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The myofibroblast: Paradigm for a mechanically active cell

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ABSTRACT

Tissues lose mechanical integrity when our body is injured. To rapidly restore mechanical stability a multitude of cell types can jump into action by acquiring a reparative phenotype—the myofibroblast. Here, I review the known biomechanics of myofibroblast differentiation and action and speculate on underlying mechanisms. Hallmarks of the myofibroblast are secretion of extracellular matrix, development of adhesion structures with the substrate, and formation of contractile bundles composed of actin and myosin. These cytoskeletal features not only enable the myofibroblast to remodel and contract the extracellular matrix but to adapt its activity to changes in the mechanical microenvironment. Rapid repair comes at the cost of tissue contracture due to the inability of the myofibroblast to regenerate tissue. If contracture and ECM remodeling become progressive and manifests as organ fibrosis, the outcome of myofibroblast activity will have more severe consequences than the initial damage. Whereas the pathological consequences of myofibroblast occurrence are of great interest for physicians, their mechano-responsive features render them attractive for physicists and bioengineers. Their well developed cytoskeleton and responsiveness to a plethora of cytokines fascinate cell biologists and biochemists. Finally, the question of the myofibroblast origin intrigues stem cell biologists and developmental biologists—what else can you ask from a truly interdisciplinary cell?

1. Introduction

This special issue of the Journal of Biomechanics covers a variety of tissue and cell types that are subject to different mechanical challenges and that actively play various mechanical roles. In normal tissues under physiological conditions the residing cells experience specific mechanical signals within a distinct range of magnitudes. Typical examples are vascular endothelial cells and leukocytes, exposed to shear stress, epithelial cells to shearing and stretching, smooth muscle cells to stretch, striated muscle cells to stretch and compression, osteoblasts and chondrocytes to compression. In other words, most cells in the adult organism live in a 'mechanical niche' and it is generally acknowledged that this defined set of mechanical cues is crucial to maintain their identity (Discher et al., 2005; Janmey and McCulloch, 2007). Similarly, during development the constantly changing mechanical environment is a major determinant of cell fate (Krieg et al., 2008). At this stage cells are more plastic and adapt/contribute to complex mechanical patterns present in the in early embryo. Adult cells can (re-)gain some level of plasticity after tissue injury and during repair, conditions that in many respects can resemble the situation in the embryo. Tissue boundaries are disintegrated and the mechano-protective architecture of the extracellular matrix (ECM) is disturbed. In addition to this dramatic imbalance in their mechanical equilibrium cells become exposed to an overwhelming cocktail of cytokines, initially deriving from damaged and inflammatory cells (Gurtner et al., 2008).

Activated by mechanical stress and cytokines, many cells of predominantly mesenchymal origin differentiate into myofibroblasts which drive tissue repair by secreting collagen and reorganizing (contracting) the ECM (Tomasek et al., 2002). Despite the fact that acquisition of the myofibroblast phenotype is generally called 'differentiation' it may be more appropriate to consider this cell being less differentiated than its precursor and rather primitive. Primitive is here used in the positive sense of the word: 'being of origin' or 'being of simple character' and several characteristics of the myofibroblast support this point of view. Typical molecular features of the differentiated myofibroblast are neo-expression of α-smooth muscle actin (α-SMA) and of the fibronectin (FN) splice variant ectodomain (ED)-A FN. Phylogenetically and during embryogenesis, α-SMA is one of the earlier

Abbreviations: AFM, atomic force microscopy; α-SMA, α-smooth muscle actin; ECM, extracellular matrix; ED-A, extra domain A; FA, focal adhesion; FN, fibronectin; LAP, latency associated protein; LTBP, latent transforming growth factor β binding protein; MRTF, myocardin-related transcription factor; TGFβ, transforming growth factor β

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expressed muscle actins. During heart development for instance, cardiomyocytes first transiently express α-SMA, followed by α-skeletal actin, which is finally replaced by α-cardiac actin persisting in the in the adult heart muscle (Clement et al., 2007). Similarly, the ED-A FN splice variant is characteristic for embryonic development, becomes down-regulated in most adult tissues and re-appears during tissue repair in the context of myofibroblast development (Ffrench-Constant et al., 1989; Serini et al., 1998). Also on the functional level, myofibroblasts are rather poor construction workers. They effectively repair defects and re-establish mechanical tissue integrity but never truly regenerate the damaged tissue. The resulting formation of a collagenous and stiff scar leads to reduced tissue function and even organ failure if myofibroblast repair becomes chronic such as during progression of fibrosis (Hinz, 2009).

Together, these features render the myofibroblast an interesting cell type for the study of mechanobiology: (1) it is highly relevant for physiological and pathological tissue remodeling, (2) it is mechanically active and contributes to alterations in overall tissue mechanics, (3) it is mechano-sensitive and mechanically inducible, and (4) the fundamental character of the myofibroblast allows for studying basic mechanical principles and pathways.

2. A beginner's guide to the myofibroblast

The myofibroblast was discovered by Gabbiani and coworkers in the early 1970s and first shown to actively promote dermal wound contraction (Gabbiani et al., 1971). Since then, this cell has been on the rise and its importance demonstrated for many pathophysiological processes that include tissue repair and remodeling. Myofibroblast activity is beneficial for dermal wound closure and for restoring the mechanical stability of injured organs against rupture. De-regulated and chronic myofibroblast activity however generates tissue deformation by contracture and impedes organ function. Tissue contractures are clearly visible in skin hypertrophic scars such as those developing after burns (Atiyeh et al., 2005), in scleroderma (Strehlow and Korn, 1998; Varga and Abraham, 2007) and in the palmar fibromatosis of Dupuytren's disease (Tomasek et al., 1999). Myofibroblast-generated contractures are also fundamental in organ fibrosis, affecting liver (Gressner and Weiskirchen, 2006; Iredale, 2007), heart (Baudino et al., 2006; Brown R.D. et al., 2005), lung (Phan, 2002; Thannickal et al., 2004) and kidney (Liu, 2006) with often lethal consequences. Myofibroblasts are instrumental in creating tissue constrictions around solid body implants (Comut et al., 2000), they contract silicone breast implants (Rudolph et al., 1978; Siggelkow et al., 2003) and are activated by different implanted biomaterials in a fibrotic host reaction (Anderson et al., 2008; Li et al., 2007). Myofibroblasts further contribute to the evolution of atheromatous plaque after blood vessel injury (Bochaton-Piallat and Gabbiani, 2006) and play a crucial role in the stroma reaction to epithelial tumors (De Wever et al., 2008; Desmouliere et al., 2004). The finding that cancer progression is stimulated by the myofibroblast-created environment has exposed the tumor-associated myofibroblast as an important target for anti-cancer therapy (Albini and Sporn, 2007).

