Biochimica et Biophysica Acta 1793 (2009) 911–920

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

From mechanotransduction to extracellular matrix gene expression in fibroblasts

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ARTICLE INFO

Article history:
Received 25 January 2008
Received in revised form 12 January 2009
Accepted 22 January 2009
Available online 31 January 2009

Keywords:
Mechanotransduction
Fibroblast
Focal adhesion
Extracellular matrix
Cytoskeleton
RhoA
Integrin linked kinase
p130Cas
Megakaryocytic leukemia protein
Tenascin-C

ABSTRACT

Tissue mechanics provide an important context for tissue growth, maintenance and function. On the level of organs, external mechanical forces largely influence the control of tissue homeostasis by endo- and paracrine factors. On the cellular level, it is well known that most normal cell types depend on physical interactions with their extracellular matrix in order to respond efficiently to growth factors. Fibroblasts and other adherent cells sense changes in physical parameters in their extracellular matrix environment, transduce mechanical into chemical information, and integrate these signals with growth factor derived stimuli to achieve specific changes in gene expression. For connective tissue cells, production of the extracellular matrix is a prominent response to changes in mechanical load. We will review the evidence that integrin-containing cell–matrix adhesion contacts are essential for force transmission from the extracellular matrix to the cytoskeleton, and describe novel experiments indicating that mechanotransduction in fibroblasts depends on focal adhesion adaptor proteins that might function as molecular springs. We will stress the importance of the contractile actin cytoskeleton in balancing external with internal forces, and describe new results linking force-controlled actin dynamics directly to the expression of specific genes, among them the extracellular matrix protein tenascin-C. As assembly lines for diverse signaling pathways, matrix adhesion contacts are now recognized as the major sites of crosstalk between mechanical and chemical stimuli, with important consequences for cell growth and differentiation.

1. Introduction: mechanical stress and tissue homeostasis

The extracellular matrix (ECM) of bones and connective tissues bears most of the physical loads that act on our body, and it shields embedded cells from adverse effects of excess mechanical forces [1]. During normal walking or running, for example, the force acting on the human Achilles tendon can reach several kilonewtons, corresponding to a tensile stress (defined as force per area) in the order of megapascals [2]. Due to the stiffness of the ECM, however, resulting tissue strains (deformations) are modest, ranging from a small fraction of one percent in bone [3] up to several percent in tendon [2]. Relevant are those strains of the tissue that are transmitted from the ECM to the embedded cells via their matrix adhesion sites [4], and current cell and tissue culture models aim at imitating the strains measured in vivo [5,6]. Intracellularly, ECM substrate strains of a few percent translate into very small forces (in the nanonewton range) that mainly arise from resistance and/or contraction of the cytoskeleton [7,8]. These tiny mechanical stimuli can be transduced into chemical signals, eliciting a multitude of cellular responses [9–12]. Muscles, bones, connective tissues and blood vessels need to respond adequately to external mechanical stress in order to adapt to changes in load [13]. In the absence of physiological load levels (e.g. upon forced bed rest or prolonged microgravity conditions), these tissues suffer atrophy even if nutrition is optimal [14]; conversely, excess load can induce their hypertrophy [15]. If the ECM is damaged and weakened, e.g. after injury of a tendon, higher tissue strains result in excess forces acting on the embedded tendon fibroblasts, causing inflammation, degeneration and apoptosis [16]. Alternatively and depending on conditions, tensile strain might induce fibrosis e.g. during skin wound healing [17]. Thus, there is much evidence from physiology that tissue mechanics provide an important context for the function and effectiveness of other stimuli such as nutrition, hormones and growth factors.

However, mechanical stimuli are not only permissive for growth and differentiation; they can also be instructive. Not surprisingly, specific changes in ECM synthesis and degradation are an important part of cellular responses to mechanical stress. It is known for more than a century that bone constantly remodels its trabecular structure according to the amount and direction of applied forces [18]. Connective tissue cells are able to distinguish between various modes of mechanical stress: compressive (e.g. in cartilage [1]), tensile (e.g. in tendons [9]) and shear (e.g. in blood vessel walls [19]). In fact, the type of stress can influence cell differentiation: in embryonic development as well as during adult fracture healing, the ratio between tension and compression can tip the balance between cartilage and bone formation [20]. Tendons mostly consist of dense parallel collagen bundles specialized to bear tensile stress, but fibrocartilage or sesamoid bones develop within tendons where they bend over joints; formation of the latter structures strictly

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doi:10.1016/j.bbamcr.2009.01.012

depends on compressive forces that in tendons occur only at these locations [21]. Thus, connective tissue cells clearly sense the mode, magnitude and direction of mechanical stresses, and are able to translate this information into specific adaptive responses.

Here we focus on recent advances in understanding how adherent cells sense strains in their extracellular environment and transduce the resulting mechanical forces into chemical signaling. In addition, we will argue that local mechanical parameters provide an important context for specific cellular responses to other stimuli. We will discuss new results indicating how fibroblasts might integrate mechanical with growth factor derived signals, in order to achieve specific changes in gene expression. This brief review is not meant to cover the relevant literature exhaustively; we are aware of being rather selective in choosing examples. For additional and more comprehensive information on various aspects of this fascinating topic, the reader is referred to several excellent reviews that have appeared recently [4,22–26].

