved in all cell-matrix, and some cell-cell adhesions, both their expression and activation must be well controlled. For instance, integrins are often deregulated in cancer, as they determine the ease by which cells migrate or can anchor in distant tissues. Activity regulation occurs by conformational changes, integrin clustering, lateral diffusion or cytoskeletal changes, and the cytoplasmic tails have been shown to be essential for this [8, 9]. Not only can this 'inside-out' signalling activate integrins, adhesion to a substrate itself can also increase the affinity, and thus the activity, of the integrins present [10]. It is likely that there are several intermediate states of activation as well, where full activation is required to separate the two cytoplasmic tails which facilitates the integrin's own signalling function. Certain aspects of this 'outside-in' signalling will be the main focus later on.

I.2 *Integrins – intracellular domain*

The properties of a cell to adhere to a certain substrate is not only dictated by the type of integrin expressed and its activation state, but the manner of integrin distribution over a cell membrane plus the associated proteins on the cytoplasmic face are of importance as well. Integrins can be loosely dispersed over the membrane, or are present in adhesive complexes termed focal complexes (FC) or the more mature and stronger focal adhesions (FA). While the extracellular domain binds to ECM components, the intracellular domain is connected to the actin cytoskeleton. Most integrins possess a short cytoplasmic tail, which is associated with up to 50 different proteins on the cytoplasmic side, serving different functions [11].

One class of these proteins are scaffolds which link integrins to the actin cytoskeleton, examples of which are talin, vinculin, filamin and α-actinin. The actin cytoskeleton can exist as dispersed networks or as contractile filament bundles, and functions to provide cell shape and generate an inward-directed tension. A second type of proteins within FA's are actin binding proteins, which get recruited by the first class and play an important role in actin polymerization and FA assembly. Examples are tensin, profilin, zyxin and VASP. Finally, the focal adhesion contains several signalling elements, which facilitate integrin signal transduction, such as FAK, paxillin, p130cas and Src kinases [12, 13].

Integrins are present in focal complexes which can mature into focal adhesions, which takes about a full hour to complete. FA's are elongated streak-like

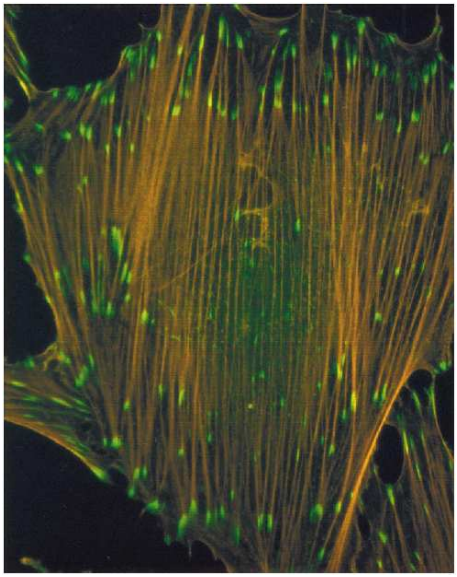


Figure 2 *– Image of a rat fibroblast, showing the focal adhesions (green) and actin stress fibers connected to them (red). These are aligned to the tension in which the cell exists [2].*

structures of 3-10 μm in length, and associated with actin stress fibers (**figure 2**) which exert a contractile force upon the adhesive complex. They are found on cells which are spread out and adhering tightly to the underlying substrate. FC's in contrast are 0.5-1 μm sized clusters of integrins, which mediate weaker adhesive forces and are often found on membrane extensions of migrating cells. In addition, FA's contain more cytoplasmic proteins than focal complexes, although talin and vinculin are still the first to get recruited to adhesive sites [14, 15]. We will look at the formation of FC's and FA's and their increase in adhesive strength in chapter III, and discuss the role of both intra-and extracellular force in these processes.

Because of their association with both the ECM and the cytoskeleton, as well as various signalling molecules, integrins play an important role in transferring tension which can be generated both intra- and extracellularly. This is termed 'mechanotransduction', as a mechanical signal is converted into a biochemical one within the cells. Conveying these forces then mediates the appropriate cellular response, which can be cell shape changes, adhesion modulation, migration and even growth or apoptosis. We depict integrins in adhesive complexes as bi-directional signalling components, which can alter endogenous tension and cell fate. Before we go into the results that support the role of integrins as mechanotransducers, and signalling mechanisms by which integrins adjust cellular responses, we will first describe how cells exist in a state of tension, balancing their intracellular force with that encountered in the environment.

I.3 *Tensegrity*

Virtually all cells experience forms of mechanical stress from their environment, and have to constantly adjust to these changes in the extracellular matrix by reorganizing their cytoskeleton and signalling pathways within the cell. Several models have been proposed which envisage how cells sense and respond to mechanical forces, often depicting the cell membrane or cortex as the major load-bearing component, while the cytoplasm is viewed as a viscous, elastic substance [16]. Another model is that of the 'tensional integrity', or tensegrity, which we will focus on during the rest of this work. This envisages the cell as an integrated system, where the different cytoskeletal components are linked together and forces applied locally are conducted through the entire cell. The properties of individual molecules are of importance, but it is equally significant to realize how they work together in a hierarchical, integrated system. Tensegrity views systems, and thus cells, as structures 'that stabilize their shape by continuous tensions or tensional integrity rather than continuous compression' [16], which means that continuous tensile forces are balanced by local compressional struts, which maintains the shape of the structure because it is in a state of balanced stress, called 'prestress'. In a model in which compression-bearing elements are supported by tension, global shape change will occur when force is

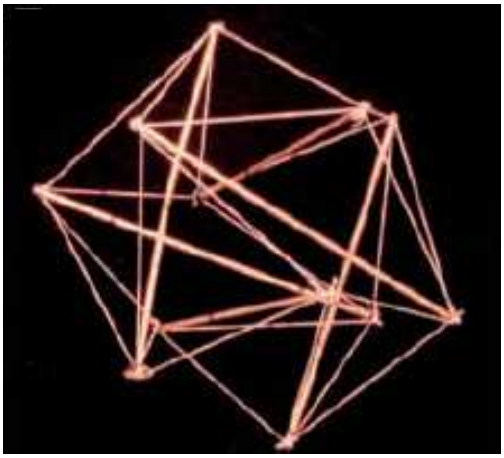


Figure 3 *– Schematic representation of a simple tensegrity structure, consisting of 6 struts bearing compression and 24 elastic cables under tension, where local deformation will result in global shape change. This structure can be used to model the cellular tensegrity, where actin microfilaments are represented by elastic cables, and microtubules by the struts [3].*

applied locally, for instance when a model as in **figure 3** is stretched or suspended from one end, while the tensional balance within the entire structure remains intact.

The cytoskeleton consists of components which fulfil the criteria of the tensegrity model: microtubules are rigid, compression bearing elements, while actin microfilaments can bear tension and actively prestress the entire cell by actomyosin contractions. Intermediate filaments interconnect the other two elements throughout the entire cell and provide stiffness. Finally, cell adhesions to the ECM as well as osmotic pressure further stabilize the cell and add to the prestress in the structure. The cytoskeleton in the cell and its extracellular adhesions are dynamic systems, where filaments are constantly (de)polymerizing and new connections are being formed and broken down, however, as long as local compression and continuous tension are required for stability, it will remain a tensegrity structure. In this manner, cells exist in a state of isometric tension, balancing mechanical loads imposed on it [16].

I.4 *Cytoskeletal prestress*

Microtubules are the most likely candidates to act as internal compression struts balancing the generated tension, because they are inherently rigid and stiff due to their hollow organization [5]. While microtubules *in vitro* can appear as rigid, straight structures, in cells they usually buckle near the cell cortex as they polymerize [17]. To confirm that the MT's indeed buckle because they bear compression due to the inward-directed actin contractility, laser incision of a bent microtubule fiber shows it snaps back into a rigid position when it loses its end-on impingement [18]. Furthermore, MT buckling increases when actomyosin contractility is stimulated, and decreases when the tensile actin network is disrupted, which is more evidence that MTs function as compression struts in living cells [17].

The actin cytoskeleton is responsible for generating the contractile forces which continuously tense the entire cell, a force balanced by the microtubules. Actin fibers adhere to contacts with the ECM such as focal adhesions, and generate an inward-directed tension via actomyosin contractility. Indeed, several experiments measuring the amount of pulling forces a cell exerts on the ECM, show that in the absence of MTs this tensile force is increased. In contrast, disrupting actomyosin contraction decreases it, which then leads to a decreased cellular prestress and stiffness [17, 19, 20]. Cells which are spread out over a large area have been shown to generate a higher intracellular tension due to higher actin contractility. Thus, the level of prestress and also the 'stiffness' within a cell correlates with cell

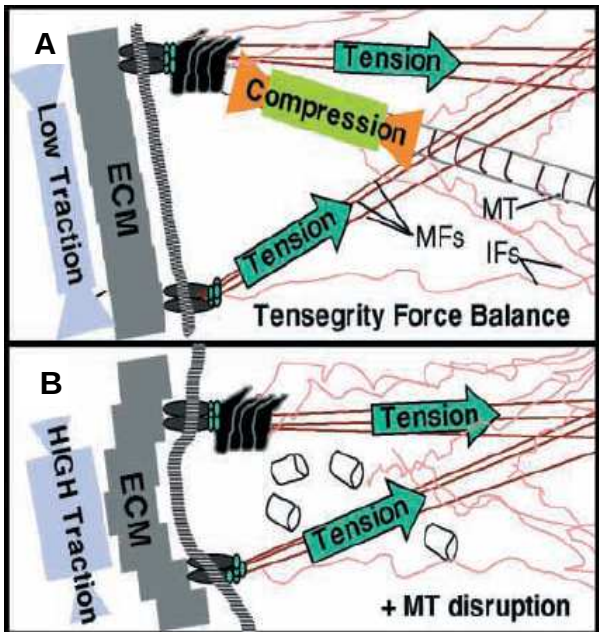


Figure 4 *– Drawing of the different cytoskeletal components and their role in prestressing the cell. In (A), intact microtubules outwardly stabilize the cell and balance the inward directed tension generated by the contractile actin cytoskeleton. The cell therefore generates low traction (pulling) forces on the ECM, while in (B) MTs are disrupted, leading to a net increase in contractile tension, resulting in the cell to exert a greater tractional force on the ECM via its adhesions [6].*

spreading [21]. As we will discuss in a later chapter, this can have profound effects on cell fate.

Finally, intermediate filaments have been shown to structurally integrate different subcellular structures as well as tensionally stiffening and stabilizing microtubules [5].

I.5 *Forces and tension*

We have now confirmed that cells exist in a continuous balanced state of tension called prestress, which responds to forces exerted onto it by changing this tensional balance and adjusting cell shape. Two types of forces can be distinguished: intracellular and extracellular.

Intracellular forces – Actin contractility

Intracellular forces are generated by the contractile actin cytoskeleton, via the actions of the motor protein myosin II. Actin-myosin interactions promote filament sliding, bundling of actin filaments into stress fibres, and thus tension generation. The key molecule involved in this process is the small GTPase Rho. Its two family members, Rac and Cdc42, play antagonizing roles and function in actin polymerization and membrane extensions, instead of the inward-directed contractile forces generated by Rho [4]. Intracellular force generation determines the cellular prestress, which leads to focal adhesion remodelling and influences cell fate (see chapters III and IV).