Another reason for the attractiveness of the myofibroblast for a broad scientific and clinical audience is the large panel of cells that can develop this phenotype upon activation. It appears that myofibroblasts can be recruited from whatever local cell type is suitable to rapidly repair injured tissue (Hinz et al., 2007). Local fibroblasts residing in different tissue locations are considered the most prominent source of myofibroblasts. However, a variety of other precursor cells contribute to the myofibroblast population depending on the nature of the injured tissue and the particular microenvironment. The incomplete list, in no particular order, includes chondrocytes, osteoblasts, hepatic stellate cells, smooth muscle cells, pericytes, fibrocytes, mesenchymal stem cells, epithelial cells undergoing epithelial-to-mesenchymal transition, and possibly astrocytes (for more a more detailed evaluation of myofibroblast precursors, see (Hinz, in press; Hinz et al., 2007 and references therein).

The amazingly heterogeneous selection of possible progenitors raises the question: What are the criteria that identify the myofibroblast? Today, neo-expression of the smooth muscle actin isoform α-SMA is the most widely used myofibroblast marker in research and clinical diagnostics. Myofibroblasts are usually negative for desmin, smooth muscle myosin heavy chain, h-caldesmon, and smoothelin, distinguishing them from normal smooth muscle cells (Schurch et al., 2007). The convenience of using α-SMA as molecular marker may have contributed to the misconception that a myofibroblast must express α-SMA to be a myofibroblast. A priori however, the definition of the myofibroblast is based on its contractile function reflected in its well chosen name. Myofibroblasts combine ultrastructural and functional features of smooth muscle (myo-) by forming contractile actin/myosin-containing stress fibers, with the extensive endoplasmic reticulum of synthetically active fibroblasts (Gabbiani et al., 1971). To highlight the fact that the contractile cytoskeleton is not a feature of normal tissue fibroblasts, we previously introduced the term 'proto-myofibroblast' for stress fiber-containing, but α-SMA-negative fibroblasts. 'Differentiated myofibroblast' designates cells with α-SMA-positive stress fibers (Tomasek et al., 2002). This distinction is more than semantic finesse because both phenotypes can co-exist *in vitro* and *in vivo* and perform different functions. For instance, in the early granulation tissue of open rat wounds, α-SMA-negative proto-myofibroblasts, identified by Phalloidin decoration of stress fibers, emerge after 6 d of healing. Proto-myofibroblasts lay down the first collagen bundles and pre-organize the provisional ECM by exerting comparably small traction forces. Consecutive appearance of α-SMA-positive differentiated myofibroblasts in 9 d-old wounds then hallmarks the contractile phase of wound closure (Fig. 1) (Hinz et al., 2001b).

3. Mechanical control of the myofibroblast phenotype—it is in the matrix

Mechanics play a pivotal role in controlling myofibroblast differentiation and function. The goal of myofibroblast activity is to rapidly re-establish tissue integrity by secreting and organizing new ECM; this process is precisely controlled through a mechanical feedback from the ECM. The provisional ECM laid down after acute tissue injury, e.g., the fibrin clot of dermal wounds, is estimated to be very compliant with a Young's modulus of 10–1000 Pa (Fig. 2). Under comparable conditions *in vitro*, such as growth on very soft two-dimensional polyacrylamide gels and in three-dimensional soft collagen gels, development of stress fibers by fibroblasts is suppressed. Fibroblasts without stress fibers form only very small and immature adhesions with the ECM that are called focal complexes or nascent adhesions (Tamariz and Grinnell, 2002; Yeung et al., 2005) (Fig. 3). The proto-myofibroblast phenotype is only produced on stiffer culture substrates exhibiting an elastic modulus of at least 3000 Pa; these cells form α-SMA-negative stress fibers that terminate in mature focal adhesions (FAs) (Figs. 1–3) (Yeung et al., 2005). A stiffness of ~18,000 Pa has been measured in 7 d-old rat wound granulation tissue which is mainly populated by proto-myofibroblasts (Figs. 1 and 2). Even stiffer culture substrates with a Young's modulus of ~20,000 Pa and higher are required to permit further myofibroblast differentiation. Expression of α-SMA in stress fibers on stiff substrates is associated with the formation of large supermature FAs (Goffin et al., 2006; Wells, 2005) (Figs. 1–3).