2. Cell–matrix adhesion contacts as sites of mechanosensation

2.1. Force transmission between the extracellular matrix and the cell interior

Fibroblasts firmly attach to their ECM substrate via matrix adhesion contacts on their cell surface. It should be mentioned that although fibroblasts are the main focus of this article, many of the studies described below have been performed separately or in parallel with other strongly adherent cells of mesenchymal origin (e.g. myofibroblasts, smooth muscle cells, cardiomyocytes, endothelial cells), with very similar results (see e.g. [7,8]). For optical reasons, adhesion contacts have been studied most extensively in cells cultured on flat, rigid surfaces. In fibroblasts grown on two-dimensional substrates, three different types of matrix contacts can be distinguished, namely focal complexes, focal adhesions (also called focal contacts), and fibrillar adhesions (Fig. 1). All matrix contacts contain integrin receptors as their major transmembrane proteins. With their extracellular domains, distinct integrin heterodimers (e.g. $\alpha5\beta1$) bind to specific ECM components (e.g. fibronectin), whereas their cytoplasmic tails firmly interact with adaptor proteins that link them to the actin cytoskeleton [27,28]. Focal complexes (Fig. 1A) are the first contacts that form when a fibroblast interacts with ECM during cell adhesion and migration; they are small (<1 μm diameter) and dynamic. The major integrin of focal complexes is the so-called "vitronectin receptor" $\alpha v\beta3$, which recognizes various ECM proteins via their RGD peptide motifs. To establish new contacts with ECM, cells first have to activate integrins at their surface, which occurs mostly by signaling from within the cell [29,30]. Inside-out activation of the integrin heterodimer involves conformational changes of its ectodomain that are triggered by binding of adaptor proteins talin [31] and/or kindlin [32] to its cytoplasmic tails. Activated integrins then bind to their ECM ligands, and once a mechanically stable contact can be established, focal complexes mature into larger (several μm diameter) focal adhesions (Fig. 1B). This process involves the co-clustering of several integrin types (notably of $\alpha v\beta3$ with the fibronectin receptor $\alpha5\beta1$), as well as the recruitment of adaptor proteins such as talin, vinculin, α-actinin and paxillin that mediate the connection to actin stress fibers. Interestingly, mechanical stress is an important signal for the maturation and maintenance of focal adhesions: their size is roughly proportional to the local mechanical stress, which in turn depends on substrate stiffness and cytoskeletal pulling forces at a specific site ([7,8]; see also next paragraph). Finally, fibrillar adhesions (Fig. 1C) are pulled out and separated from focal adhesions by actomyosin-generated force, leading to partial sorting of specific components: compared to focal contacts, fibrillar adhesions are enriched in the fibronectin receptor $\alpha5\beta1$ and the adaptor protein tensin [33]. Fibroblasts use fibrillar adhesions for assembling (or "spinning") secreted fibronectin monomers into matrix fibrils [33,34].

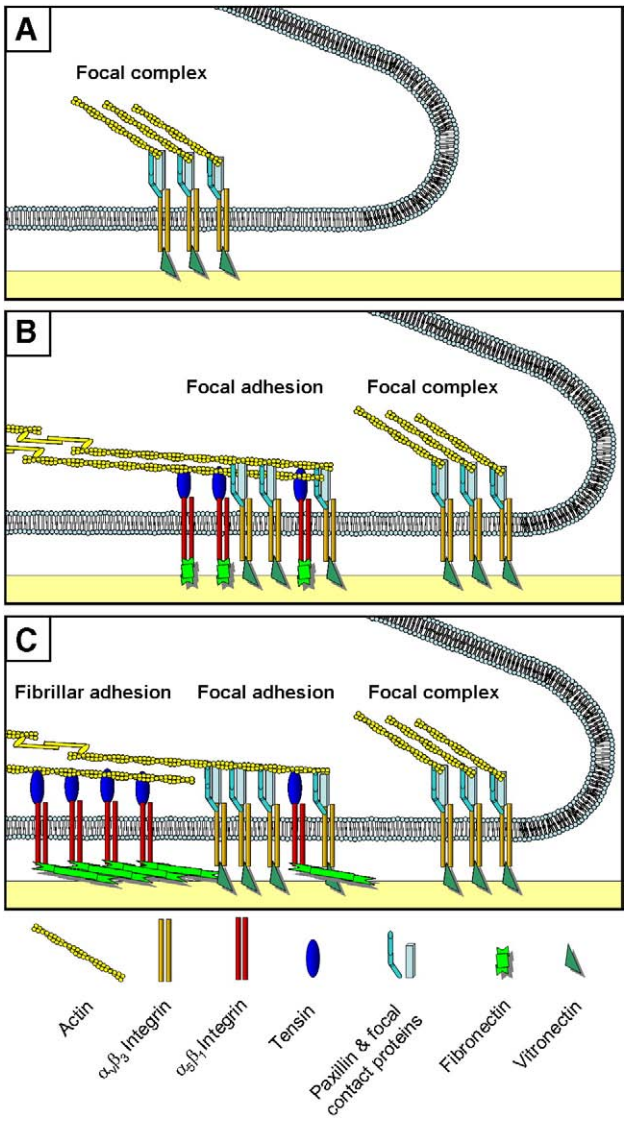

Fig. 1. Schematic representation of matrix adhesion contacts commonly found in fibroblasts cultured on two-dimensional substrates. (A) Focal complexes are small and dynamic contacts at the margins of a cell in the process of spreading on a substrate, or at the borders of lamellipodia in a migrating cell. (B) Focal adhesions (or focal contacts) mature from focal complexes if a physically stable contact with the substrate can be established; they are characterized by a firm connection to actin stress fibers. (C) Fibrillar adhesions are "pulled out" from focal contacts in the process of extracellular fibronectin matrix assembly. For further explanations, see text.

A distinguishing feature of fibrillar adhesions is their lateral (parallel) association with the cellular actin meshwork, in contrast to focal adhesions where stress fibers insert head on.

Fibroblasts embedded in a three-dimensional ECM meshwork in vivo or in 3D culture assume a bipolar or stellate instead of a spread shape. Under these conditions, the distinction between different types of matrix contacts is less clear and their dynamics are more complicated [35–37]. However, focal adhesions are not simply culture artifacts since structures are found with similar composition and function in vivo, e.g. in myofibroblasts of wound granulation tissue [38,39]. Another prominent example is the myotendinous junction of skeletal muscle fibers, which transmits actomyosin-generated force onto the tendon collagen bundles, and which is packed with integrins and other typical focal adhesion proteins essential for its function [40,41].

Obviously, the described physical link between the ECM and the cytoskeleton across the cell surface (via integrins in adhesion sites) allows propagating mechanical forces in both directions. Mechanical connectivity continues inside the cell. Plakins are a family of proteins

that couple the various cytoskeletal networks – actin, cytokeratin and tubulin – to the cell membrane and to each other [42]. At their inner end, cytoskeletal elements are hooked up to the nuclear lamina via nuclear membrane proteins. Nesprins span the outer nuclear membrane and link the actin and cytokeratin networks to inner nuclear membrane proteins such as sun1 and sun2, which bind to nuclear lamins [43,44]. The connectivity of structural elements all the way from the ECM to the nucleus is evident even after detergent extraction of intact cells or tissues [45]. It is responsible for the changes in nuclear shape that are observed when external forces are applied, or during cell spreading and movements [22]. Thus, mechanical forces are readily propagated throughout the cell, and mutations that result in structural disintegration at any of the described levels can cause severe developmental or degenerative phenotypes [46].