Different intra-and extracellular cues modulate Rho activity, and likely these mechanisms work in concert to ensure full activation of the small GTPase when it is required [22]. Rho activates Rho associated kinase/ROCK and Dia1, of which the former can be linked directly to the activation of myosin II driven contractility. ROCK is required for the phosphorylation and activity of myosin light chain (MLC), and relieves its repression by inhibiting MLC phosphatase [23]. In addition, it indirectly activates profilin, which is necessary for actin stabilization [24]. Dia1 plays a more complex role, mediating the Rho signal under circumstances of external force application. Studies from yeast show that this protein

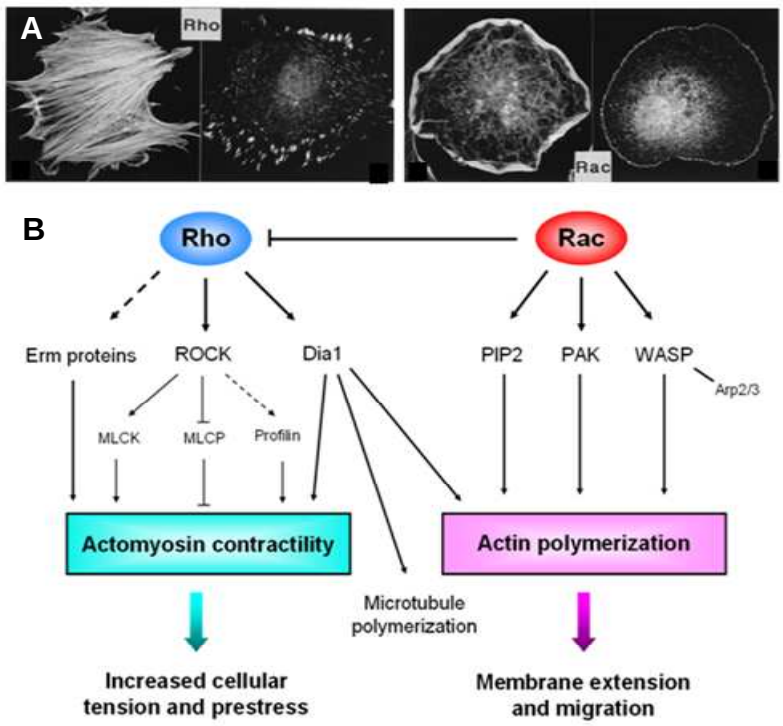


Figure 5 *– Cytoskeletal effects of small GTPases Rho and Rac. (A) Cells stained for actin filaments (left) and the FA marker vinculin (right). Rho activation induces stress fiber and FA assembly (left panel), while cells containing ectopically active Rac extend lamellipodia (right panel) [4]. (B) Schematic overview of downstream effectors of and interplay between Rho and Rac. In general, Rho signals toward actomyosin contractility while Rac favours actin polymerization. Rho thus induces cellular tension, leading to an increase in prestress, while Rac acts more locally to extend membrane processes, playing an important role in migration.*

induces linear actin polymerization, but Dia1 can also regulate microtubule dynamics, stabilizing the ends. So while ROCK induces contractility and thus intracellular force generation, Dia1 plays a more dual part, also mediating negative feedback [14]. Though not direct targets of Rho, proteins of the ERM family have also been shown to play a role in actin contractility and stress fiber assembly, possibly by linking the actin filaments to the cell membrane [4].

Rho antagonist, the small GTPase Rac, may function to inhibit Rho directly and therefore reduce actin contractility, favouring membrane extension instead of contraction [14]. The mechanism by which Rac transduces its signal for actin cytoskeletal organization is not entirely clear. Several possible targets are PIP2, WASP proteins and PAK, all involved in actin polymerization and lamellipodial extension: PIP2 induces focal complex formation and actin polymerization, WASP cooperates with the FC component Arp2/3 and also stimulates actin polymerization, while PAK inhibits actin depolymerizing proteins [4, 25]. In addition, PAK mediates stress fiber and focal adhesion disassembly, and will be addressed shortly in a later section. A scheme depicting the general downstream actions of Rho and Rac onto the actin cytoskeleton is shown in **figure 5.**

Extracellular forces – Extracellular matrix

Cells are constantly exposed to forces from the environment. An example is the shear stress mediated by bloodflow upon endothelial cells, but the ECM itself also generates tension, which is efficiently transduced into the cells via adhesions. In addition, manipulating cells *in vitro* by allowing cells to bind to beads or microneedles and subsequently pushing, pulling or twisting these, also mimics forces exerted upon them. Examples of these techniques will follow in the next chapter, and we will now shortly focus on forces generated by the ECM itself.

Rigid cell environments exert higher tension on cell-matrix adhesions than compliant, softer tissues. Integrins as mechanotransducers can sense the force a rigid ECM exhibits, and remodel their cytoskeleton to adjust the intracellular tension to that encountered on the outside, a process which again depends on Rho [26-28]. It has been shown that cells cultured on rigid matrices express and activate integrins at a higher level, thus leading to increased focal adhesion assembly and increased Rho-dependent cytoskeletal contractility. A positive feedback loop between ECM rigidity, Rho, cell contractility and subsequent cellular behaviour then ensues [29]. This has been efficiently shown in vitro by coating cells on matrices of increasing rigidity (**figure 6**).

A rigid ECM exerts stronger forces upon adhesive sites, which results in a higher intracellular tension and thus in greater cell spreading and FA assembly [30]. Therefore, one can imagine cell migration is directed by matrix compliance. Indeed this has been shown to be the case,

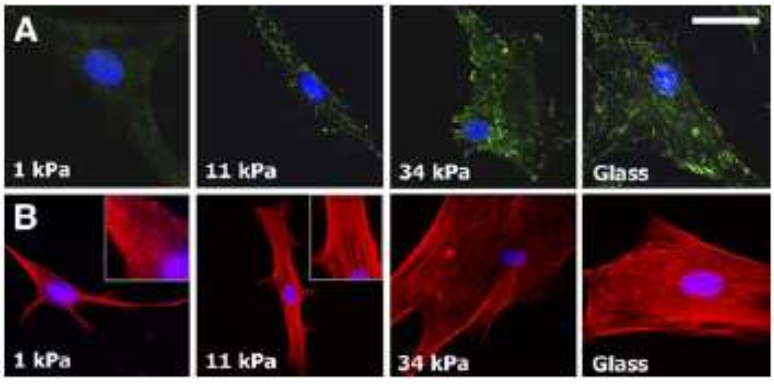


Figure 6 *– Extracellular matrix rigidity increases cellular tension. (A) Cells grown on matrices of increasing rigidity exihibit more and larger focal adhesions, as shown by staining for paxillin (green). (B) In addition, actin contractility increases, exemplified by the change from dispersed actin into stress fibers (red staining) [6].*

as cells tend to migrate towards stiffer matrices, and direction of movement can be manipulated by applying strain to one side of the cell. This process is called durotaxis [31].

I.6 *Integrins as mechanotransducers*

Thus far, we have described integrins as cell-ECM adhesion molecules, which can be expressed in a variety of subunit combinations which dictate ligand specificity and cell-type expression. Their intracellular association with the actin cytoskeleton and many proteins within focal complexes and focal adhesions facilitates their role in strengthening adhesive sites and propagating intra – or extracellular mechanical forces, contributing to a balanced prestressed state of the entire cell.

For a cell to behave like a tensegrity structure, forces must be transmitted throughout the entire cytoskeleton, as far as the nucleus and ECM, so local stresses result in global rearrangements in order to reach a new equilibrium in the compression-tension lattice and maintain cell shape. Adhesions and interactions with the ECM are critical for a cell to balance the different forces and exist in a so-called state of 'isometric tension'. These connections are provided by integrins, which we propose function efficiently as mechanotransducers of any force exerted upon the cell from the environment, thereby mediating a global structural and biochemical response to a local stress applied to it [16]. Several studies have addressed this mechanotransduction in more detail, and shall be considered in the next chapter.

Chapter II – Integrins as Mechanotransducers

As discussed in the previous chapter, integrins are good candidates to act as transducers of mechanical stress, coming both from within the cell itself due to actomyosin contractility, and from forces from the cellular environment. It has been shown that focal adhesions are dependent on either type of force, and their size generally correlates with the magnitude of the tension exerted onto them [25]. These forces are transduced throughout the entire cell, which occurs via cytoskeletal connections, and the cell can respond by both stiffening this cytoskeleton, as well as strengthening the adhesions with the ECM. Therefore, both global and local effects are observed when integrins, but not other transmembrane receptors, are manipulated by exerting different types of forces upon them.

II.1 *Mechanotransduction and global effects*

Integrins can transduce externally applied mechanical stresses to the cytoskeleton and influence the state of prestress of the cell. Using magnetic beads coated with integrin ligands, it is possible to measure the force exerted by the cell onto the bead, and thus the cytoskeletal stiffness. Mechanical shear stresses can be applied when these beads are bound by integrins by magnetically twisting them, after which prestress can be measured by the degree in which cells allow the beads to be twisted. It was found that efficient transmembrane force transfer takes place, as the cells become stiffer and resist mechanical deformation at higher levels of applied stress. In addition, focal adhesions are formed when integrins bind to RGD-coated beads, the substrate recognition sequence All three cytoskeletal components appear to be necessary for efficient force transfer, which suggests that the linear response in cytoskeletal stiffening to applied stress is an integrated response [32].

If local tension applied via focal adhesions on the cell surface is indeed transduced throughout the entire cytoskeleton, this should reach the nucleus as well. Indeed, using the same magnetic bead twisting device to apply shear stresses to integrins, displacement of intra-nuclear structures can be observed. These displacements correlate with the level of prestress, and thus actin stress fibers are important for the force transfer to take place [33]. Similar results are obtained when ECM-coated micropipettes are used, which bind integrins and apply stress when these are pulled away. This leads to nuclear movement and shape changes, while the actin cytoskeleton aligns along the direction of stress. Taken together, the results suggest that integrins act as mechanotransducers, propagating local stresses via the cytoskeleton towards to the nucleus. [34].

Another way to visualize whether local stresses applied to focal adhesions result in global cytoskeletal arrangements, is to look at movement of fluorescent cytoskeletal components, or indirectly, microtubule-associated mitochondria. These structures are indeed displaced, even when they are on the other side of the cell from the point where the integrin-bound RGD coated beads are pulled. This indicates that forces are transmitted via integrins onto the actin cytoskeleton and subsequently via interconnection onto the microtubules, and are then transferred throughout the entire cell [17, 35].

II.2 *Local strengthening responses to applied forces*

The examples thus far indicate that local force application on integrins is efficiently transduced throughout the entire cell and has effects on the state of tension of the cell, a process dependent on the interconnection of all cytoskeletal components. However, applying mechanical stresses on integrins also has local effects, notably in the maturation and formation of focal adhesions and thus their strengthening. Focal complex formation can be induced by either internal or external forces. For instance, allowing integrins to bind to large beads induces actomyosin contractility, followed by an enrichment of FC marker vinculin at the adhesive sites, as well as a strengthening in adhesive force. Using small beads but applying mechanical tension upon these by optically trapping them with a laser, has the same effect. Thus, initial interactions mediate force transmission from either inside or outside of the cell, which leads to FC maturation and subsequent strengthening of the ECM-integrin-cytoskeleton linkage [36].