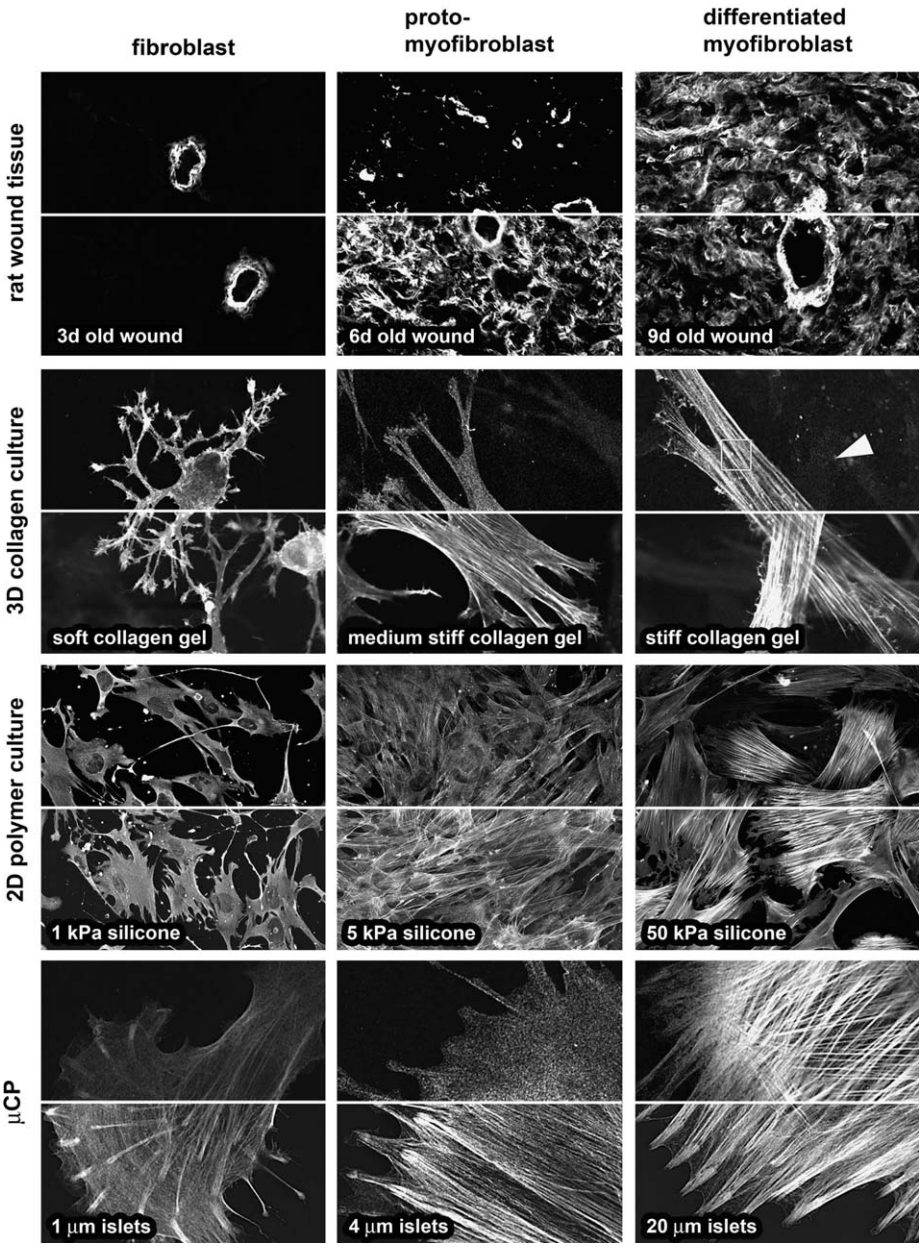

Fig. 1. *ECM stiffness controls myofibroblast differentiation.* Unlike non-contractile tissue fibroblasts, proto-myofibroblasts form contractile stress fibers that are α-SMA-positive in differentiated myofibroblasts. Co-immunostaining for F-actin (Phalloidin, all lower image parts) and for α-SMA (all upper image parts) discriminates between all three phenotypes. In 3 d-old rat open wound granulation tissue, only smooth muscle cells of small vessels stain for F-actin and α-SMA; α-SMA-negative proto-myofibroblasts emerge after 6 d and α-SMA-positive differentiated myofibroblasts hallmark contractile 9 d-old wounds (Hinz et al., 2001b). Newly polymerized mechanically restrained collagen gels are soft after 2 h, medium stiff after 5 h and stiff after 72 h culture as a consequence of active collagen remodeling by the resident cells (Hinz, 2006). Collagen stiffness determines fibroblast morphology and the organization level of F-actin and α-SMA similar to wound granulation tissue. Myofibroblasts cultured for 24 h on differently stiff silicone culture substrates (elastic modulus indicated in kPa) attain differentiated myofibroblast characteristics on stiff polymers, form α-SMA-negative stress fibers on medium stiff substrates and no contractile bundles on very soft surfaces (Goffin et al., 2006). Similar control is achieved by growing myofibroblasts on microcontact-printed arrays of focal complex—small (1 μm length), FA—long (4 μm) and supermature-sized (20 μm) adhesion islets (Goffin et al., 2006). Increasing the available adhesion spot size increases the level of myofibroblast differentiation. Note that differentiated rat lung myofibroblasts, subjected to different mechanical culture conditions, were always cultured in the presence of the myofibroblast-inducer TGFβ1.

Corresponding to the threshold stiffness for myofibroblast differentiation *in vitro*, fibrotic tissues and contracting wound granulation tissue were shown to exhibit a stiffness of 25,000–50,000 Pa (Fig. 2) (Hinz, 2009).

3.1. Mechanical control of α-SMA expression-transforming growth factor β1, what else?

Mechanoregulation of α-SMA, myofibroblast function and differentiation occurs on different levels. First, stress directly modulates the bioactivity of transforming growth factor (TGF) β1. TGFβ1 is the major cytokine inducing myofibroblast differentiation and binding of the TGFβ receptor type II triggers a cascade of signaling events that ultimately lead to myofibroblast differentiation (Hinz, 2007). Notably, TGFβ1 up-regulates the expression of α-SMA and other components of the myofibroblast contractile cytoskeleton (Malmstrom et al., 2004) as well as collagen production (Breen, 2000). Myofibroblasts themselves secrete biologically latent TGFβ1 in complex with the latency associated peptide (LAP). LAP and TGFβ1 form the large latent complex

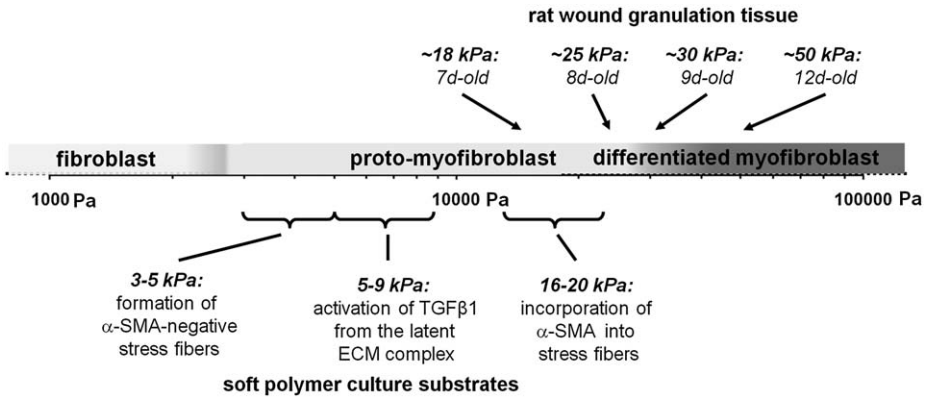

Fig. 2. *A stiffness map for the myofibroblast.* This scheme situates fibroblast-to-myofibroblast differentiation as a function of substrate stiffness. Please note that the indicated Young's modulus values have been determined using AFM and are displayed on a logarithmic scale; absolute values may indeed vary according to the methods used for quantification.