2.2. Matrix adhesion contacts as sensors for substrate rigidity

As outlined above, matrix adhesion contacts transmit forces derived from the ECM substrate to the interior, and cytoskeleton-generated forces to the exterior of the cells. In a stationary cell firmly attached to its ECM substrate, external and internal forces cancel out each other, whereas tipping the force balance in either direction results in movements such as cell contraction, extension or translocation [22,47]. Due to these dynamic interactions, fibroblasts can in addition use their adhesion contacts as sensors to probe the mechanical properties of their extracellular environment [7,24,48,49]. By actively pulling on an adhesion contact, cells gain information about the softness or rigidity (i.e. elasticity) of the ECM substrate at this point [23]. The sensing mechanism thus depends on actomyosin contractility, which is largely controlled by the RhoA/ROCK pathway: the small GTPase RhoA is known to induce actin polymerization via its effector mDia1, and to stimulate actomyosin contraction by activating Rho-dependent kinase ROCK I/II. The latter enzyme increases myosin II activity mainly by inhibiting myosin light chain phosphatase [50]. As already mentioned in the last paragraph, mechanosensation in turn strongly influences the formation and turnover of adhesion contacts themselves: resistance to applied cellular force indicates a stiff ECM substrate and thus stable mechanical conditions, and this acts as a signal for the growth and reinforcement of an adhesion contact at this position [7,8,48,49]. Conversely, unstable mechanical conditions (too much, too little, or varying stress) lead to disassembly of focal adhesions. Key signaling components that control focal adhesion dynamics are focal adhesion kinase (FAK) and Src family kinases, and rigidity sensing apparently involves phosphorylation of focal adhesion components such as α-actinin, paxillin and vinculin by these enzymes [24].

Elucidation of these cellular mechanisms has been made possible by exciting developments in nanotechnology in the last decade (Fig. 2). For example, the group of M.P. Sheetz [49] coated micrometer-sized translucent beads with integrin ligands (fibronectin or RGD peptide) and allowed them to bind to fibroblast surfaces. They then used a laser as "optical tweezers" to pull on the beads with defined and adjustable forces in the piconewton range (Fig. 2A). They found that loosely bound beads were moved centripetally on the cell surface by a very small cellular force. However, when a surface-bound bead was trapped and immobilized by the optical tweezers, thus mimicking a stiff extracellular environment, the cell started to "pull back" on this bead with a much stronger force. This happened within minutes, and was accompanied by the recruitment of integrins, focal adhesion proteins and polymerized actin at these locations. Another recent technological advance for this type of studies is provided by micro-patterned cell culture substrates that are fabricated from elastic polymers. Pelham and Wang [48] prepared collagen-coated polyacrylamide gels that varied in compliance, and demonstrated that 3T3 fibroblasts and rat kidney cells preferentially formed small and dynamic focal complexes on soft gels, whereas only stiffer substrate induced large, stable focal adhesions and a more spread phenotype. By embedding fluorescent marker beads into the polyacrylamide gels, the same authors were then able to visualize distortions of the elastic substrate by cellular traction forces [51]. B. Geiger and colleagues [7,52], perfected this method by printing a grid of sub-micrometer-sized fluorescent dots onto the surface of elastic silicone rubbers (Fig. 2B). When such substrates were coated with fibronectin, primary rat cardiac fibroblasts attached, formed matrix adhesions, and exerted contractile forces that distorted the elastic substrate. Knowing the elastic module of the polymer, cytoskeletal forces could be calculated exactly from measuring displacements of the fluorescent marker dots on the substrate underneath the cell. A different method to measure cellular forces was developed by the lab of C.S. Chen [8,53,54] who used arrays of micrometer-sized silicone posts as culture substrates (Fig. 2C). The top

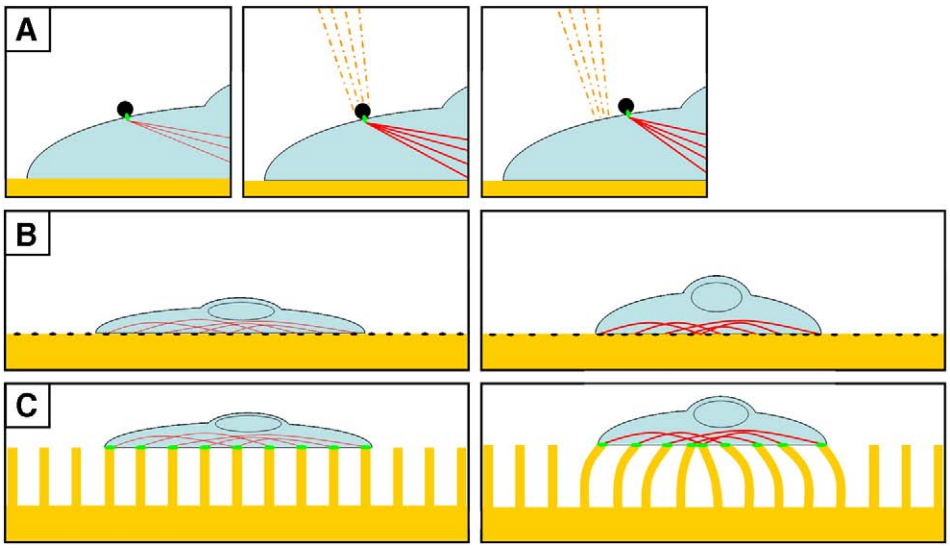

Fig. 2. Various published methods to quantify cytoskeletal force with which a cell pulls on its matrix adhesion contacts in response to substrate rigidity or external force. (A) Optical trapping of a fibronectin-coated bead bound to integrin receptors on the cell surface. A small cellular force moves a free bead. Upon trapping the bead with a laser beam, the cell applies more force, eventually pulling the bead out of the trap. Holding force is calculated from laser intensity and bead optical properties [49]. (B) Cells are cultured on elastomer surfaces marked with a grid of visible dots. The attached, contracting cell distorts the surface with the grid [51,52], (C) Cells are grown on a "bed of needles" molded from silicone elastomer. The attached, contracting cell distorts the needles [8]. In both (B) and (C), forces can be calculated from the geometry and the material properties.

