Formation and Function of the Myofibroblast during Tissue Repair

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It is generally accepted that fibroblast-to-myofibroblast differentiation represents a key event during wound healing and tissue repair. The high contractile force generated by myofibroblasts is beneficial for physiological tissue remodeling but detrimental for tissue function when it becomes excessive such as in hypertrophic scars, in virtually all fibrotic diseases and during stroma reaction to tumors. Specific molecular features as well as factors that control myofibroblast differentiation are potential targets to counteract its development, function, and survival. Such targets include α-smooth muscle actin and more recently discovered markers of the myofibroblast cytoskeleton, membrane surface proteins, and the extracellular matrix. Moreover, intervening with myofibroblast stress perception and transmission offers novel strategies to reduce tissue contracture; stress release leads to the instant loss of contraction and promotes apoptosis.

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Introduction

It is generally accepted that myofibroblasts represent key players in the physiological reconstruction of connective tissue after injury and in generating the pathological tissue deformations that characterize fibrosis (Gabbiani, 2003; Desmouliere *et al.*, 2005). In previous reviews, we have focused on the role of cell–cell and cell–matrix contacts in myofibroblast development and function (Hinz and Gabbiani, 2003a, b; Hinz, 2006). Here, I draw a more general picture how the mechanical and chemical microenvironments integrate to promote tissue remodeling by myofibroblast. Differentiation of fibroblasts into myofibroblasts can be understood as a two-step process: (1) to re-populate damaged tissues, fibroblasts acquire a migratory phenotype by *de novo* developing contractile bundles. These *in vivo* stress fibers are first composed of cytoplasmic actins and generate comparably small traction forces (Hinz *et al.*, 2001b). We have recently proposed the term ''proto-myofibroblast'' to discriminate such activated fibroblasts from quiescent fibroblasts that are devoid of any contractile apparatus in most intact tissues (Tomasek *et al.*, 2002). This first phenotypic change occurs in response to changes in the composition, organization, and mechanical property of the extracellular matrix (ECM) (Hinz and Gabbiani, 2003b) and to cytokines locally released by inflammatory and resident cells (Werner and Grose, 2003). (2) With increasing stress in the ECM resulting from their own remodeling activity, proto-myofibroblasts further develop into ''differentiated myofibroblasts'' by neo-expressing α-smooth muscle actin (α-SMA), the most widely used myofibroblast marker. Expression of α-SMA is precisely controlled by the joint action of growth factors like transforming growth factor (TGFβ1), of specialized ECM proteins like the fibronectin (FN) splice variant ED-A FN, and of the mechanical microenvironment (Tomasek *et al.*, 2002). Incorporation of α-SMA into stress fibers significantly augments the contractile activity of fibroblastic cells and hallmarks the contraction phase of connective tissue remodeling (Hinz *et al.*, 2001a).

It has to be noted that the contribution of myofibroblast contraction to physiological tissue remodeling has been questioned by a study reporting normal closure of pig full thickness wounds despite repeated excision of the central and peripheral granulation tissue (Gross *et al.*, 1995). However, rat wounds that were kept open for 10 days with a plastic frame and have then been released from the splint contract to ~50% of their initial size within 6 hours; this cannot be explained by enhanced proliferation of fibroblasts and keratinocytes at the wound edge. Hence, the porcine skin may be able to compensate for the experimentally induced chronic loss of granulation tissue by mechanisms that have not yet been elucidated. Such a compensatory mechanism appears to function in α-SMA-knockout mice that macroscopically exhibit almost normal wound closure (personal communication, J.J. Tomasek, University of Oklahoma Health

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Abbreviations: α-SMA, α-smooth muscle actin; ECM, extracellular matrix; FA, focal adhesion; Fizz, found in inflammatory zone; FN, fibronectin; LAP, latency-associated protein; LTBP-1, latent TGFβ1-binding protein; SMC, smooth muscle cell; TGF, transforming growth factor

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Sciences Center, Oklahoma City, OK). These mice appear to substitute for α-SMA by *de novo* expressing other muscle actin isoforms like γ-smooth muscle and α-skeletal actin, which also explains why blood pressure is surprisingly little affected by the lack of α-SMA in vascular smooth muscle cells (SMCs) (Schildmeyer *et al.*, 2000). In contrast to these chronic conditions, acute inhibition of myofibroblast contraction by intracellular delivery of the specific N-terminal sequence AcEEED of α-SMA significantly reduces wound contraction (Hinz *et al.*, 2002). It will be interesting to test whether conditional α-SMA-knockout mice or mice treated with α-SMA RNAi will exhibit similarly impaired wound closure.