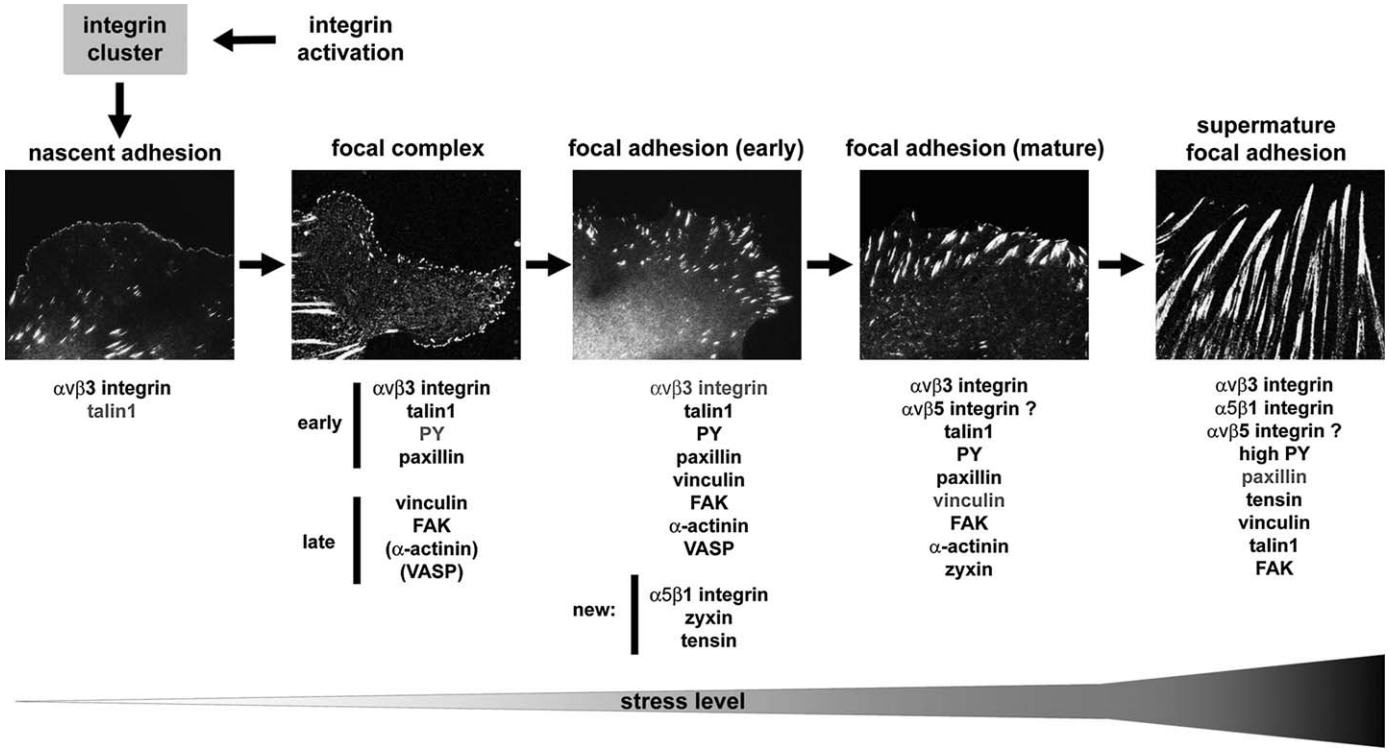

Fig. 3. *Maturation and molecular composition of actin-associated adhesions.* Cell–ECM adhesions formed by fibroblasts on two dimensional culture substrates undergo stress-dependent maturation. Activated integrins (here: αvβ3) bind talin1 that directly bridges between the cytoplasmic tail of integrins and actin. Actin nucleation and polymerization is then locally initiated by recruiting Arp2/3 and members of the Diaphanous/formin family of proteins. Within lamellipodia such nascent adhesions transit into focal complexes by accumulating tyrosine phosphorylated residues (PY) and by incorporating additional cytoplasmic proteins, including paxillin and later vinculin and focal adhesion kinase (FAK); a small population also stains for α-actinin and VASP. Stress application to focal complexes generates early FAs and leads to the accumulation of zyxin, α5β1 integrin and tensin. The latter two proteins relocate towards the cell center in a stress-dependent mechanism and generate fibrillar adhesions, which are FN organizing organelles (not shown). Proteins remaining associated with αvβ3 integrin in the cell periphery constitute mature FAs. Under conditions of extraordinary high stress, mature FA transform into supermature FAs. The here indicated proteins include only hallmark components of the respective adhesion structure; the list is far from being comprehensive. Proteins that have been immuno-localized for the pictures are indicated in grey. Bar: 10 μm. Figure modified and reproduced with permission (Hinz, 2008).