When integrins in focal complexes bind ECM components, this leads to elongation of the structures and maturation into focal adhesions, a process dependent on actin polymerization [37]. Integrin clustering and activation leads to increased stiffness of integrin-cytoskeleton connections, as can be seen by the ease by which RGD-coated beads can be pulled away from the adhesion site with a magnetic microneedle. This stiffness depends on the recruitment of FA and cytoskeletal proteins to the site of attachment, thus adjusting the micromechanical properties of individual focal adhesions [38]. Other researchers also found that integrin binding to fibronectin stiffens the link between integrins and the cytoskeleton, in proportion to the density of the substrates. This reinforcement was shown to be a local effect, and depended on occupancy of the integrin-fibronectin binding site [39].

These different experiments indicate that integrins transduce forces coming from both outside and within the cell, and respond by stiffening their link with the cytoskeleton, as well as forming mature focal adhesions. This ensures that cell shape and the tensional balance is maintained when local stresses are applied.

II.3 *Force and cell spreading*

Cell spreading, cytoskeletal prestress and focal adhesion assembly are tightly linked, as one is the prerequisite for the others via Rho-mediated actomyosin contractility. As an example, it was found that not the density of ECM substrates, but the total area over which a cell is spread dictates total FA assembly, which was shown by coating individual cells on islands of differing sizes, thereby controlling cell spreading. In addition, individual focal adhesions were organized in an asymmetric manner, with a higher density of FA proteins on the distal side of the ring-like structure, relative to the cell centre. This is likely due to a greater pulling force being exerted further away from the cell centre, leading to positioning of actin stress fibers to the sites of highest tension. Note that in this experimental setup, intracellular forces generated by actomyosin contractility dictate focal adhesion formation. This shows that cell shape controls level and direction of cytoskeletal tension, which modulates FA assembly [40].

Looking further into the asymmetry of individual focal adhesions, growth and formation of FA's has been observed to take place in the direction in which the stress is applied, whether this originates from the ECM or comes from force generated by cytoskeletal contraction. This suggests that elastic deformation of adhesion sites due to either internal or external mechanical forces induces a biochemical reaction which leads to the (dis)assembly of focal adhesion components in

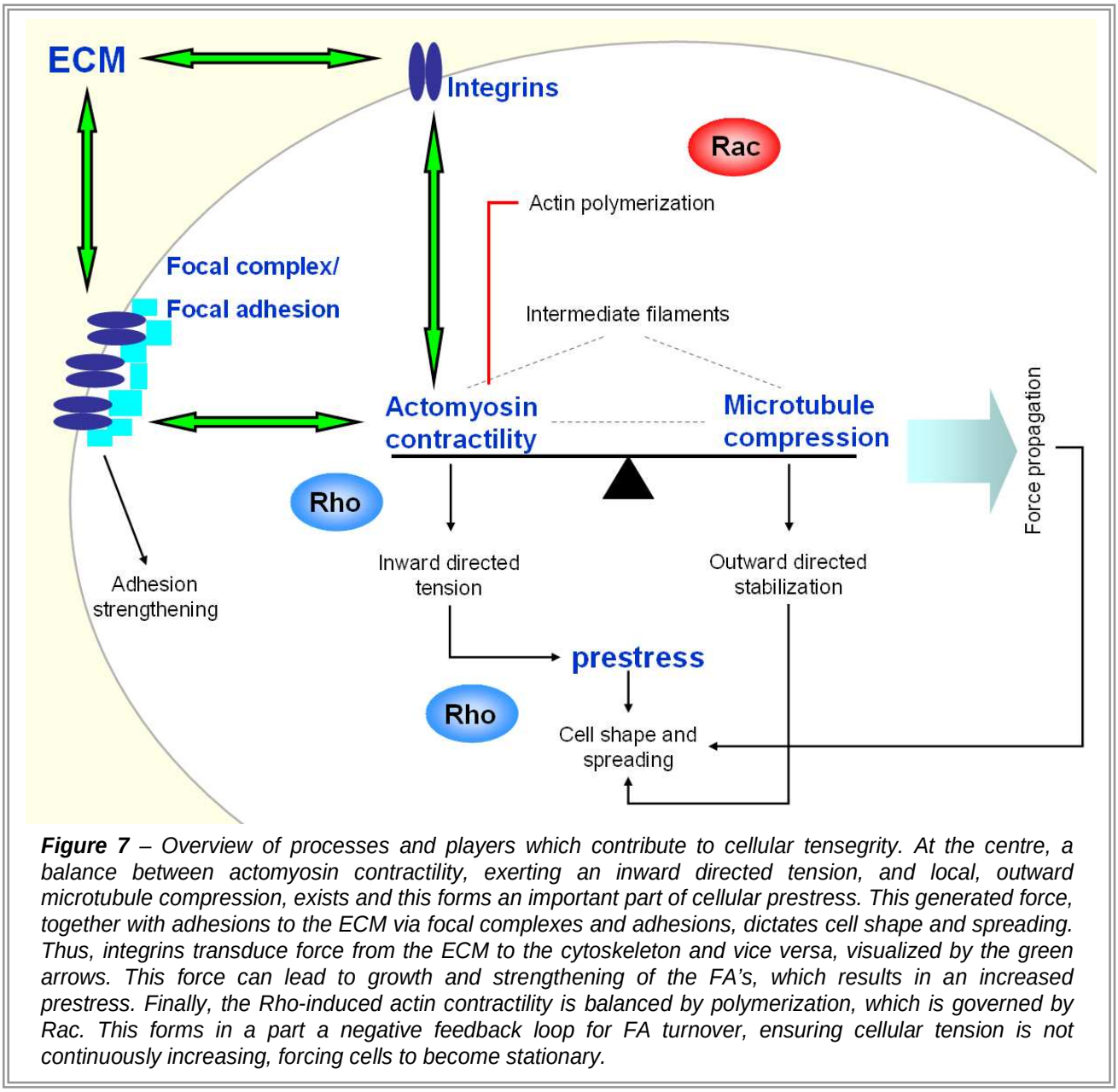


Figure 7 *– Overview of processes and players which contribute to cellular tensegrity. At the centre, a balance between actomyosin contractility, exerting an inward directed tension, and local, outward microtubule compression, exists and this forms an important part of cellular prestress. This generated force, together with adhesions to the ECM via focal complexes and adhesions, dictates cell shape and spreading. Thus, integrins transduce force from the ECM to the cytoskeleton and vice versa, visualized by the green arrows. This force can lead to growth and strengthening of the FA's, which results in an increased prestress. Finally, the Rho-induced actin contractility is balanced by polymerization, which is governed by Rac. This forms in a part a negative feedback loop for FA turnover, ensuring cellular tension is not continuously increasing, forcing cells to become stationary.*

the direction of the applied force [41]. Taken together, intracellular force generated by cell spreading induces FA formation, with an internal asymmetry which aligns the adhesions to the tension.

The results described above indicate that indeed integrins function as mechanoreceptors and transmit applied forces to the interconnected cytoskeleton of the entire cell. While many of these experiments show that stresses are transmitted from the ECM to the cytoskeleton in either a local or global tension-dependent response, some also indicate that intracellular forces generated within the contractile cytoskeleton can be exerted on adhesions with the ECM, influencing cell spreading. In both cases, integrins within focal adhesions act as the key mechanotransducers and ensure the maintenance of the cellular state of isometric tension, thus governing cell shape. By adhering to the ECM, they add to the continuous transmission of tensional forces within the contractile cytoskeleton and between the cell and its environment, a tension which is balanced by compression

bearing elements, mostly in the form of microtubules. These findings are schematically summarized in **figure 7**, which shows the interplay between the main components, maintaining and adjusting cellular prestress.

One of the effects of exerted force upon integrins is their subsequent strengthening by maturing into a FC or FA. This shows that a mechanical cue is converted into a biochemical signal, which via a signalling cascade leads to (dis)assembly of FA's, ensuring maintenance of the tensional balance within the cell. In the next chapter, we discuss the signalling components involved in more detail.

Chapter III - Mechanotransduction and Signalling: Focal Adhesion Dynamics

Focal adhesions have been shown to be dynamic structures, continuously (dis)assembling and being sorted into different integrins and intracellular proteins. Focal adhesions depend on the mechanical forces exerted on them, generated by actomyosin contraction. Similarly, they depend on forces from the ECM, which can be determined by the relative rigidity of the substrate that the cells are grown on [22]. As has been mentioned in the previous chapter, both intra- and extracellular forces applied to integrins mediate focal adhesion assembly and adhesion strengthening. The mechanical stresses are transduced through the cytoskeleton and signalling machinery to induce integrin clustering and recruitment of FA proteins to the sites of applied force. While initial responses such as strengthening of integrin-cytoskeletal linkages or resistance against mechanical bead displacement can be passive, later adjustments always involve biochemical changes within the cell [38]. For instance, integrin-cytoskeleton strengthening can be blocked by the use of a phosphatase antagonist, implying the need for dephosphorylation steps and thus signalling cascades in this process [39].

Individual focal adhesions can be envisaged to be self-accelerating, growing with applied force and leading to the incorporation and connection of more contractile actin fibers, thus increasing the tension even more. In contrast, migrating cells receive mechanical signals from the environment, yet need to break down strong adhesions in order to be able to move over the extracellular matrix. Therefore this process is highly regulated and dependent on several molecules, among which are small GTPases regulating actomyosin contractility [14]. While multiple signalling inputs activate these, we will focus on the role of integrins and microtubules on the activation of small GTPases Rho and Rac, and how these signals lead to focal adhesion strengthening or disassembly.

III.1 *The role and regulation of Rho GTPases*

Cytoskeletal contractility and polymerization is regulated by small GTPases Rho, Rac and Cdc42. Mechanical stresses acting upon integrins influence especially the first two, where strong forces will eventually lead to Rho-mediated contractility and strengthening of focal adhesions. However, the mechanism by which integrins activate Rho is complex, as this depends on integrin and cell type. It appears that the activation is biphasic: initial contacts with the ECM inhibit Rho activity but activate Rac and Cdc42, which is desirable when nascent contacts with the ECM are made during membrane extension; while strong attachments later on during cell spreading correlate with high Rho activity [22]. In general, it appears that the relatively weak and small focal complexes depend on Rac, while the large and stronger focal adhesions, associated with contractile actin stress fibers, are induced by Rho.

Rho stimulates contractility, the formation of stress fibers, integrin redistribution and the phosphorylation of focal adhesion proteins [42]. As discussed in the first chapter, the downstream signalling of Rho to activate Myosin II occurs through ROCK and Dia1, which in turn can lead to focal adhesion strengthening due to the generated intracellular force. In contrast, both Rho and ROCK generated actomyosin contractility can be bypassed by the application of external force upon integrins, thus still mediating adhesion maturation. This indicates that Dia1 can drive FA assembly

induced by both cellular and external forces. This could indicate a parallel pathway to myosin-driven actin contractility, possibly via Dia1's functions in actin polymerization or microtubule-associated protein delivery to maturing focal adhesions [37].