surfaces of the posts were contact-printed with fibronectin. When bovine endothelial cells or mouse 3T3 fibroblasts were plated on such "beds of micro-needles", each cell spread on the top surfaces of several adjacent posts. The cellular contractile forces exerted on each post started bending them individually, and the force per post could again be calculated from optical displacement measurements. Since cells essentially formed a single focal contact on each silicone post, the cellular force exerted by individual matrix adhesions onto the ECM substrate could be estimated by this method. It is a key feature of all methods described here that substrate rigidity could be modulated (by varying the force applied to cell surface-bound beads [49], the elastic modulus of the substrate [48], or the length of the silicone posts [8], respectively). As already indicated above, these studies made clear that fibroblasts use cytoskeletal forces and matrix adhesion contacts to constantly probe the mechanical properties of their ECM substrate. In turn, cells use this information for dynamic remodeling of their physical interactions with the environment, and hence of their shape and movements [24]. As outlined in more detail below (Section 3.3), the intracellular signals evoked by sensing substrate features can have profound effects on cell differentiation and function [55,56].

2.3. Role of matrix adhesion contacts in mechanotransduction

In the last paragraph, some of the immediate cellular responses to substrate-derived mechanical stimuli have been exemplified. In its narrow sense, mechanotransduction is defined as the process by which physical stimulation is converted intracellularly into various types of electrical or chemical signals. The process has been studied originally in excitable mechanosensory cells. In neurons and muscle, stretch-activated membrane cation channels transduce mechanical forces into electrical action potentials, which are either propagated or transformed into chemical signals [57]. For non-excitable cells such as fibroblasts and other cells of mesenchymal origin, there is increasing evidence that mechanical stimulation can be converted directly into chemical signaling [23]. We described above how mechanical forces are transmitted from the ECM to the cytoskeleton. Integrins constitute one of the key links, and pulling on integrins via their extracellular ligands has long been shown to trigger a variety of signaling pathways within the cell [49,58]. One possibility by which mechanical stress at the cell surface could translate into a chemical signal is via the opening of stretch-activated cation channels [59] that are associated with adhesion contacts. Indeed, calcium influx and calcium-mediated intracellular signaling has been observed in fibroblasts [6,60,61] and various other cell types [58,62] in response to mechanical stimulation. Alternatively, tension on focal adhesions could change the binding kinetics between structural and signaling components, as it has been reported for the interaction between integrin and zyxin [63]. According to another interesting hypothesis, proteins associated with focal adhesions might act as "strain gauges" that sense mechanical stresses both of external (ECM) and internal (cytoskeletal) origin. Stretch has recently been shown to induce conformational changes in the adaptor protein p130Cas, exposing hidden phosphorylation sites that become targets of Src family kinases [64]. The newly modified sites are recognized by other signaling proteins, which in turn activate small GTPases of the Rho and Ras families [45]. As described below, the latter are known to have multiple effects on cytoskeletal dynamics, gene transcription, cell division and differentiation [50].

3. Cellular responses to mechanical stimulation

3.1. Major signaling pathways triggered by mechanical stimuli

As a consequence of mechanotransduction as described in the last paragraph, a multitude of intracellular signaling pathways can be triggered in fibroblasts and other adherent cells in response to various mechanical stimuli (tension, compression, shear). This topic has been covered in previous reviews for various cell types [5,6,10,19,65–70], and only a brief summary of common features is given here. Among the prominent signaling cascades elicited immediately in adherent cell types at cell-ECM adhesion contacts by mechanical stress are:

(1) Calcium influx through stretch-activated cation channels and activation of calcium-dependent signaling [6,60,61,71,72];
(2) Generation of active oxygen species and activation of nuclear factor kappa-B (NFκB) [65,73–75];
(3) Stimulation of small GTPases of the Ras family and of mitogen-activated protein kinases (MAPKs) [9,58,76–78];
(4) Changes in the activity of small GTPases of the Rho family and of cytoskeletal dynamics [71,79,80].

Not surprisingly, among the immediate early genes induced by mechanical stimulation are certain transcription factors that are regulated via the same pathways by other extracellular signals. In endothelial cells, for example, the gene for transcription factor Egr-1 is activated within minutes by fluid shear stress via integrin-dependent activation of the Ras/Erk-1/2 pathway [77]. Egr-1 might in turn be responsible for the delayed up-regulation of many secondary mechanoresponsive genes (see Section 4.1). In fibroblasts c-Fos, an AP-1 transcription factor and classical immediate early gene in the response to serum and growth factors, is rapidly induced by cyclic substrate strain [81,82]. The question remains how the RhoA/ROCK pathway, which is primarily involved in controlling actin dynamics, can relay mechanical stress sensing to changes in gene expression. As described in a previous paragraph, a typical immediate reaction of many cells to tensile strain is an integrin- and RhoA/ROCK dependent increase in actin stress fiber formation [65]. A possible link to gene regulation via this pathway is megakaryocytic leukemia protein MAL/MKL1/MRTF-A, a myocardin-related transcriptional co-activator of serum response factor SRF [83]. In serum-starved cells, MAL is found associated with monomeric actin in the cytoplasm. Upon RhoA-dependent actin assembly triggered by serum or growth factors, MAL is thought to be released from G-actin and to translocate to the nucleus, where it can stimulate SRF-dependent gene expression [84,85]. Thus, cytoskeleton-associated molecules like MAL might be involved, directly or indirectly, in the transduction of a mechanical into a chemical signal. One recent publication [86] reported that mechanical stimulation of smooth muscle cells (by pulling on collagen-coated magnetic beads bound to their surface) induced nuclear translocation of MAL, as well as MAL/SRF-dependent activation of the αSM-actin gene promoter. Induction of the connective tissue growth factor (*CCN2*) gene by cyclic strain also requires increased actin polymerization [87], and in this case the response is mediated by an NFκB binding site in its proximal promoter. Indeed, the same authors claim that strain-induced NFκB translocation to the nucleus depends on the increase in actin polymerization; the mechanism is not clear, however. In any case, these and other findings indicate that, in addition to transcription factors activated or induced via MAPK pathways, MAL/SRF and NfκB are the likely candidates for mediating immediate gene induction by mechanical stress.