together with the latent TGFβ1 binding protein-1 (LTBP-1) (Fig. 4). LTBP-1 binds to other proteins in the ECM and there provides a reservoir of latent TGFβ1 (Annes et al., 2004; Jenkins, 2008; Wipff and Hinz, 2008). The different mechanisms of how cells can dissociate and thus activate TGFβ1 from the latent complex are subject of comprehensive recent reviews (Jenkins, 2008; Sheppard, 2005; Wipff and Hinz, 2008); here, I will briefly discuss how mechanics play a role in latent TGFβ1 activation. The LAP portion of the ECM-bound latent TGFβ1 provides binding sites for a variety of myofibroblast integrins, including αvβ5, αvβ3, αvβ8, which intracellularly connect to the contractile cytoskeleton (Sheppard, 2005; Wipff and Hinz, 2008). Stress applied to integrin αvβ5, either by stretching the ECM or by inducing cell contraction renders the active TGFβ1 available for its cell-membrane bound receptor, by a putative conformational change in the latent complex (Wipff et al., 2007) (Fig. 4). A similar

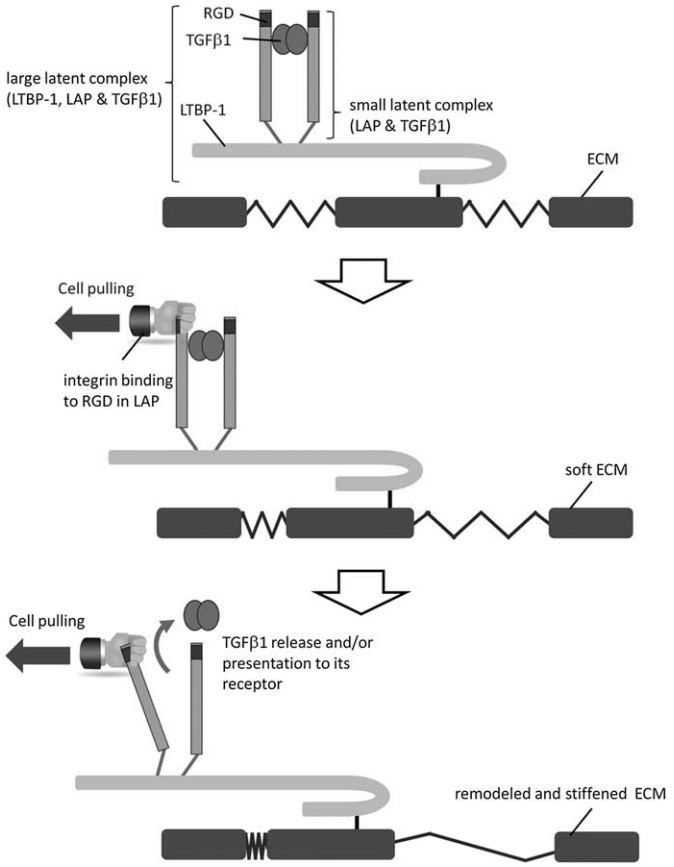

Fig. 4. *A highly schematic view on latent TGFβ1 activation by cell traction.* Integrin binding to a specific RGD site in the LAP portion transmits intracellular force to the latent TGFβ1 complex. In the context of a soft ECM (unloaded springs) cell pulling will simply drag the latent complex. When bound to a remodeled stiff ECM (loaded springs) integrin-mediated force exertion can trigger a conformation change in the LAP and make TGFβ1 available for its receptor.

mechanism appears to be in place for epithelial cells to activate TGFβ1 via αvβ6 integrin (Jenkins et al., 2006). In fact, αvβ6 was the first integrin shown to mediate latent TGFβ1 activation in a process that requires polymerized actin, paving the road for a mechanical activation model (Munger et al., 1999). Importantly, mechanical activation of TGFβ1 by myofibroblasts is not operational in the context of a compliant ECM with Young's modulus of ≤ 5000 kPa (Wipff et al., 2007) (Fig. 2). This is explained by a model in which the cell pulling force transmitted to LAP is counteracted by anchoring the latent complex via the LTBP-1 to a mechanically resistant ECM (Fig. 4). Consistently, deletion of the ECM-binding sequence from LTBP-1 abolishes latent TGFβ1 activation via integrins as shown for αvβ6 integrin (Annes et al., 2004). It is noteworthy that the threshold ECM stiffness for latent TGFβ1 activation is lower than the minimal stiffness required for expression and incorporation of α-SMA into stress fibers (Fig. 2). Hence, mechanical activation of TGFβ1 can provide a first control point in the progression of tissue remodeling by translating the level of ECM stiffness (organization) into a pro-fibrotic signal.

Second, stress can directly modulate α-SMA protein expression. It is intriguing that development of differentiated myofibroblasts is restricted to later phases of wound healing although blood platelets and inflammatory cells provide abundant active TGFβ1 during the first days following injury (Blakytny et al., 2004; Werner and Grose, 2003). Insufficient mechanical conditioning of the ECM at this stage can be one explanation. Consistently, growth on soft ECM suppresses the differentiated myofibroblast phenotype even in the presence of super-physiological concentrations of active TGFβ1 (Arora et al., 1999; Goffin et al., 2006). This suggests a link between the levels of mechanical stress and of myofibroblast differentiation that is not regulated by the amount of active TGFβ1. Application of force at sites of integrin adhesions formed with collagen-coated magnetite beads was demonstrated to up-regulate the promoter activity of α-SMA in cardiac fibroblasts and osteoblasts. This effect involves binding of serum response factor to the CArGB box in the α-SMA promoter (Wang et al., 2002, 2003). More recently, it has been shown that stress-regulated activity of the α-SMA promoter further requires Rho/Rho kinase activity and involves translocation of the myocardin-related transcription factor (MRTF) A/MAL to the nucleus (Zhao et al., 2007). It is worth mentioning that mechanical stress alone is not sufficient to induce myofibroblast differentiation in the absence of active TGFβ1. Concomitantly, myofibroblast differentiation and α-SMA expression on rigid plastic substrates or in mechanically stressed wounds are suppressed by inhibition of TGFβ1 (Hinz et al., 2001a, 2001b). It remains to be shown how mechanical factors cooperate with the TGFβ1 signaling pathway to regulate α-SMA expression. Interestingly, mechanical stress and TGFβ1 also collaborate to up-regulate expression of collagen, another hallmark of the myofibroblast during tissue repair and fibrosis development (Lindahl et al., 2002).