Rac has antagonistic functions to Rho in FA assembly and contractility, favouring membrane extension instead of contraction. The fact that Rac is associated with cytoskeletal relaxation implies that integrins could initially activate this GTPase upon ECM binding, and somehow in later stages switch their signalling to activate Rho. Indeed, integrins have been shown to activate Rac locally via several guanine exchange factors, and facilitate coupling to its effectors [43]. Cross-talk between Rac and the formation of new adhesive sites at the leading edge of migrating cells exists [14]. In addition, Rac is responsible for shear stress-mediated cytoskeletal alignment to the direction of the flow, which is mediated by integrins in focal adhesions [44]. Rac is mainly involved in the formation of focal complexes (FC), as was shown by accumulation of the FC marker vinculin upon addition of large fibronectin-coated beads to cells. The binding of integrins to the beads induced intracellular contractility, thereby strengthing ECM-cytoskeletal linkages via integrins in a Rac-dependent manner [36].

Taken together, integrins can activate both Rac and Rho, but likely do so in a sequential order to ensure different responses. In early stages, Rac stimulates membrane extensions by focal complex formation and actin polymerization, but counteracts the functions of Rho, which gets activated at a later time point. These encompass stimulating actomyosin contractility via ROCK and Dia1 and focal adhesion formation, so likely the increased intracellular forces on integrins in FA's stimulate further Rho activation. We will now look into further detail at the signalling components associated with the mechanosensory function of integrins, and how these lead to the strengthening or turnover of adhesive sites via Rho and Rac.

III.2 *Integrin signalling and FA dynamics*

Integrins mediate mechanical signals from the environment and influence cellular signalling pathways to induce the appropriate reaction. This requires binding to ECM ligands, inducing a conformational change in integrins to activate several downstream signalling components [45]. Both intra- and extracellular forces applied to integrins can then be converted into a biochemical signal, which influences maturation, strengthening or disassembly of the adhesive sites. While focal adhesions consist of more than 50 different proteins, a few of the signalling functions in mediating focal adhesion dynamics have been elucidated.

FA's contain several tyrosine kinases and phosphatases, as well as scaffolding proteins and molecules that link integrins to the actin cytoskeleton. Applying strain onto integrins leads to phosphorylation of FAK, Src, p130cas and paxillin, and these can transduce the mechanical signal to the cytoskeletal machinery and FA components to influence the (dis)assembly of these structures, the strengthening of the connection and mediate migration. These responses mostly occur via the small GTPases Rho or Rac. It appears that applying mechanical force upon integrins has contrasting effects on adhesion strengthening of disassembly, where the cell type and signalling context determines the exact outcome. However, we briefly outline a few important molecules in either process, which is schematically depicted in **figure 8.**

Rac activation and FA turnover

Several of the integrin-associated kinases such as Src and FAK have been shown to induce FA disassembly [14, 22]. FAK is assumed to be the canonical kinase which gets recruited to activated integrins and autophosphorylated upon their clustering and binding to the ECM. FAK activity correlates with cell spreading, and thus with cytoskeletal tension [46]. This facilitates Src binding, which can then phosphorylate FAK at other sites, and together these kinases can phosphorylate several other substrates [47]. An example of Rac activation via integrins occurs by recruiting paxillin to activated FAK, and both this protein and Src have been shown to be upstream signalling components of several Rac GEFs [48]. Indeed, integrins are able to activate Rac locally and facilitate coupling to its effectors [43], several possible GEFs in this case being Vav2, DOCK180 and PIX [48].

FAK can inhibit contractility and FA formation by inhibiting Rho: Through Src, integrin binding activates the GTPase activating protein p190RhoGAP, which mediates Rho conversion to the inactive, GDP-bound state [49]. FAK knockout cells show prominent stress fibers and aberrations in cell shape and migration, and these defects are due to an inability of the cell to disassemble focal adhesions. Without FAK, Src is not activated or recruited to focal adhesions, nor is p190RhoGAP. This therefore leads to constitutive Rho-ROCK-MLC activity, and the subsequent increased contractility would then result in focal adhesion formation and their inability to turn over [50].

In addition, FAK directs PAK to focal adhesions, where it inhibits contractility [47]. PAK is a kinase mediating stress fiber and focal adhesion disassembly, which forms a complex with, among others, a Rac GEF and can then inactivate MLC kinase. Indeed, without FAK, PAK fails to be recruited or activated at FA's [50]. Another mechanism for PAK localization and activation occurs downstream of Rac, which gets localized in response to adhesions to the ECM [43]. In summary, FAK and Rho appear to be balance each other, as active rho induces focal adhesions and FAK signalling, while FAK can negatively regulate Rho, and in addition inhibit myosin II via PAK,

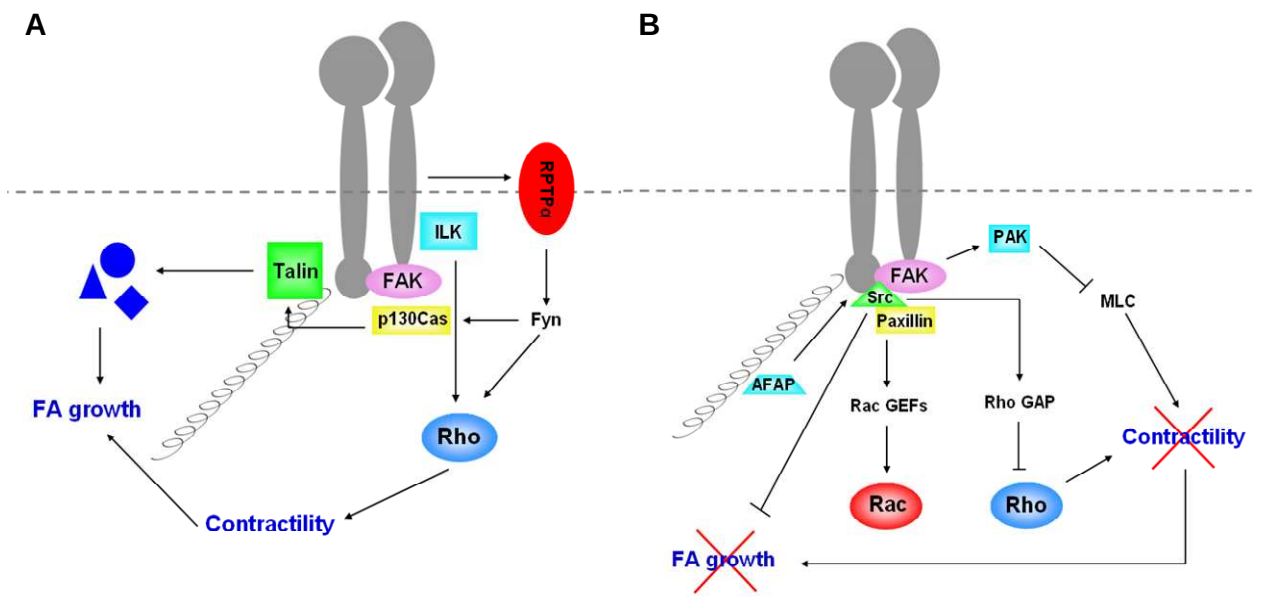


Figure 8 *– Integrin signaling towards FA turnover or strengthening. (A) Integrins can signal via associated kinases, phosphatases or structural proteins, which indirectly activate Rho. Rho then induces cytoskeletal contractility and FA growth, leading to an increased prestress. (B) Cytoskeletal contraction and FA formation can also be inhibited by integrin-associated components. This occurs either directly or via stimulation of Rac, but also via inhibition of Rho and it's downstream signals.*

thereby decreasing actin contractility.

Turning to another integrin-associated kinase, members of the Src family also play a role in focal adhesion disassembly. Using laser trapping experiments of vitronectin-coated beads, it was found that c-Src inhibits vinculin assembly in growing focal complexes, indicating that it negatively regulates strengthening of the integrin-cytoskeletal connection upon external force application [36]. Similar results were obtained by Felsenfeld et al., who showed that in the absence of Src, cells coated on vitronectin reduced spreading and motility, while adhesion and cytoskeletal force was increased. While Src is thus an inhibitor of force generation through vitronectin receptors, integrin binding to fibronectin is not affected [51]. c-Src activation can occur via FAK, however another possible candidate is AFAP, an actin-associated molecule which has been shown to bind directly to c-Src and is necessary for its stretch-induced activation [52]. Alternatively, Src might interact directly with clustered β integrin tails, which exposes its activation site [53].

Another protein kinase which gets activated downstream of integrins is PKA, and this occurs via release of cAMP, dependent on mechanotransduction via integrins and G-protein signals [54]. PKA is responsible for decreased actin contractility via the inhibition of both Rho and myosin light chain. Indirectly, it also activates Rac and Cdc42, thus further counteracting the contractile effect of Rho [48].

Adhesion strengthening

A feature of integrin signalling which appears to be in contrast with the ones described above is the strengthening of cell-ECM adhesions and focal complex maturation. Nascent binding of integrins to the ECM leads to strengthening of these initial interactions, and can grow into Rho-dependent focal adhesions. Depending on integrin type and cellular context, mechanical signals can thus also increase adhesive forces, and several of the signalling compounds playing a role in this process have been identified.

One of the Src family members, p130Cas, was found to activate Rap1 in a force-dependent manner [55], a GTPase which can signal through RIAM and Talin to adjust integrin confirmations, thus facilitating adhesion strengthening in response to applied force [56]. Talin is one of the scaffolds linking integrins to the actin cytoskeleton. In its absence focal complex formation in response to contacts with the ECM is delayed, and reinforcement of cytoskeleton-integrin connections does not take place. This is likely due to a failure of vinculin and paxillin to be recruited to the sites of adhesion, indicating that Talin is necessary for force-induced early adhesion formation and strengthening [57].

As mentioned in the first chapter, downstream effectors of Rac (PIP_2, WASP and vinculin/Arp2/3) are involved in actin polymerization, and act to recruit proteins to FA's which mediate adhesion strengthening. These molecules can also be activated directly downstream of activated integrins bound to ECM components [4, 48]. While it appears that integrins have several ways of activating Rac, it is not this GTPase, but Rho which induces strong FA assembly. A possible activating candidate is integrin linked kinase (ILK), which was shown to activate Rho and subsequent contractility and stress fiber formation after application of cyclic stress. However, not in all cases of contractility stimulation is ILK needed, indicating the existence of parallel pathways [58].

Not only tyrosine kinases, but also tyrosine phosphatases contribute to downstream integrin signalling.. These have many targets and in some cases appear to activate Rho and stimulate FA assembly, and in other experiments appear to have an inhibitory function. However, one example might explain the delayed Rho-stimulating effect of mechanosensation by integrins: RPTPα, a

receptor tyrosine phosphatase which associates with integrins, promotes cell spreading, focal complex assembly and adhesion strengthening. While initially c-Src is activated, which inhibits Rho via p190RhoGAP phosphorylation, after 45 minutes RPTPα activates Fyn, which indirectly stimulates Rho activity [48, 59]. Fyn, a Src family kinase, has been shown to be recruited to migrating cells' leading edges, where it can phosphorylate p130Cas, strengthening adhesive sites and facilitating cell spreading [26].