3.2. Cooperation between growth factor and integrin signaling

Some of the best examples for "context-dependent signaling", the theme of this special section, are provided by the long known phenomena called "anchorage-dependent growth" and "anoikis". Anchorage-dependent growth describes the observation that most normal cells of mesenchymal and epithelial origin (in contrast to transformed or tumor cells) are not able to proliferate when cultured either in suspension or in nonadhesive gels such as soft agar, even in the presence of high concentrations of serum or growth factors [88–92]. Anoikis (homelessness) is a special type of apoptosis (regulated cell death) that is often triggered as a consequence of normal cells

being suspended for a prolonged period of time, i.e. when they lack a firm adhesive substrate [93–95]. In the last decade it became clear that an important mechanistic basis for these phenomena is the close cooperation between growth factor receptors and integrins in triggering intracellular signaling cascades [96–105]. The functional interplay between these two components is facilitated by their close proximity in matrix adhesion contacts: physical interactions between various types of integrins and growth factor receptors have been reported that can be both direct [103] and indirect, i.e. via distinct adaptor proteins [106–108]. Essentially, matrix adhesion contacts function as assembly lines for intracellular signaling pathways [109,110]. Engagement of integrins with ECM ligands not only leads to their clustering and linking to the actin cytoskeleton as described above, but also to the recruitment of many signaling components to matrix adhesion contacts. Among them are direct effectors of activated integrins (e.g. protein kinases such as FAK, Src, PI3K), but in addition growth factor receptors and their downstream targets such as guanine nucleotide exchange factors, G-proteins and small GTPases [109,110]. Integrin-mediated clustering of receptor kinases together with their downstream targets vastly amplifies the signaling of growth factors to MAPK and other pathways involved in cell growth and differentiation. Moreover, physical and functional interactions of shared components allow the crosstalk between growth factor- and integrin-triggered signaling pathways at many levels [111–113]. Although various integrins clearly respond to specific chemical cues due to their interactions with distinct ECM ligands [114], their main function lies in force transmission and force sensing [23,115]. Hence, the association of integrins with growth factor receptors at matrix adhesion contacts allows cells to integrate mechanical with growth factor derived signals, resulting in context-dependent cellular phenomena such as anchorage-dependent growth and anoikis.

3.3. Mechanical control of cell differentiation and growth

When considering the sustained effects of mechanical signaling on the function of adhesion-dependent cells, we will first turn to the precursors of fibroblasts, namely mesenchymal stem cells. A few years ago, the group of C.S. Chen [55] published a hallmark paper demonstrating that adhesion-mediated mechanical context, in combination with specific growth factors, can act as a switch controlling the differentiation of mesenchymal stem cells towards either the osteoblast or the adipocyte lineage. Mesenchymal stem cells were plated on fibronectin squares of different areas (1000–10,000 μm^2) that were contact-printed onto culture dishes. Interestingly, cells that became attached to large adhesive squares, and thus were able to spread extensively, started to express osteoblastic markers such as alkaline phosphatase. In contrast, cells on small squares were restricted in their spreading and forced to assume a more rounded shape; these cells accumulated lipid droplets typical for adipocyte differentiation. This differentiation switch was mediated by cell shape-dependent activation of the RhoA/ROCK pathway. Because integrin-dependent focal contact formation also stimulates actin assembly and contraction (see above), activation of the RhoA/ROCK pathway very much depends on the extent of cell spreading. Notably, McBeath et al. [55] found that when they inhibited ROCK activity in mesenchymal stem cells spread on large fibronectin squares, these cells switched from an osteoblastic to an adipocytic phenotype; conversely, viral transduction with constitutively active ROCK induced the osteoblastic marker even in rounded cells attached to small squares. The authors concluded that mechanical cues experienced in a developmental context are essential for the commitment of stem cell fate. In accordance with these results, the lab of D.E. Discher [56] reported recently that varying the substrate elasticity can exert a similar instructive effect on mesenchymal stem cell differentiation: soft gels favoring a round cell shape induce neurogenic marker genes, substrates of intermediate elasticity promote myogenesis, and stiff culture surfaces allowing maximal cell spreading lead to osteoblastic differentiation as expected.

Fibroblasts of course have a much more restricted developmental potential than their mesenchymal precursors. Nevertheless, their differentiation to myofibroblasts e.g. during wound healing involves prominent changes in gene expression, cytoskeletal structure, and function [116]. Interestingly, myofibroblast differentiation seems to be governed by very similar RhoA/ROCK-dependent mechanisms as those described above for mesenchymal stem cells. Specifically, tensile stress in combination with TGF-β [117] is required for the expression of myofibroblast specific genes, e.g. α-smooth muscle actin [86].

Substrate-determined mechanical features also have a large influence on cell proliferation, both of single cells and of tissue-like aggregates. C.S. Chen et al. [118] had shown previously that endothelial cells attached to large fibronectin squares divided more rapidly than those on small patches, in agreement with anchorage-dependent growth of many cell types (see above). Recently, these authors investigated the behavior of entire sheets of ca. 100 endothelial cells on large (250×250 μm) fibronectin squares [119]. Since endothelial cells (in contrast to fibroblasts) form extensive cell–cell contacts, they pull on each other when they individually contract their actin cytoskeleton. The resulting mechanical stress propagates throughout the cell sheet. It can be calculated by finite element analysis that cells at the edges of the sheet experience more tensile stress than those in the center, and that tension is highest at the corners of the square. Interestingly, cells at edges and corners not only showed increased actin stress fibers, but also significantly higher incorporation of BrdUrd into DNA. Inhibition of ROCK not only dissipated the tensile stress within the cell sheet, but also abolished the site-specific differences in DNA synthesis. Thus, spatial features such as edges and corners lead to an uneven (anisotropic) distribution of tensile stress in multicellular aggregates or tissues, and via a RhoA/ROCK dependent mechanism this in turn affects cytoskeletal organization as well as the rate of cell division. The described experiments might be special for endothelial cells because cultured fibroblasts do not form cell–cell adhesions or cell sheets. However, for fibroblasts embedded in 3D extracellular matrix, forces are transmitted from cell to cell via the intervening ECM, and it has been shown that fibroblast proliferation in 3D culture and in tissue depends on local mechanical differences as well [120,121].