3.2. *Mechanical control of α-SMA localization—to be or not to be in the stress fiber*

A third level of mechanical control comprises the (re-)localization of cytosolic α-SMA to stress fibers. Culture experiments revealed that transferring differentiated lung myofibroblasts from stiff plastic culture dishes (Young's modulus of MPa) to soft silicone substrates ($\leq 16{,}000$ kPa) results in the selective dislocation of α-SMA from persisting β-cytoplasmic actin stress fibers within hours (Fig. 1). A similar effect is achieved by relaxing differentiated myofibroblasts with Rho kinase inhibitors on stiff culture substrates (Goffin et al., 2006). This function of α-SMA as a mechano-sensitive/responsive protein is still mysterious but it stimulates some interesting thoughts:

(a) Stress fibers of differentiated myofibroblasts must be constructed in a way that removal of α-SMA will not result in their disintegration. To this end it is speculative that stress fibers composed of β-cytoplasmic and possibly other actin isoforms serve as a template for subsequent recruitment of α-SMA. It is unclear why cellular α-SMA should not copolymerize with β-cytoplasmic actin. No structural particularities are obviously preventing the formation of actin isoform mixed filaments in cellular systems although small differences have been reported for the mechanics of different actin isoform filaments (Allen et al., 1996).

(b) The differential localization of α-SMA, either cytoplasmic or in stress fibers, possibly mediates different functions. Previous works have shown that α-SMA localization in stress fibers augments fibroblast contractile activity; its selective removal from these structures eliminates the extra contraction (Hinz et al., 2001a, 2002). But is there also a function for monomeric α-SMA? Generally, the ratio of monomeric G-actin and polymerized F-actin provides important clues about the mechanical state of the cell. G-actin monomers control gene transcription through interaction with MRTF/MAL (Miralles et al., 2003; Posern and Treisman, 2006; Vartiainen et al., 2007). The molecular basis of G-actin binding to MRTF/MAL is

beginning to be understood (Mouilleron et al., 2008) but is presently unknown whether the actin isoform-specific N-terminus has any relevance for this interaction. Yet it appears that at least β-cytoplasmic actin, α-cardiac and γ-cytoplasmic actin exhibit similar binding affinities to MRTF/MAL (Posern et al., 2004). Future studies may reveal whether monomeric α-SMA can mediate an actin isoform specific function through this pathway.

(c) The observation that cytosolic α-SMA only recruits to stress fibers under mechanical load suggests a mechanosensitive element within the contractile bundles that specifically binds to -SMA (Goffin et al., 2006). The nature of this hypothetical binding partner is unclear; its existence however is supported by the fact that cytoplasmic delivery of the α-SMA specific N-terminal sequence Ac-EEED as a 'competitive' peptide selectively removes α-SMA from stress fibers (Chaponnier et al., 1995; Clement et al., 2005; Hinz et al., 2002). Proteins like zyxin, myosin and α-actinin have been demonstrated to exhibit a similar dependence of their location in stress fibers on mechanical load (Colombelli et al., 2009; Hervy et al., 2006; Peterson et al., 2004). Moreover, stress fibers under tension have been shown to reveal cryptic cysteine residues detected by *in vivo* labeling (Johnson et al., 2007). Whereas relatively little is known about mechanosensitive elements in the stress fiber, studies on FAs provide insight into basic mechanisms of how mechanical cues are translated into chemical signals as discussed further below.

4. Myofibroblasts reveal the power and the danger of culture models

Studies on myofibroblast differentiation clearly demonstrate the pitfalls of conventional rigid plastic dish culture. In fact, virtually all fibroblasts growing in conventional dishes in standard culture conditions are myofibroblasts by definition because contact with the stiff plastic surface inevitably triggers formation of contractile stress fibers. The capacity of any particular fibroblast type to secrete and to activate TGFβ1 may then finally determine the percentage of differentiated, α-SMA-positive myofibroblasts in the population. With this respect the development of synthetic substrates with tunable elastic modulus represented a major advance. Two-dimensional compliant substrates are mainly produced from polyacrylamide-, polyvinyl alcohol-, or silicone-based elastomers, providing tissue-like stiffness and optical properties that match tissue culture plastic (Brown X.Q. et al., 2005; Pelham and Wang, 1997; Zajaczkowski et al., 2003). Using this methodology, the mechanical property of the substrate was shown to represent a major influence on the behavior and differentiation of cancer cells (Paszek et al., 2005), mesenchymal stem cells (Engler et al., 2006), neurons (Georges et al., 2006), myotubes (Engler et al., 2004), cardiomyocytes (Engler et al., 2008) and fibroblasts (Goffin et al., 2006), with the list of substrate-responsive cell types continuously growing.

Nevertheless, the use of two-dimensional compliant culture substrates has limits that have to be taken into consideration. Two-dimensional compliant substrates cannot mimic three dimensional tissue environments, which is a major challenge for biomaterials and tissue engineering (Lutolf and Hubbell, 2005). Moreover, polyacrylamide and silicone substrates are not biodegradable and thus cannot be remodeled by the cells. Hence, the mechanical qualities of elastic synthetic polymers and of rather viscoelastic biopolymers are very different on the cellular perception level (Storm et al., 2005). This feature may be appropriate to simulate the relatively constant mechanical microenvironment of established adult tissues but not the dynamic reorganization taking place during tissue repair and regeneration. In fact, fibroblasts in repair and fibrotic processes *in vivo* face and react to an increasingly stiff environment because they make it stiffer themselves (Hinz, 2009; Tomasek et al., 2002). Another danger is the possibility that cells find their ways to circumvent the direct mechanical input from the compliant substrate surface. Cells will secrete their own ECM that may eventually reach a cross-linking and stiffness grade exceeding that of the underlying polymer. However, this effect will likely only occur over prolonged culture time and not influence the outcome most short-term experiments. Alternatively, cells form cell–cell adherens and tight junctions that link the contractile stress fiber system in confluent cultures. Under these conditions the significance of the substrate may be low compared with the mechanical influence of the neighboring cells (Yeung et al., 2005). Furthermore, elastic polymer substrates are homogeneous. Isotropic stress distribution is without doubt a great advantage for the reproducibility of culture experiments but hardly reproduces the complex and anisotropic lines of stress in a 'real' tissue matrix.