Taken together, it appears that integrins use the same signalling principles, and even some similar proteins, to induce either focal adhesions strengthening or disassembly. While the former process requires rho activation, inducting cytoskeletal contractility, FA turnover uses Rac-induced actin polymerization, which plays an important role during cell migration.

III.3 *Involvement of Microtubules*

Microtubules act as compression struts to counteract the contractile function of the actin cytoskeleton. While mechanical forces are transmitted via integrins leading to increased contractility, this also means microtubule dynamics are altered and these in turn can feed back to focal adhesion strengthening or disassembly. Microtubules have the ability to suppress contractility via a biochemical pathway converging upon Rho and Rac [60], so a mechanism by which localized MT polymerization regulates focal adhesion turnover appears likely.

Microtubule depolymerisation is associated with increased contractility via MLC phosphorylation [30]. Disruption of the MT's appears to activate Rho and thus stimulates contractility. This might occur via the release of MT-associated Rho GEFs such as Vav1, p190RhoGEF and GEF-H1, which are normally sequestered when MTs are stable [22, 61].

In contrast to contractility induction by microtubule depolymerisation, growing MTs can counteract this process. In migrating cells, membrane extensions are associated with high tension and thus focal adhesion assembly, which can increase tension and adhesive strengthening. However, when microtubules then grow into the extension, this interrupts the positive feedback loop and allows focal adhesions to be broken down, thereby facilitating migration. This relationship between microtubules and focal adhesions can be emphasized by the finding that stable MTs are associated with Rac activity, which is recruited by MT tip-binding proteins. Thus, local FA disassembly is partly due to the antagonistic relationship between Rac and Rho, which are provided by growing or shrinking microtubules, respectively [22, 61].

Microtubule growth and Rac activation stimulates lamellipodial extensions, which in combination with the finding that MTs can locally induce FA disassembly, aids in the polarized migration of cells. Feedback between focal adhesions, actomyosin contractility and microtubule polymerization exists via the actions of the Rho effector Dia1, which has been shown to induce MT polymerization along actin fibers into focal adhesions [61].

III.4 *Movement and regulation of focal adhesions during migration*

A clear example of a process involving FA turnover regulation is cell migration, where adhesions to the substrate are necessary, but adhesive complexes need to be constantly broken down and formed anew. In addition, cytoskeletal forces are necessary for a cell to propel itself forward over the ECM. In migrating cells, FA's usually remain static and fixed in one place while the cell moves over them, while in stationary cells focal adhesions are observed to move towards the cell centre due to

contractility in the cytoskeleton. The disparity of FA's in migrating cells is polarized, in a manner where they are formed at the leading edge, followed by a persistence zone in which they grow and mature, and turn over at the cell tail [22]. Adhesive sites at the lateral sides are motile and integrins are actively transported towards the extending lamellipodium [61, 62].

Membrane protrusions in the cell front, the lamellipodia, are regulated by Rac-induced actin polymerization. More towards the centre, traction forces due to Rho activity help the cell propel forward, while in the rear Rho plays a role in tail-retraction, which requires focal adhesions in that region [23, 61]. Cell-matrix adhesions in moving cells consist mostly of focal complexes instead of the larger and stronger focal adhesions [61]. The density of integrins within adhesive sites, as well as their dynamics and turnover, is dependent on cytoskeletal tension and small GTPases. Sites of high actomyosin contractility, mediated by Rho, correlate with high integrin density, high turnover and a sliding behaviour of the focal contacts at the cell rear of migrating cells. In contrast, the adhesive sites at the cell front, where Rac induces actin polymerization but cytoskeletal tension is lower, contain fewer integrins and these focal contacts remain stationary [63].

Chapter IV - Mechanotransduction and Signalling: Cell Fate

Mechanotransduction via integrins not only affects cytoskeletal organization and adhesion strength, but force-mediated signalling regulates processes as widespread as proliferation, differentiation and apoptosis. Besides signal transduction originating from activated integrins, cytoskeletal tension and cell spreading are also essential in mediating cellular responses to stimuli from the micro-environment. Because of their direct association with many signalling molecules, their close coupling to the cytoskeleton, and their ability to be modulated by inside-out signalling, integrins can integrate biochemical signals, adhesion, cytoskeletal organization and mechanical forces.

In this chapter, we discuss how cytoskeletal tension and integrin signalling affects cell fate decisions, independent of or in cooperation with biochemical signals emanating for the cell's microenvironment. Cell shape and adhesion can influence control over proliferation and death, and a role for integrins in dictating cell fate is in the modulation of signalling pathways which can have different outcomes depending on cell context, and thus integrin expression. So in addition, several concepts by which integrins act together with growth factor receptor signalling are outlined.

IV.1 *Cell adhesion and cytoskeletal tension dictate cell fate*

It has long been known that cells need anchorage to a substrate to proliferate, so cells in suspension become quiescent or undergo apoptosis. Cells which are adherent to soft gels, but do not display stress fibers and focal adhesions, also do not progress through the cell cycle. In contrast, adhesion together with cell spreading mediates a survival and proliferative response.

To further define to which extend cell spreading is necessary for life-death decisions, Chen et al. used a micropatterning technique. Cells can be cultured on adhesive islands of a defined size and shape, which are coated with a saturating density of ECM ligands such as fibronectin to which cells will adhere precicely (**figure 9**). When endothelial cells cultured in this manner are then subjected to soluble mitogens, those that are spread over the largest area and thus exhibiting the greatest tension, will grow and undergo cell cycle progression. In contrast, the cells that are cultured on a small island and thus prevented from spreading will undergo apoptosis. Those cultured on intermediate size islands will become quiescent and differentiate, indicating that

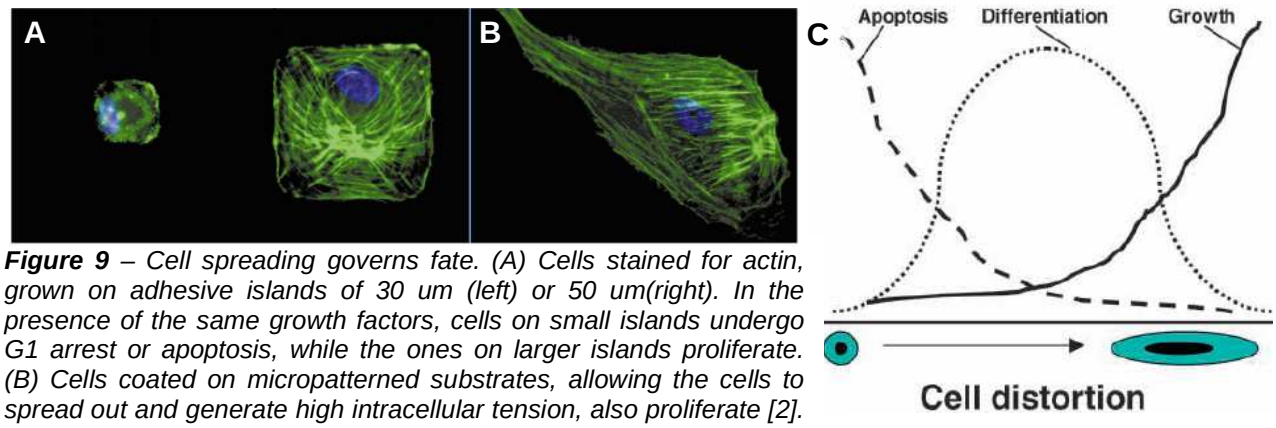


Figure 9 *– Cell spreading governs fate. (A) Cells stained for actin, grown on adhesive islands of 30 um (left) or 50 um(right). In the presence of the same growth factors, cells on small islands undergo G1 arrest or apoptosis, while the ones on larger islands proliferate. (B) Cells coated on micropatterned substrates, allowing the cells to spread out and generate high intracellular tension, also proliferate [2]. (C) Graph showing the correlation between cell fate and degree of spreading [6].*

mechanical distension is a critical determinant dictating cell behaviour to the same mitogen, though the nature of the adhesive substrate appears to modulate the response somewhat as well [46].

Thus, adhesion alone is not sufficient to induce cells to proliferate, but Rho-mediated cytoskeletal tension induced by cell spreading is needed in addition [2, 5]. Besides adhesion-mediated signalling, integrin-associated components can also be activated by mitogens, and these too result in activation of Rho and subsequent cell cycle progression [8]. It appears that both integrin ligand binding, cytoskeletal tension and an external growth stimulus are required to generate a proliferative output [2].

IV.2 *Integrins and apoptosis*

Most cells which do not adhere to a substrate enter apoptosis, a phenomenon termed anoikis for death due to anchorage loss. Apoptosis of fibroblasts can be induced by locally degrading the ECM, showing that the tension this exerts on the cells is essential for viability [64]. Furthermore, decreasing tension on collagen gels also induces cell death, a response which can be inhibited by activity of β1 integrins [65]. Thus, integrin signalling is important in modulating apoptotic signals, as expression or absence of certain subunits can increase or decrease anoikis rates depending on the presence of different extracellular signals. To further add complexity, the exact response is also highly dependent on cell type.

Apoptosis susceptibility depends on a balance between survival signalling pathways such as PKB/Akt or NFκB, and apoptosis-inducing factors originating from death receptor pathways and caspases; these can all be modified by adhesion-mediated signals. Several researchers have shown that in general, FAK signalling downstream of integrins is essential to mediate the anchorage-signal in normal cells and thus prevent apoptosis, a pathway which is often circumvented in tumour cells which have gained the ability to grow in suspension [8, 65, 66]. In addition, integrin-mediated adhesion activates survival pathways [65], and upregulates anti-apoptotic proteins of the bcl-2 family. Two of these are associated with the actin cytoskeleton, and may thus play a role in preventing spread cells from entering programmed cell death [3].

In contrast to protecting against cell death, studies in endothelial cells have found that some integrins can induce apoptosis, for instance by activating caspases when unbound to ligand. Another way by which integrins can suppress mitogenic signals could be through trans-dominant integrin inhibition, where occupancy of one type of integrin can inhibit the activity of other integrins. For example, ligand binding of αvβ3 actively downregulates α5β1, which is a potent suppressor of apoptosis through activation of the PKB survival pathway, making these cells more susceptible to cell death [67]. In vascular smooth muscle cells, which are exposed to shear stress, increased loads of tension activates Rac through β1 integrins, which can have pro-apoptotic effects on the cell via upregulation of Fas ligand or p53 [68].

These results are only a few examples of the vast amount of data available on adhesion and apoptosis, and thus shows that the exact signalling pathway and response is dependent on integrin and cell type, as well as cellular context.

IV.3 *Mechanotransduction in cell cycle progression*

As has already been emphasized, adhesion and cell spreading cooperate with growth factor signalling and can induce cells to proliferate. Studies have shown that integrin clustering via

binding to fibronectin is essential for cell cycle progression via upregulation of several immediate-early genes, a response growth factor stimulation alone can not elicit. Furthermore, adhesion alone in the absence of growth factors can even be sufficient for the cells to express these genes and progress through G1 [69]. We will explore the mechanosensitive signalling routes which mediate G1 progression in further detail.