4. Regulation of extracellular matrix genes by mechanical signaling

4.1. Indirect regulation of major extracellular matrix components by mechanical stress

As outlined earlier, ECM production is a major response of fibroblasts to increased tensile stress, as e.g. observed during wound healing and fibrosis [17,116], and various major ECM components have been shown to be induced by static or dynamic substrate strain in cultured fibroblasts (reviewed in [5]). However, in many cases the induction of gene expression is slow and apparently indirect. In cultured primary fibroblasts, fibronectin is moderately induced by cyclic strain after 24 h but not at earlier time points [122]. One possible mechanism of indirect regulation involves the prior synthesis of a transcription factor that in turn induces secondary mechanoresponsive genes such as fibronectin. Transcription factor Egr-1 was mentioned earlier as an immediate early gene induced in endothelial cells in response to fluid shear stress [77]. In glioblastoma cells, Egr-1 has been shown to transactivate the fibronectin gene directly by binding to specific sites in its proximal promoter [123]. Although comparable studies with fibroblasts are missing, tensile strain is likely inducing the fibronectin gene indirectly in these cells, presumably via prior synthesis and activation of Egr-1. Another indirect way by which mechanical stress can regulate ECM genes is via the secretion and

activation of TGF-β, a prominent growth factor required for myofibroblast differentiation and ECM production [17]. Mechanical stress can lead to the paracrine release of TGF-β from fibroblasts [124], or to the activation of preexisting TGF-β in the ECM of myofibroblasts [117]. The released and activated TGF-β will then induce target genes such as procollagen α1(I) via classical signaling pathways [124]. Thus, increased bulk production of ECM should probably be viewed as a sustained differentiation process, rather than as an immediate response to changes in mechanical conditions. However, at least one ECM component seems to be regulated by mechanical stress via a direct mechanism: the mRNA for tenascin-C is increased within 1 h of applying cyclic strain to cultured fibroblasts [82], and this induction occurs even in the absence of protein synthesis [122]. Because of these interesting features, the mechanotransduction pathway regulating tenascin-C expression is described in more detail in the next section.

4.2. Tenascin-C: a mechanoresponsive modulator of cell adhesion

Tenascin-C is a large ECM glycoprotein with structural relationship to fibronectin, however with opposite function (see accompanying article by R.P. Tucker and R. Chiquet-Ehrismann). In contrast to fibronectin, tenascin-C on its own is weakly or not adhesive for most cells, and it inhibits fibronectin-mediated cell spreading when the two proteins are mixed [125]. Tenascin-C was reported to interfere with cell spreading by inhibiting binding of fibronectin to its co-receptor syndecan-4 [126]. Consequently, integrin α5β1 signaling to FAK and RhoA is disturbed, focal adhesions are diminished, and the cells round up [127]. Interestingly, tenascin-C is a prominent ECM protein whose expression is regulated by mechanical stress in vivo and in vitro (for review, see [128]). During development and in adulthood, tenascin-C expression is mostly confined to tissues experiencing high tensile stress, such as ligaments, tendons and smooth muscle [13,129]. We showed in a chick embryo model that leg muscle immobilization suppressed tenascin-C accumulation in developing tendons [130]. Conversely, fixing small weights to the wings of young chickens strongly induced tenascin-C mRNA and protein in endomysial fibroblasts of the affected holding muscle [131]. Tenascin-C was over-expressed in arterial smooth muscle in a hypertensive rat model [132] and in the periosteum of rat ulnae loaded in vivo [133], and it is an early marker for overload-induced juvenile osteoarthritis [134]. Conversely, tenascin-C expression was diminished in the osteotendinous junctions of immobilized rat legs [135]. One might speculate that in tissues under high mechanical load, secretion of tenascin-C helps cells to loosen their matrix adhesion contacts if required, in order to avoid overstretching. Irrespectively of its precise mode of action, tenascin-C seems an ideal target to study mechanical regulation of gene expression in vitro.

4.3. Rapid induction of tenascin-C by combination of cyclic strain and growth factors

For studying signaling pathways that lead to the induction of a specific gene by mechanical stimuli, several in vitro systems are being used. Growing fibroblasts on either stretched or relaxed collagen gels can simulate different states of static tension [136,137]. One way to apply dynamic strain to cultured cells is to plate them on ECM-coated elastomer membranes that are stretched and relaxed cyclically using a suitable device [122,138,139]. Tenascin-C expression by cultured fibroblasts responds to both static and dynamic (cyclic) strain. Tenascin-C mRNA and protein levels in fibroblasts were shown to be high on stretched and low on relaxed collagen matrices [136,137]. When we cultured chick embryo fibroblasts on fibronectin-coated silicone membranes and subjected them to 10% strain at 0.3 Hz, tenascin-C mRNA and protein was robustly induced within 3–6 h [122]. This response was not blocked by cycloheximide, an inhibitor of protein synthesis. Thus, tenascin-C induction by tensile stress does not seem to depend on the prior synthesis of a transcription factor. There was no evidence for a paracrine mechanism either: incubation of cells with conditioned medium from cyclically stretched cells did not induce tenascin-C in resting cells [122]. The growth factors TGF-β and PDGF increased tenascin-C mRNA levels as reported previously [128], and this stimulation was inhibited to various degrees by MAPK antagonists. Surprisingly, MAPK inhibitors had no effect on tenascin-C mRNA induction by cyclic strain, whereas inhibition of ROCK, the target kinase of RhoA, suppressed this response [5,122]. This indicated that distinct pathways regulate the induction of tenascin-C by growth factors and by mechanical stimulation, respectively (although cross-talk is likely since e.g. PDGF stimulates both MAPK and RhoA pathways [140]). Supporting this notion, the combination of growth factors with cyclic strain had additive effects on tenascin-C expression over a wide range of conditions. There is evidence from gene promoter studies that integration of the various signals occurs in part at the level of gene transcription for tenascin-C: distinct cis-acting elements on the gene promoter seem to be responsible for the response to growth factors and mechanical stress, respectively [136,141].