The latter has very practical implications when comparing the stiffness of a polymer with that of tissues. Many of the above mentioned polymer stiffness values have lately been determined with atomic force microscopy (AFM) which is extremely powerful to determine the Young's modulus of homogeneous materials. When used on heterogeneous tissues however, AFM delivers average stiffness values with considerable standard deviations. AFM enthusiasts may be pleased to read that this is the fault of the tissue and not of the instrument. Not surprisingly, other methods to determine material stiffness provide congruent values for chemical polymers but cause heated discussions among scientists when it comes to determine the Young's modulus of a 'simple' cell-free collagen gel; not to mention gels with cells. Methods like bulk shear rheometry, macro-indentation, polymer expansion, ultrasound palpatation, and AFM assess material properties at very different levels of sensitivity. Consequently, the values for the effective Young's modulus of a given tissue can sometimes vary by orders of magnitude depending on the method of quantification. At present, there is no simple solution to this problem and the adequateness of an experimental tissue stiffness value will always depend on how stress and/or tissue stiffness are perceived on the (sub-) cellular level. To give only one example: individual collagen bundles were shown to resist longitudinal extensions up to the million Pascal range. The stiffness of surface attached gels made of such collagen bundles ranges from hundreds to few thousands of Pascal as determined by rheometry or AFM indentation. Now, what is the stiffness received and integrated by fibroblasts in the gel? Is it the overall gel's modulus or the modulus of single collagen bundles that are stabilized with the rigid culture support? And what happens when a fibroblast fights a tug-of-war with another fibroblast competing for the same collagen bundle?

5. How myofibroblasts are feeling

Fibroblasts form FAs to attach to the ECM (and to have a tug-of-war). FAs not only anchor stress fibers to the substratum but provide myofibroblasts with information about substrate mechanics and chemistry. Compared with the classical FAs of proto-myofibroblasts in culture (~6 μm long), differentiated myofibroblasts develop extraordinary large 'supermature' FAs (10–30 μm long) (Fig. 3) (Dugina et al., 2001; Hinz et al., 2003). Supermature FAs withstand and exert 3–4-times higher stress (~12 nN/μm^2) compared with their little FA brothers in the proto-myofibroblast (Fig. 3). It is presently unclear how this higher

stress is achieved on the molecular level but the specific composition of the supermature FA may provide one explanation (Goffin et al., 2006; Hinz, 2006). The sheer size of supermature FAs controls the transition between proto- to differentiated myofibroblast. When differentiated myofibroblasts are forced to grow on arrays of ECM islets with the length of classical FAs ($\leq 6\,\mu m$), α-SMA disassembles from stress fibers (Goffin et al., 2006) (Fig. 1). Because supermature FAs only develop on stiff substrates it has been proposed that information about substrate stiffness is at least partly encoded in the size of adhesions (Hinz, 2006).

5.1. The mechanosensing function of matrix adhesions—practical aspects

The relation between size maturation of FAs and the capability of cells to sensing stress is not unique in myofibroblasts; it has been shown in different experimental settings that both features are the Yin and Yang of mechanosensing (Geiger et al., 2009; Puklin-Faucher and Sheetz, 2009; Wang, 2007). Stress-induced maturation of FAs starts with the single integrin. Computational simulations suggested that force applied to αvβ3 integrin via the 10th type III FN module ($FnIII_{10}$) can shift the integrin to its active conformation (Puklin-Faucher et al., 2006). Experimentally, a switch from weak to strong integrin interaction with FN has recently been demonstrated for α5β1 integrin under intracellular and extracellular traction. Inhibition of either cell contractile activity or reducing extracellular resistance by growth on soft substrates both suppress the integrin high adhesion switch (Friedland et al., 2009). In another study, application of force to single α5β1 integrins using AFM cantilevers grafted with FN fragments increased the bond lifetime—a defining feature of catch bonds (Kong et al., 2009). A similar increase of adhesion (friction with the cytoskeleton) appears to take place on the cytoplasmic side of integrins under stress. Laser-tweezers-pulling of single integrins generates a 2 pN force-resisting slip bond with the actin cytoskeleton (Jiang et al., 2003). This slip bond, provided by the cytoplasmic protein talin1, has been described as the initial event in force-dependent FA maturation and clustering. Forces in the range of 2–12 pN were further shown to potentially unfold the talin molecule and revealing cryptic binding sites for another FA protein, vinculin (del Rio et al., 2009; Papagrigoriou et al., 2004). This finding can explain how actin/myosin generated tension leads to the recruitment of vinculin to nascent adhesions (Delanoe-Ayari et al., 2004; Galbraith et al., 2002), an event that further strengthens the adhesion structure (Gallant et al., 2005). At this level of maturation, adhesion sites are detectable at the light microscopy level as $\sim 1\,\mu m^2$ large focal complexes (Fig. 3) (Choi et al., 2008).

Transition of such nascent adhesions into mature FAs is also driven by mechanical force exerted by contractile actin–myosin stress fibers and a mechanically resistant ECM (Bershadsky et al., 2003; Chen et al., 2004). FA enlargement along the lines of stress occurs upon application of external force to entire cells or to single contact sites (Li et al., 2002; Riveline et al., 2001; Sawada and Sheetz, 2002). At constant extracellular stress, FA growth is driven by intracellular stress generated by retrograde actin network flow and stress fiber contraction (Alexandrova et al., 2008; Giannone et al., 2007; Hotulainen and Lappalainen, 2006). Culturing cells on polymer substrates with different stiffness reveals a direct correlation between the size of FAs and substrate stiffness. On highly compliant substrates adhesion formation is arrested at the level of focal complexes even when inducing cell contraction (Pelham and Wang, 1997; Yeung et al., 2005). Inversely, FA size is reduced by reducing intracellular and/or extracellular stress even if cells are grown on rigid glass or plastic substrates (Ballestrem et al., 2001). Stress-mediated transition of focal complexes into FAs and subsequent size increase is associated with the recruitment of new components into the adhesion plaque (Fig. 3). Proteins that are selectively enriched in FA upon stress application include vinculin (Riveline et al., 2001), paxillin (Sawada and Sheetz, 2002; Zaidel-Bar et al., 2007), zyxin (Lele et al., 2006; Yoshigi et al., 2005) and a number of proteins that participate in phosphotyrosine and small GTPase signaling cascades. Tyrosine phosphatase and kinase signaling are suggested to playing central roles in FA mechanosensing and the reader is referred to recent reviews on this subject (Giannone and Sheetz, 2006; Vogel and Sheetz, 2009).