Cells enter division by integrating mitogenic cues from the microenvironment, which converge on the transcriptional upregulation of cyclin D1. This protein binds to Cdk4/6 and inhibits Rb and Cip/Kip proteins, thereby initiating S phase. Studies have shown that the ERK signalling cascade is needed for Cyclin D1 induction and a sustained ERK activity of several hours is essential. Both growth factors and integrins impinge on this pathway, of which the canonical signalling occurs via Ras-Raf-MEK-ERK [2, 47]. While growth factors can signal to activate Ras, integrin signalling enhances Raf-MEK-ERK activation, and thus amplifies the signal, even though obtained results are disputed on the exact point in the pathway where integrin signalling acts [70]. Just like sustained GF signalling is essential to ensure progression through G1, sustained adhesion-mediated integrin signalling is also necessary. Among the proteins phosphorylated and/or activated for a prolonged time during adhesion are FAK, p130Cas, Paxillin and PIP5k [70]. Below, we outline the role of several integrin-mediated signals in cell cycle progression, summarized in **figure 10**.

Integrin-associated signalling components

FAK is a multifunctional molecule which can enhance growth factor signalling via various downstream components. Integrin matrix binding results in the activation of FAK and Src, which can phosphorylate p130Cas. A downstream target of this molecule is Jun kinase, which has been linked to Cyclin D1 gene expression. FAK can also regulate ERK signalling directly [71]. In addition, FAK has been reported to stimulate Ets-like transcription factors and KLF8, which upregulate Cyclin D1 mRNA downstream of ERK. Other ways by which FAK induces cell cycle progression is via the growth signalling pathway of PI3K-PKB/Akt, which can be activated downstream of Cdc42 and Rac [47].

Integrin signalling also affects cell cycle components downstream of cyclin D1: non-adherent cells have high expression of Cip/Kip proteins, which inhibit Cyclin E/Cdk2 activity. Integrins, via FAK, reduce these Cip/Kip levels by regulating transcription and translation of Skp2, the substrate specificity factor of the ubiquitin ligase which induces Cip/Kip degradation [47].

Another integrin-associated kinase, ILK, also regulates PKB/Akt, forming another possible pathway via which integrins mediate cell cycle progression [71]. ILK becomes active upon integrin-ECM adhesions and PIP3 signalling, and can directly activate PKB, thus inducing cell growth and survival. ILK also inhibits GSK3β, releasing β catenin into the nucleus where it stimulates proliferation. Furthermore, ILK can decrease E-cadherin mediated cell-cell adhesion, and as discussed in the previous chapter, phosphorylates MLC, increasing contractility. Adding to this the fact that it indirectly activates ERK, it is thus not surprising that this kinase has been implicated in cancer progression [72].

Cytoskeletal contractility and small GTPases

While growth factors will activate ERK in all attached cells, cells prevented from spreading will not increase Cyclin D transcription, p27 levels remain high, and Rb hypophosphorylated, thus mediating growth arrest. Disrupting the actin cytoskeleton in spread cells has the same effect, indicating that actomyosin generated tension via Rho-ROCK signalling during cell spreading is

essential for cell cycle progression [73]. This is necessary for sustained ERK activity and thus Cyclin D1 induction [47].

Both cell spreading and GF-induced Ras activity mediate Rho activation [67], which signals to balance its effectors ROCK and Dia1 to increase the activity of Skp2, which subsequently downregulates p21/p27 and thus facilitates cell cycle progression [74, 75] [2, 71]. Also downstream of integrins, Rho antagonist Rac1 can activate Cyclin D1 transcription via PAK, which regulates MEK-ERK interactions [75] and can directly activate RAF and MEK [71]. Joyce et al. have shown that Rac signals via the NFκB pathway, as components of this route directly bind the cyclin D1 promoter [76]. Finally, Rac induction by integrins can influence cyclin D1 mRNA translation via ERK-independent pathways, thereby forming another level of control of cell proliferation [77].

Altering ECM rigidity to manipulate cell spreading and integrin signalling support the idea that high intracellular tension induces cell cycle progression via Rho and ERK. In contrast, when cultured on compliant matrices, cells do not activate FAK, and no Rho or ERK signalling occurs, leading the cells to become quiescent. At substrates of intermediate rigidity, Rac is activated which can partly induce cyclin D1, yet multiple inputs are needed for these cells to proliferate [47]. Indeed, as was discussed in the previous chapter, activated integrins initially tend to signal to Rac and not Rho. This indicates that adhesion alone can already convey some growth-inducing signals, while cytoskeletal tension needs to be generated for full G1 progression. Therefore it might be likely that adhesion-mediated integrin signalling is important during early G1, while during mid/late-G1 contractility and Rho activity is necessary [78].

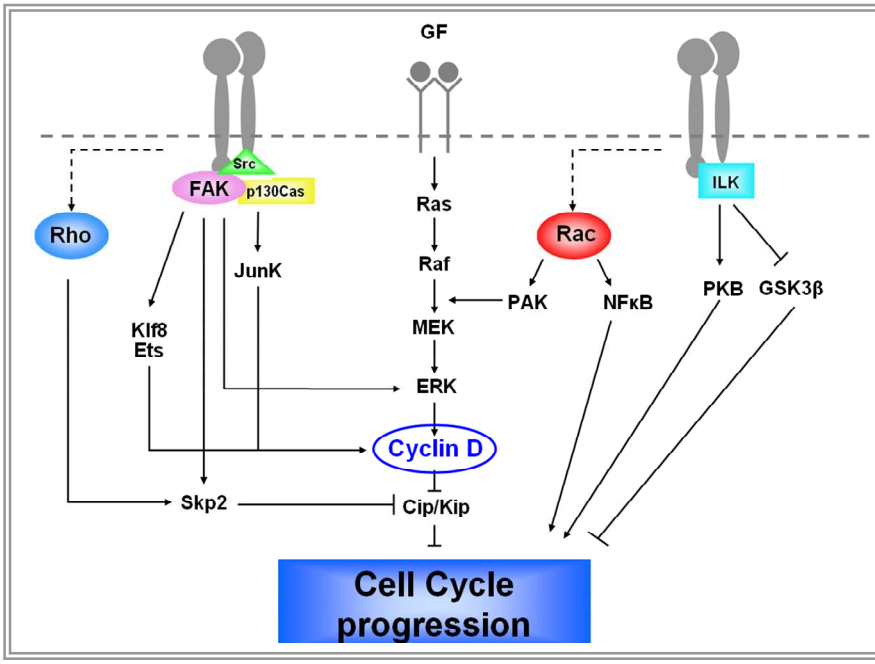


Figure 10 *– Crosstalk between GF and integrin signalling pathways. The canonical activation of Cyclin D occurs via Ras-ERK, and integrins downstream signalling components impinge on this pathway at various levels. In addition, via Skp2, NFkB, PKB and GSK3B, cell cycle components downstream of CycD are also regulated. This indicates that adhesion-mediated signalling is important to elicit a full GF-dependent response in the form of cell cycle progresssion*

IV.4 *Integrin crosstalk with growth factor signalling*

It is clear that mechanotransduction cooperates with mitogenic pathways to influence proliferation and apoptosis. Several principles by which integrins crosstalk with growth factor receptor (GFR) pathways can be distinguished (see also **figure 11**). (1) Integrins associate directly with GFRs in complexes, leading to amplification of both downstream signalling pathways [67]. (2) Downstream signals of integrins impinge on GFR pathways, such as RAS-ERK which was discussed above. (3)

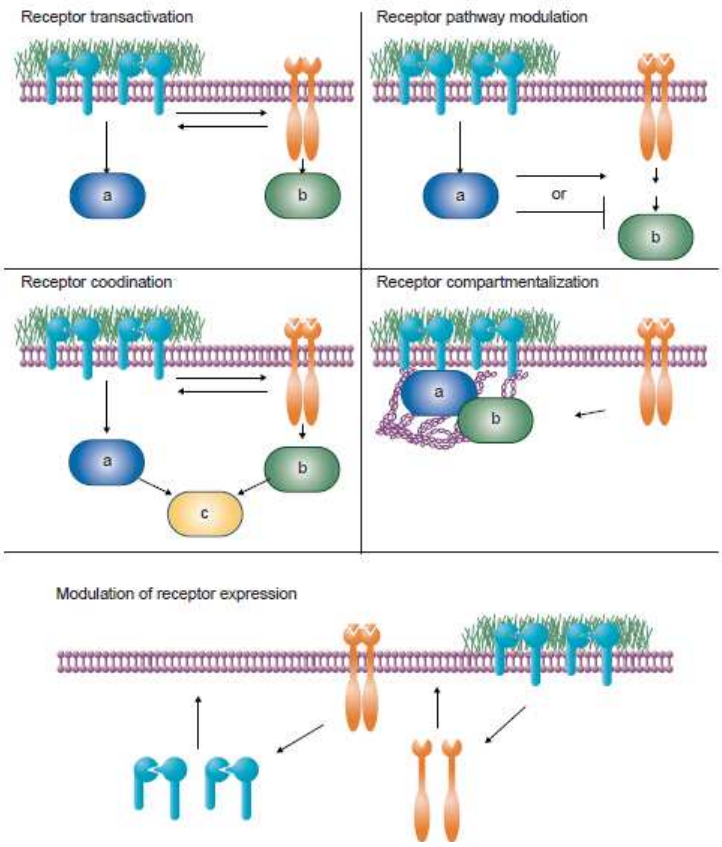


Figure 11 *– principles of crosstalk between integrins and GF signaling. (A) direct association between receptors (B) downstream signals from integrins modulate components of GF pathway (C) downstream pathways cooperate (D) Scaffolds spatially regulate GF signaling components (E) integrin signaling regulates GF receptor expression [3].*

Integrin signalling can produce or activate components which are substrates for other pathways. (4) The many scaffolding proteins and cytoskeletal associations of focal adhesions facilitate compartmentalization of GF signalling pathways. (5) Activation of either integrins or GF receptors can induce the expressional upregulation of the other, so signals can be strengthened further [3].

A combination of these crosstalk mechanisms was shown to occur with the EGF receptor: adhesion stimulates the association of integrins with EGF receptors, due to the actions of c-Src and p130Cas. In addition, EGF receptors undergo phosphorylation in response to the formation of this macromolecular complex, and finally the amount of EGF receptors on the cell surface is increased in response to integrin-mediated adhesions [79]. There appears to be a bi-directional signalling relationship between EGF receptors and integrins, as both influence the activity and localization of the other, thus further strengthening the cooperativity between adhesion and growth factor signalling pathways [80].

IV.5 *Integrin influence on the gene level*

While the function of several focal adhesion signalling molecules has at least in part been elucidated, mechanical stress upon integrins also leads to effects far away from the adhesive site. Indeed, in chapter II it was already discussed that forces can be propagated towards the nucleus, so it makes sense that integrins somehow signal to influence transcriptional and translational events.