4.4. Role of cytoskeletal dynamics for gene induction by cyclic strain

Since ROCK inhibition suppressed tenascin-C induction by cyclic strain (see above), we further investigated the role of RhoA/ROCK signaling in controlling this process (Fig. 3). We found that cyclic strain (10%, 0.3 Hz) stimulated RhoA activity in mouse embryo fibroblasts within 5 min, followed by cell contraction and redistribution of actin stress fibers (Fig. 3A) as well as focal adhesions to the cell margins. Tenascin-C mRNA levels increased after 1 to 6 h of strain. All these processes were attenuated by inhibition of either ROCK or myosin II activity. Conversely, chemical activators of RhoA (lysophosphatidic acid or thrombin) strongly enhanced the effect of cyclic strain on cell contractility as well as on tenascin-C mRNA levels. These responses were found to depend on an intact cytoskeleton: disruption of the actin network abolished induction of tenascin-C mRNA by both cyclic strain and chemical RhoA activators [139]. It can be concluded that the expression level of the tenascin-C gene in fibroblasts is directly coupled to the mechanosensory function of a dynamic actin cytoskeleton. The data suggest the interesting possibility that previous activation of RhoA/ROCK, e.g. by inflammatory mediators during wound healing, increases the cytoskeletal pre-stress and thus renders fibroblasts more sensitive to subsequent mechanical stimulation [142]. This would be an example where the growth factor milieu in turn tunes the mechanical signaling, making it context-dependent.

We considered the possibility that MAL/MKL1/MRTF-A (see Section 3.1) might provide a link between actin dynamics and gene expression also in response to cyclic strain. Using a novel monoclonal antibody, we could indeed demonstrate that MAL translocated to the nucleus in 60–90% of fibroblasts subjected to cyclic strain (10%, 0.3 Hz) for 1 h (Fig. 3B; [82]). The mouse tenascin-C gene promoter does not possess a canonical SRF binding site (i.e. a serum response element [83]), and we currently investigate whether tenascin-C induction by cyclic strain (Fig. 3C) depends on MAL directly or indirectly. However, one other publication [86] has recently shown that a mechanical stimulus can trigger RhoA activation, actin polymerization as well as MAL nuclear translocation in smooth muscle cells. In these experiments, collagen-coated beads were allowed to bind to the cell surface, and force was applied with a magnet. The mechanical stimulation activated the α-smooth muscle actin gene promoter, and this response was suppressed by mutation of the serum response element in the promoter as well as by co-transfection with dominant negative MAL. Thus, at least for this gene important for smooth muscle differentiation, a direct link from mechanical stimulation to transcriptional control via a RhoA/actin/MAL/SRF pathway could be demonstrated. Because of the prominent *de novo* expression of tenascin-C in wound healing and fibrosis [128], we expect that its RhoA-dependent