5.2. The mechanosensing function of matrix adhesions—theoretical aspects

An alternative approach to explain the mechano-sensing mechanisms of molecular complex structures such as FAs is made using theoretical models (Bershadsky et al., 2006). In the 'thermodynamic model', Shemesh and coworkers postulate that elastic stress generated within the adhesion protein aggregate decreases the chemical potential of the aggregate molecules versus the free molecules. This will thermodynamically favor the recruitment of new components into the aggregate which is modeled as a one-dimensional adhesion plaque and anchored to a substrate (Shemesh et al., 2005).

The 'strain-sensor' model of Safran and coworkers is based on elasticity theory. In this model FA mechanosensing relies on force-induced elastic deformations of the ECM-attached integrin layer but not of single proteins within the adhesion plaque (Besser and Safran, 2006; Nicolas et al., 2004). Contractile actin bundles inserting into the layer of cytosolic FA plaque proteins will compress the lower layer of ECM-engaged integrins and increase its affinity for new components. Indeed, experiments show that the transition of focal complexes into stress fiber-associated FAs requires lateral integrin spacing of under 70 nm (Arnold et al., 2004; Cavalcanti-Adam et al., 2007) and depends on a critical density of extracellular ligands (Koo et al., 2002). The model is further supported by the fact that FAs under stress display higher protein density compared with relaxed contacts (Ballestrem et al., 2001). In contrast to the thermodynamic model that predicts isotropic FA growth and incorporation of new components over the entire stretched adhesion site, the elastic strain model predicts anisotropic FA growth. Under elastic strain new FA components would preferentially recruit to the stress fiber-connecting and compressed FA portion; proteins should leave the plaque preferably at their distal end at the cell periphery. By extending their model, the authors recently provided theoretical explanation of how matrix elasticity governs FA size (Nicolas et al., 2008; Nicolas and Safran, 2006).

The 'elastic stress-sensor' model proposes the presence of proteins within the adhesion plaque that function as molecular switches by exhibiting stress-activated open (on) and relaxed-closed (off) conformations for the binding of specific partners (Giannone and Sheetz, 2006). Indeed, a number of FA proteins bear the structural potential to act as mechanical switch like integrins (Arnaout et al., 2005; Luo et al., 2007), vinculin (Cohen et al., 2006; Ziegler et al., 2006), talin (del Rio et al., 2009; Gingras et al., 2006), Src (Wang et al., 2005), and p130CAS (Sawada et al., 2006). In the elastic stress-sensor model the position of the molecular switch within the adhesion site will determine the direction and mode of FA growth. Future experiments will show which of these models matches best reality. Each model predicts a different site for the incorporation of new proteins to FA under

mechanical load. Hence, the theoretical prediction can be tested in experiments, i.e., by determining dynamic close protein interactions in the FA using fluorescence energy resonance transfer or by analyzing protein density distributions within stressed and relaxed FAs (Ballestrem et al., 2006). With little doubt the very large supermature FAs of the myofibroblast are a great model to study local protein recruitment and turnover with optical methods such as fluorescence recovery after photobleaching (FRAP). Given the fact that validity of each model is not yet decided for general cell mechanosensing it is highly speculative to say which mechanism is used by the myofibroblast. One can expect that myofibroblasts plays along the rules and utilize FA maturation and mechanosensing principles that are valid for other cells. The higher contractile activity of this phenotype however can make an important difference. In the elastic stress-sensor model for instance, one may speculate on molecular switches that are only activated at higher forces, not produced by the average fibroblast and creating the particular molecular composition of myofibroblast FAs.

6. Conclusion

The contractile and ECM remodeling activity of the myofibroblast has important clinical impact on virtually all fibrotic diseases, in tumor progression, atheromatous plaque formation and wound healing. One of the major clinical problems is the often progressive character of the myofibroblast-supported disease, leading to gradual replacement of functional tissue with a fibrotic scar. Once the myofibroblast gets started it is apparently difficult to control and to terminate its harmful activity. Partly, this is caused by the constant mechanical feedback that the cells receive from the ECM: stiffer matrix leads to higher myofibroblast contraction and ECM secretion, which again leads to further ECM stiffening. Consequently, fibrotic scars are stiffer compared with the tissue compliance of the most non-fibrotic organs. This stiff character in itself dramatically impedes the function of organs whose function relies on mechanics, such as in fibrotic heart and lung and skin.

One big challenge of the future will be to therapeutically intercept the myofibroblast contraction—ECM stiffening loop, either by targeting force generation, ECM adhesion or stress perception. Many questions remain open on how the myofibroblast is mechanically controlled. What starts the mechanical feedback loop with the ECM—are fibroblast first becoming contractile to stiffen the ECM or is ECM stiffening preceding the fibrotic process, as recently suggested (Wells, 2008)? Considering the heterogeneity of their precursors, are all myofibroblasts equally responsive to mechanical factors (and chemical cues)? This may not be the case; unpublished findings in our laboratory show that rat cardiac myofibroblasts cultured on compliant substrates preserve the differentiated phenotype longer than lung or subcutaneous fibroblasts. Related to this is the question of whether myofibroblasts (or cells in general) possess a ‘mechanic memory’. In other words, does it matter which mechanical condition cells have experienced before becoming exposed to a new mechanical challenge? Further, is myofibroblast differentiation in response to continuously increasing ECM stiffness a gradual process or do cells perceive ‘stiffness’ thresholds’? Obviously, much is left to be done and it will never become a boring task to work with the myofibroblast.

Conflict of interest

None.

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