Transcriptional control

The exact pathways by which integrins signal to regulate transcriptional events have not been entirely elucidated. It is clear that applying stress on integrins modulates gene expression, a response dependent on phosphorylation events, stretch-activated ion channels and cytoskeletal tension [81]. In addition, there appears to be a role for reactive oxygen species (ROS). Integrins in

fibroblasts activate Rac1, which can produce ROS, subsequently activating NFκB signalling. Another manner in which ROS acts downstream of adhesions is via mitochondria, as cyclic strain on endothelial cells induces actin-dependent ROS release from mitochondria. NFκB signalling affects the expression of several genes, for instance important in ECM remodelling and adhesion [82, 83].

Integrin-ECM contacts can modify actin dynamics, and so regulate for instance the nuclear accumulation of transcription factors like SRF, a serum-inducible factor. SRF activity has been shown to be regulated by Rho GTPases and actin treadmilling, which is likely a convergence point of Rho signalling [70, 84]. In addition, the Rho-actin pathway redistributes the SRF co-activator MAL to the nucleus, indicating that small GTPases downstream of integrins in mechanotransduction act via actin dynamics to influence transcriptional events [85].

Likely candidates to mediate signal transduction between integrins and gene transcription are members of the LIM family, which reside both at focal adhesions and the nucleus, and can shuttle between these locations. An example is Zyxin, which plays a role in actin assembly and organization in the cytoplasm. It is one of the proteins undergoing a rapid unbinding response during application of mechanical stress to focal adhesions, thus mediating the tensional signal [86]. Mechanical force on some cell types induces its translocation from the FA to the nucleus, where it mediates stretch-responsive gene expression. Zyxin interacts with p130Cas and displays transcriptional activity, though its exact signalling role is not entirely clear [87].

An interesting role has been uncovered for ICAP-1α, a protein which competes with talin for binding to β1 integrins. During adhesion, ICAP-1α inhibits Rac/Cdc42, while its role in the nucleus is to stimulate proliferation, possibly via c-Myc upregulation. However, in non-adhesive cells, ICAP-1α is mostly bound to integrins, thus playing a possible role in anchorage-dependent growth [87].

Translational control

Apart from transcriptional events, integrins also signal to influence translation. The movement of mRNA's and ribosomes to a localized compartment near adhesive sites experiencing force is a distinct mechanosensitive effect. This process is dependent on Rho, and thus involves cytoskeletal contraction and perhaps reorganization [88]. This has implications for the role of integrins in gene regulation, but may also add to a localized response in adhesion strengthening, as it has been shown that β actin transcript is present amongst the specialized mRNA pool [87]. In addition, several RNA binding proteins are found on cell edges of adhering cells preceding the formation of focal adhesions, termed spreading initiation centres [89]. Furthermore, paxillin is one of the focal adhesion proteins which mediates localization of mRNA binding proteins and their function in exporting mRNA from the nucleus. Disrupting their interaction impairs focal adhesion regulation during cell migration [90]. This indicates that localized translation is an important aspect of adhesion strengthening and cell spreading.

Related to this, work in *Drosophila* ovaries has shown that, during epithelial flattening of the follicle cells surrounding the growing oocyte, integrin signalling and mRNA localization play an important role. Mechanical tension sensing by integrins on the basolateral side activates two signalling pathways: first, Rho is recruited and is necessary for the cytoskeletal remodelling, leading to flattening of the cells; secondly, the FA protein PINCH, another FA-nucleus shuttling protein of the LIM family, is targeted to the nucleus. There, it activates transcription of dGRASP, which in turn is necessary to mediate new integrin deposition at the expanding basolateral membrane. Both dGRASP and integrin subunit αPS1 mRNA are localized near the site of mechanical tension at the

membrane, thus aiding in the localized response of upregulating adhesive sites due to epithelial flattening [91] (schotman, in press).

Chapter V – Mechanotransduction during Development and Cancer

V.1 *Mechanical control of tissue development and differentiation*

During life and development, cells within a tissue will adopt different fates, as they can migrate, grow and divide, become quiescent and differentiate, or undergo apoptosis. Apart from mitogens or morphogens, it is likely that mechanical forces play a role as well. Several developmental systems have shown that changes in forces from the ECM exerted on epithelia can change the cell shape and cytoskeletal tension in these cells, which alters their responsiveness to growth factors and enables them to progress through the cell cycle [2].

For example, endothelial cells during capillary development must adopt different fates from their neighbours to ensure correct branching points. New capillary sprouting has been shown to be due to a regional thinning of the basement membrane underneath the endothelial cells, as is depicted in **figure 12**. With the knowledge that cells are under continuous prestress and in isometric tension with their environment, this increasing compliance of the ECM can exert a force upon the cells that lie on it, which will distort their cytoskeletons and increase cell spreading. As has been mentioned, cell spreading increases growth and division rates, and in this case leads to localized budding of the endothelial layer, facilitating the outgrowth of a new vessel [5].

Integrin signalling in lung bud branching has been shown to reduce E-cadherin, and thus cells lose the epithelial phenotype to induce cleft formation and branching morphogenesis. In addition, Rho/ROCK signalling in these cells increases cytoskeletal contractility, a feature essential for proper lung development as inhibition of any part of the Rho/ROCK contractility pathway disrupts branching morphogenesis [92, 93]. This then leads to basement membrane remodelling which also increases branching, possibly by a process as described above.

Another example of ECM-integrin connections regulating developmental process is that of fibronectin in somitogenesis, however a partly opposite phenotype is induced. Fibronectin-integrin binding activates FAK, which signals to ENA/VASP to induce the mesenchymal cells to become epithelial. In addition, matrix remodelling is induced and also aids in somite boundary formation. These two developmental processes indicate that the same signals from the ECM on integrins can have opposite effects, and this can be explained because integrin signalling cooperates with many other signal transduction pathways [29].

In addition to tissue development, mechanical cues also drive single cell differentiation. Micropatterning of mesenchymal stem cells results in differentiation to adipocytes in round cells, while spread cells commit to an osteoblast fate. The adipogenic-to-osteogenic shift was shown to be dependent on cytoskeletal contractility mediated by cell spreading, and indeed active Rho and ROCK are sufficient to induce the stem cells to differentiate into osteoblasts. Furthermore, these

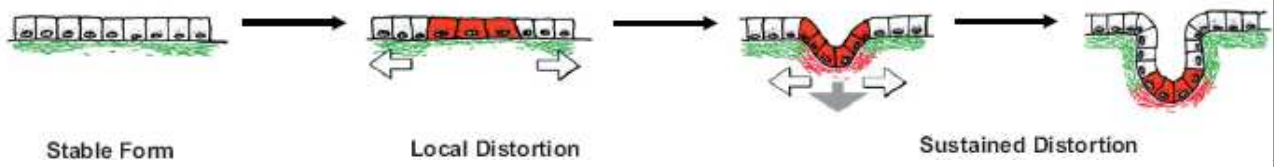


Figure 12 *– Mechanism of lung or endothelial budding. Local thinning of the ECM leads the overlying cells to experience a stretching or change in cytoskeletal tension. This will make them more sensitive to growth stimuli, and via proliferation this group of cells form an outgrowing bud, depositing new ECM and thus contributing to morphogenesis [5].*

contractility-induced signals overruled any differentiation factors present in the media [94]. A similar conclusion was drawn from experiments where mesenchymal stem cells were grown on matrices of increasing stiffness, which resemble the compliance of tissues in vivo: soft substrates induced a neurogenic commitment, intermediate compliance resulted in differentiation in myoblasts, while cells grown on the stiffest matrices gained an osteoblast fate. This was confirmed by both expression profiling and marker staining, and indeed the increase in stiffness led to greater intracellular tension, suggesting that this is what can drive stem cells to a specific lineage [95].

V.2 *Extracellular forces and cancer progression*

Hallmarks of cancer are uncontrolled proliferation and an increased susceptibility for cells to migrate and invade other tissues. Mechanosensation via integrins can influence both these processes, as tumour stroma is often more rigid than normal tissue. Greater ECM forces increase intracellular tension and integrin-mediated signal transduction, which cooperate with GF signalling and can also direct cytoskeletal contractility to influence migration.

Extracellular forces and proliferation

Several experiments have shown that cells need to be cultured on compliant matrices in order to be quiescent and differentiate. A manner to reach this is by culturing cells on top of another cell layer, which seems to be an appropriate environment for cells to differentiate [96]. Decreasing extracellular tension on cells results in their differentiation and inhibits proliferation, as activation of ERK signalling gets diminished, even when the cells are still exposed to the mitogen. In contrast, cells exposed to high tensional forces show increased levels of integrin expression and activation, thus leading to increased focal adhesion assembly and increased Rho-dependent cytoskeletal contractility of the cells. These features have a marked influence on integrin-associated changes in ERK signalling and cell behaviour [27, 30]. In addition, cells under tension will remodel the ECM further to make it more rigid, for instance by producing fibronectin. This then further increases intracellular tension and focal adhesion assembly, resulting in high FAK activity and thus high ERK, leading to proliferation [67].

As tumours are often associated with rigid stroma's, this increases integrin activation and Rho/ROCK signalling within the cells due to increased tension. Not only does this influence cell spreading and motility, but also cooperates with growth signalling pathways via ERK, leading to increased division rates and less differentiation [29]. Furthermore, activated integrins can then modulate GF receptors to activate their downstream signals, even in the absence of ligand, a process termed adhesion-dependent trans-activation [67]. Several experiments have indicated that either increasing matrix compliance or actively inhibiting cell contractility and Rho signalling, can reverse the malignant phenotype of transformed cells, indicating the prominent role of intracellular tension in tumour progression [47].

Metastasis

As extracellular tension increases, this leads to integrin-mediated signalling to actin polymerization and contractility, thus facilitating migration and hence easing metastasis of tumour cells. In

addition, cells need to break down their cell-cell adhesions in order to migrate, and must modulate cell-matrix adhesions to invade other tissues.

Research has shown that integrin-mediated cell adhesions can decrease cadherin-mediated cell-cell junctions, indicating that increased tension in cells can promote cell scattering and thus contribute to metastasis. Indeed, increasing ECM rigidity has been shown to induce cell scattering in an integrin-dependent manner [97]. β1 and β3 integrin subunits can drive transformation of cells via ILK signalling, which increases motility and detachment via E-cadherin downregulation [80]. A role for signalling via α6β4 has been uncovered in invasiveness of malignant cells, as it was shown that this integrin activates PI3K and Rac and facilitates polarity loss and migration of tumour cells. In contrast, in normal cells this transmembrane protein is necessary for tight adhesion to the basement membrane in polarized epithelia, and its absence or mislocalization is also associated with malignancy [80]. These dual and seemingly opposing functions need to be further investigated, however it is clear that increased forces upon integrins, as well as their mis-expression, results in marked changes in polarity and cell-cell adhesions, thus easing the migratory capability of these cells.

Changed integrin expression in tumours contributes largely to the phenotype, ranging from differentiation and proliferation to adhesion [98]. For instance, β6 is normally only upregulated during wound healing to facilitate migration of epithelial cells. However, it is also expressed in several cancers, probably enhancing the tumour cells' ability to migrate [13]. Deregulating integrin α6β4 function is actually able to induce non-malignant cells to acquire a malignant phenotype. In addition, expression of this adhesion molecule confers resistance to apoptosis by activating NFκB [99]. A differing integrin expression pattern may also enable the cells to adhere and grow in different environments during metastasis. The changed expression can result from changes in the ECM, as stroma rigidity can upregulate their activity. Furthermore, mechanotransduction through integrins can increase the production of lysyl oxidase by the tumour cells, which cross-links collagen and thereby increases ECM rigidity even more. Not only integrins, but also their associated signalling molecules are sometimes overactivated in tumours, examples of which are FAK, paxillin and α-actinin [13].