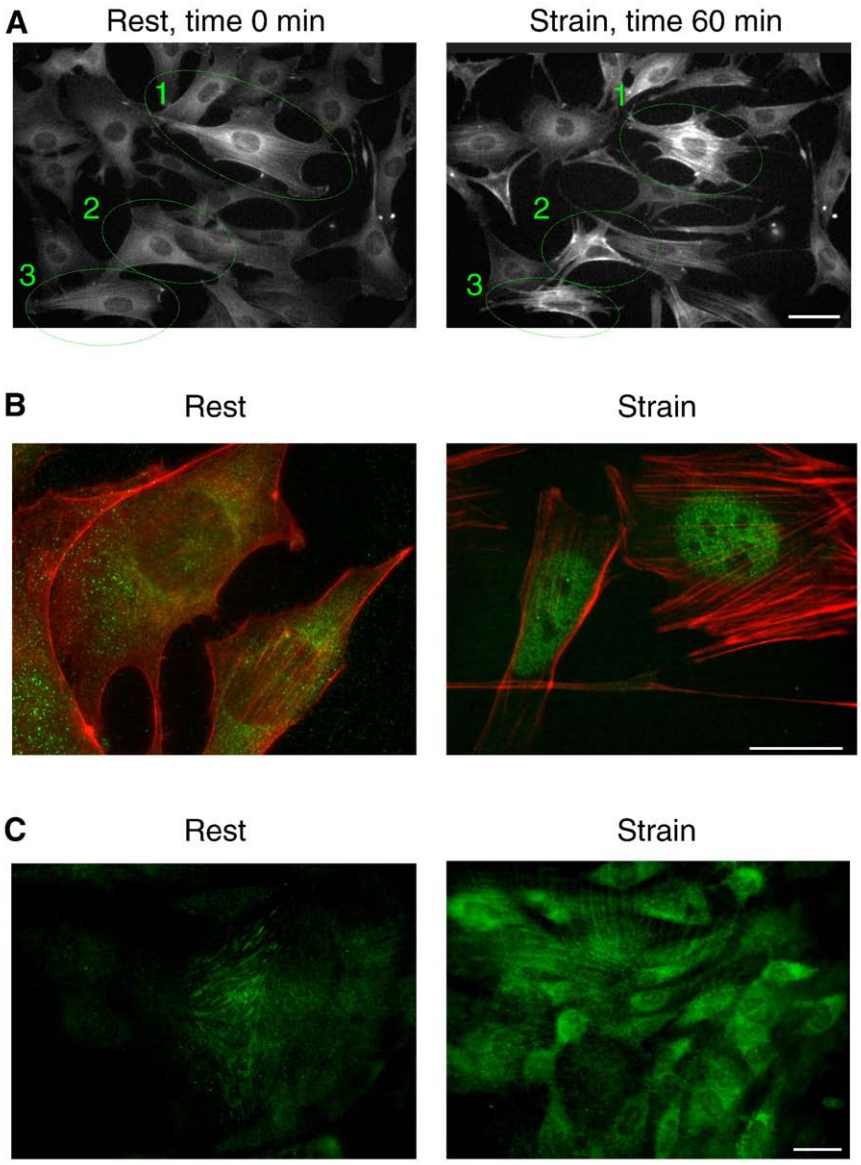

Fig. 3. Cyclic strain induces actin reorganization, nuclear translocation of MAL/MRTF-A and tenascin-C expression in fibroblasts. (A) Fibroblasts stably expressing acting-GFP were cyclically stretched (0.1 Hz, 1 h) and a picture of GFP fluorescence was taken every 2 min. The figure presents the first and last points of the experiment and circles indicate three cells where the formation of actin fiber bundles and cytoskeleton contraction is particularly important. Scale bar: 50 μm. (B) Mouse fibroblasts were cultured on fibronectin-coated silicone membranes in 0.03% serum and either left at rest or subjected to cyclic strain (10%, 0.3 Hz) for 1 h. Cells were fixed with paraformaldehyde and stained with mAb 65F13 to visualize nuclear translocation of MAL (green) and phalloidin to visualize actin stress fibers (red). The scale bar represents 20 μm. (C) Primary newborn mouse skin fibroblasts were cultured on fibronectin-coated silicone membranes in 0.3% serum and either left at rest or subjected to cyclic strain (10%, 0.3 Hz) for 6 h. Cells were fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton and stained with a 1:1 mixture of rabbit sera 473 and 474 against tenascin-C. The secondary anti-rabbit antibody was FITC-labeled and the scale bar represents 50 μm.

mechanism of regulation by mechanical stimuli be shared between fibroblasts, myofibroblasts and smooth muscle cells.

4.5. Requirement of β1-integrins and ILK for mechanical induction of tenascin-C

We asked which focal adhesion components upstream of RhoA/ROCK might be required for transducing mechanical signals into tenascin-C induction. We found that fibroblasts deficient for β1-integrins did not show any increase in tenascin-C mRNA in response to cyclic strain [68]. Although somewhat expected, this was an interesting result because β1-deficient cells still attach to fibronectin and other ECM proteins by means of their vitronectin receptor αvβ3. Concerning the response of tenascin-C to mechanical stress, however, other ECM receptors were not able to substitute for β1-integrins. Thus, in the context of mechanotransduction there is reason to focus on this class of integrins. Integrin-linked kinase (ILK) is a β1-integrin-associated adaptor protein whose enzymatic activity is disputed [108], but which is essential for the function of focal adhesions during cell polarization and migration in the embryo [143]. While in matched wild-type mouse fibroblasts tenascin-C mRNA was increased several fold after cyclic strain, it remained at base level in ILK-deficient cells. Rescue of knockout fibroblasts by stable expression of wild-type ILK restored this response to cyclic strain. Tenascin-C mRNA was still induced to some extent by serum or lysophosphatidic acid in ILK knockout fibroblasts. Therefore, signaling pathways capable to induce tenascin-C expression (see Section 4.3 and Section 4.4) were functional in the absence of ILK, but failed to be triggered by mechanical stimulation. Accordingly, cyclic strain no longer

stimulated RhoA activity, ROCK-dependent actin stress fiber reorganization and MAL translocation in ILK deficient fibroblasts, in contrast to what we observed with wild-type cells. Surprisingly however, the MAPK Erk-1/2 was still activated and mRNA for transcription factor c-Fos induced in ILK knockout cells in response to cyclic strain. Hence, mechanotransduction was not totally abolished in fibroblasts in the absence of ILK. Rather, our results indicated that ILK is selectively required for the induction of tenascin-C (and presumably a set of other genes) by mechanical stimulation via a RhoA-dependent pathway [82].

5. Conclusions and perspectives

The ability to sense mechanical forces serves two major functions in cells and tissues. The first is the capability to respond very quickly to rapid changes in mechanical load, e.g. by simultaneously adjusting the tension of many muscles to keep balance during walking. The second task of force sensing is to monitor and integrate mechanical stimuli over extended periods of time, and to use this information for structurally adapting (i.e. "training") tissues to chronic changes in mechanical load. Interestingly, distinct types of mechanotransduction mechanisms seem to have evolved that deal with fast versus slow responses to mechanical stress. The excitable mechanosensory neurons, skeletal and heart muscle cells are specialized for fast responses to mechanical stress. As is known for many years, stretch activated membrane cation channels trigger Ca^{2+} influx in these cells, leading to immediate firing of action potentials and/or myofibril contraction [57]. In contrast, mesenchymal stem cells, fibroblasts or smooth muscle cells react to mechanical stress by slower adaptive responses mainly involving changes in cytoskeletal dynamics and gene expression [68]. Mechanotransduction in these cells is being investigated only in the last decade, and a lot has been learned about an alternative mechanism that depends on force transmission from the ECM to the cytoskeleton via integrins and associated focal adhesion proteins. The most interesting feature of this type of mechanotransduction is its apparent independence from ion fluxes, since it can be reconstituted with triton-extracted "cell ghosts" that lack membranes [45]. (Of course, this does not preclude Ca^{2+} signaling from contributing to the response of living fibroblasts to mechanical stress.) In contrast to the well known stretch-sensitive membrane channels, the newly discovered cytoplasmic mechanotransducers (such as p130Cas) are thought to act as molecular springs: when force is applied, their elastic domains are stretched and partially unfolded, enabling their chemical modification. This then results in downstream signaling via classical pathways. The future challenge lies in identifying similar "strain gauges" that function upstream of other prominent signaling pathways induced by mechanical stress. Our own results indicate that upstream of the RhoA/ROCK pathway ILK might be part of a similar mechanosensor, although this needs to be investigated further. Moreover, since forces are transmitted throughout the cell, it is feasible that similar stretch sensors will be found not only in matrix adhesion contacts, but also e.g. associated with the cytoskeleton, the nuclear membrane or even the nuclear matrix, moving mechanotransduction close to the sites of gene expression [144].

The second important goal of the studies reviewed here is to elucidate how cells and tissues are able to integrate mechanical with growth factor derived signals to produce a meaningful adaptive response. In other words, we need to know how, during differentiation and regeneration, tissue mechanics provide a context for humoral control and vice versa. Our own work on the control of expression and function of the ECM protein tenascin-C provides just an isolated example; systems biological approaches are needed in the future to attack this complex problem [110]. Answers will have an impact on fields as diverse as e.g. physiotherapy, wound and fracture healing, hypertension, or tissue engineering.

Acknowledgements

Own work described in this review was funded by the M.E. Müller Foundation, the ITI Foundation, the Novartis Research Foundation, and by grants from the Swiss National Fund to MC. The authors thank Ruth Chiquet-Ehrismann for her many helpful suggestions as well as for her hospitality and continuing support.

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