ECM remodelling, changing both tension and composition of the matrix, influences malignancy. A focus lies on the nature of ECM constituents, as ligand binding can induce specific signals when bound by the corresponding integrins. In addition, the action of matrix metalloproteases, which are often secreted by tumour cells, can modify the ECM by cleaving substrates and exposing either new recognition sites, enabling activation of distinct integrins and alter their signalling pathways, or release growth and motility factors [80]. An interesting finding by Gaggioli et al. demonstrated that tumour cell invasion requires force-mediated matrix remodelling by tumour-associated fibroblasts. These cells experience Rho-mediated tension due to ECM stiffness, and help clear a path by modifying the ECM for the malignant cells to ease their invasion [100].

Breast cancer

An example of how extracellular forces contribute to tumour progression occurs in breast cancer models. Normally, these cells are quiescent and polarized, forming acini within the soft breast tissue. Cancer has been associated with an increase in tissue tension, which via Rho-mediated contractility within the cells leads to overproliferation and migration of the mammary cells.

Mammary epithelial cells in free-floating 3D collagen matrices will differentiate, a process which is initially dependent on Rho, ROCK and actomyosin contractility. However, this also leads

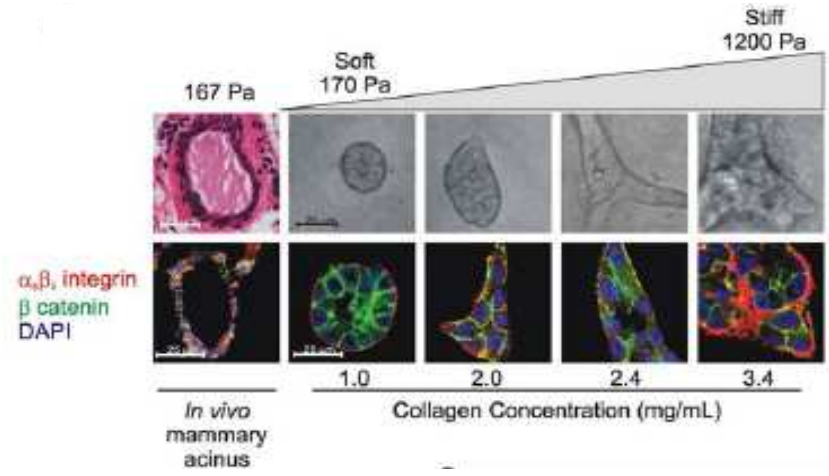


Figure 13 *– Mammary epithelial cells exhibit phenotypical changes when grown on matrices of increasing rigidity. The colonies change from quiescent acini with organized cell-cell contacts (b catenin, green) and basement membranes, into disorganized groups of undifferentiated, unpolarized cells which fail to form a lumen. These also express higher levels of integrins (red) and lose cell-cell contacts.* *[27]*

to focal adhesion downregulation, FAK inhibition and a subsequent decrease in Rho activity. This second response depends on the compliant soft matrix, and does not take place on rigid 2D substrates. This suggests that ROCK-mediated contractility sensing feeds back to diminish Rho activity and integrin signalling when the ECM exerts low forces on the cells [28].

Transformed mammary epithelial cells will contribute to an expanding mass of cells within the breast tissue, which results in a chronic, solid compressional stress being exerted upon both the tissue and cellular level. This increased force acting upon the tumour cells can enhance their proliferative rate through enhanced GF signalling, induce angiogenesis and infiltration of inflammatory cells, and can also contribute to cytoskeletal changes which affect morphology and migration [101]. In a study by Paszek and colleagues, mammary epithelial cells grown on matrices of increasing rigidity drastically perturbed tissue architecture, where loss of polarity, increased FA assembly, higher cell spreading and contractility, and increased GF-dependent ERK activation were observed (see **figure 13**). The subsequent malignant phenotype was due to an increased integrin clustering and signalling, which acts via Rho and ERK in a 'mechanoregulatory circuit'. Thus, cancer cells could be reverted by inhibiting either Rho or ERK [27].

Human breast cancer cells can be also 'normalized' of their malignant phenotype by the use of inhibitory β1 integrin antibodies. This treatment results in a functional and morphological reversion of the tumourigenic phenotype, with the formation of newly formed acini with a basement membrane, and the acquisition of differentiation and quiescence via downregulation of Cyclin D1 and upregulation of p21. Furthermore, the reverted cells engage in E-cadherin mediated cell-cell connections, and display a reorganization of the cytoskeleton, as well as integrin distribution [98]. This again emphasizes that extracellular forces of the tumour stroma are sensed by integrins leading to increased actin contractility, and mediate a response in increased proliferation rates, loss of adhesion, polarity and differentiation, and migration.

Conclusions

In this work, we have outlined several mechanisms via which integrins in cell-ECM adhesions transfer mechanical forces into a biochemical signal, eliciting the appropriate cellular response. The forces can come from within the cell, generated by actomyosin contractility, or from the environment, generally dictated by ECM rigidity. By viewing the cell as a tensegrity structure, where continuous inward directed tension is balanced by local compression, it can be explained how integrins transduce local forces throughout the entire cytoskeleton, and even influence nuclear events. Increased tension upon integrins leads to strengthening of adhesive sites, though in cases of migration, integrins must signal for adhesions to be disassembled. This occurs via several integrin-associated signalling molecules, mostly activating small GTPases downstream which remodel the cytoskeleton. In addition, integrin signalling cooperates with growth factor signalling in regulating proliferation, differentiation and apoptosis. Indeed, direct correlations between intracellular tension generation and cell fate have been found. This emphasizes the importance of mechanical tension sensing by cells during development and disease.

However, still several questions remain as to how exactly integrins act as

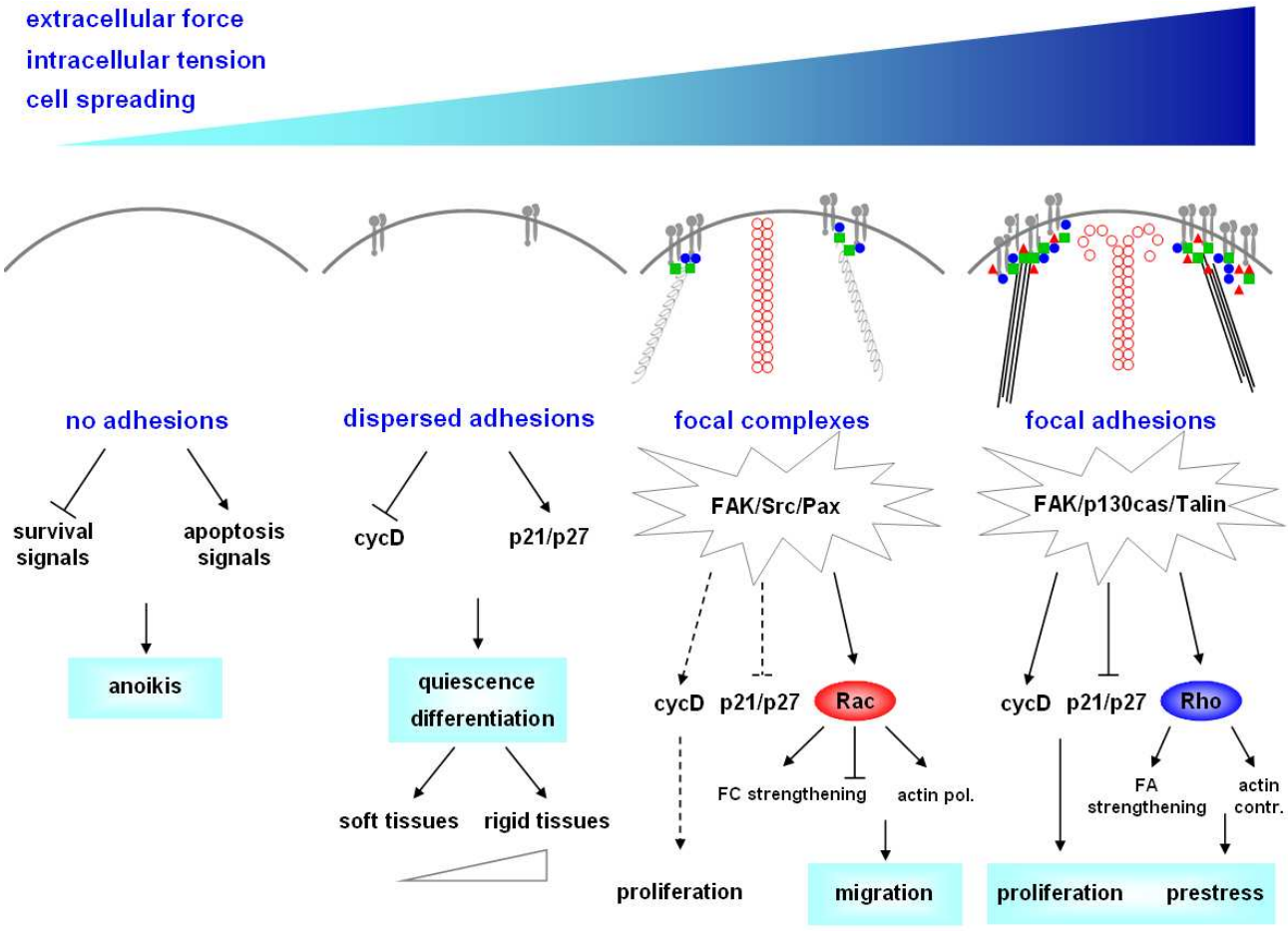


Figure 14 *– Schematic illustration on the effects of tension on cellular events. Tension can come from either extracellular forces (integrin manipulation or matrix rigidity), cytoskeletal contractility or cell spreading, and is conveyed via integrins, which act as mechanotransducers. This first leads to modulation of the cytoskeleton and adhesive sites, such as growth into focal complexes or focal adhesions, via the downstream signaling of integrins to small GTPases. Secondly, integrin-associated signaling components also influence cell fate, by contributing to cell cycle progression, apoptosis or migration events.*

mechanotransducers, and for instance convert a mechanical cue into a biochemical signal. Several suggestions have been made, such as force-induced molecular deformations exposing active sites, or stretch-activated ion channels which induce signalling cascades. In addition, a lot of information on mechanotransduction has been obtained by manipulating cells on rigid 2D substrates and exposing them to greater forces than are normally encountered *in vivo*. Recent work has focused more on 3D tissue culture systems and physiological pressure, and this might give us more insight into how adhesions are remodelled in living tissues and the exact signals which emanate from them.

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I want to thank Catherine Rabouille for supervising me during the writing process of this thesis. It has taken longer than I planned, and because of the vast amount of information available, sadly I was not able to include all the aspects of mechanotransduction. Nevetheless, I hope you are satisfied with the result, and thank you again for your patience.

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