In physiological remodeling such as during dermal wound healing, the contractile activity of myofibroblasts is terminated when the tissue is repaired; α-SMA expression then decreases and myofibroblasts disappear by apoptosis (Desmouliere *et al.*, 1995). In pathological wound healing, however, myofibroblast activity persists and leads to tissue deformation, which is particularly evident in hypertrophic scars developing after burn injury, in the fibrotic phase of scleroderma and in the palmar fibromatosis of Dupuytren's disease (Schurch *et al.*, 2006). Myofibroblast-generated contractures are also characteristic for fibrosis affecting vital organs such as the liver (Desmouliere *et al.*, 2003), heart (Virag and Murry, 2003; Brown *et al.*, 2005), lung (Phan, 2002; Thannickal *et al.*, 2004), and kidney (Lan, 2003). In addition, myofibroblast participation to the process called stroma reaction promotes cancer progression by creating a stimulating microenvironment for epithelial tumor cells (De Wever and Mareel, 2003; Desmouliere *et al.*, 2004). It becomes increasingly accepted that stroma cells represent important targets of anticancer treatments (Bissell and Radisky, 2001; Liotta and Kohn, 2001; Mueller and Fusenig, 2004). In the light of such severe consequences of myofibroblast appearance and dysfunction, the necessity of more profoundly understanding the molecular mechanisms of myofibroblast formation and function appears clear.

Myofibroblast origin

The classical view on dermal wound healing implies local recruitment of fibroblasts from the dermis of the intact adjacent skin to sites of inflammation (Desmouliere *et al.*, 2005). Pericytes and SMC from the vasculature have been proposed as another local myofibroblast source in scleroderma (Rajkumar *et al.*, 2005) as well as liver and glomerular fibrosis (Desmouliere *et al.*, 2003). In addition, fibroblasts may originate from fibrocytes, a sub-population of bone marrow-derived leukocytes with fibroblast characteristics (Abe *et al.*, 2001). This concept has recently been revisited by transplanting bone marrow from (1) male to irradiated female mice and identifying the Y chromosome by *in situ* hybridization (Direkze *et al.*, 2003) and (2) from transgenic green fluorescent mice to irradiated wild-type animals (Hashimoto *et al.*, 2004). These studies reveal a surprisingly high fraction of 30–50% of the wound myofibroblasts potentially deriving from fibrocyte progenitors (Direkze *et al.*, 2003; Ishii *et al.*, 2005; Mori *et al.*, 2005). Comparable proportions have been demonstrated for myofibroblasts appearing during fibrosis of the liver (Forbes *et al.*, 2004), kidney (Direkze *et al.*, 2003), and lung (Hashimoto *et al.*, 2004) and during the stroma reaction to epithelial tumors (Ishii *et al.*, 2003). It remains to be seen whether myofibroblasts from different origins exhibit different characteristics and functions during tissue repair as suggested for liver fibrosis (Guyot *et al.*, 2006) or whether the organism is recruiting myofibroblast precursors from several sources to satisfy the temporarily high demand of contractile cells, which may all follow a similar differentiation program.

Identification of the myofibroblast – a warrant

The question of the myofibroblast origin is closely related to the problem of its identification. Three major ultrastructural features discriminate myofibroblasts from quiescent fibroblasts in tissues: (1) bundles of contractile microfilaments, (2) extensive cell-to-matrix attachment sites, and (3) intercellular adherens and gap junctions (Eyden, 2005; Schurch *et al.*, 2006). However, this definition has its limits when myofibroblasts need to be discriminated from other contractile cell types like SMC, requiring specific molecular markers.

Cytoskeletal markers of the myofibroblast. The most frequently employed myofibroblast marker is α-SMA, which evidently fails to distinguish between myofibroblasts and SMC in situations that exhibit mixed populations. For example, remodeling of injured arteries is thought to be predominantly driven by SMC from the media but a contribution from adventitial fibroblasts has also been suggested (Sartore *et al.*, 2001; Zalewski *et al.*, 2002). Contractile SMC specifically express smooth muscle myosin heavy chain, h-caldesmon, and desmin; however, SMC loose these markers when acquiring a synthetic phenotype and after being placed in culture (Christen *et al.*, 2001). Until recently, smoothelin was suggested a late differentiation marker for SMC that is not expressed in myofibroblasts (van der Loop *et al.*, 1996). However, gene expression profiling supported by protein biochemistry revealed induction of smoothelin and other late SMC markers in TGFβ1-treated cultured lung fibroblasts (Chambers *et al.*, 2003). Very recently, the 4Ig isoform of the stress fiber protein palladin has been proposed as novel marker for myofibroblast differentiation (Rönty *et al.*, 2006), but Western blotting analysis with pan-palladin antibodies indicates expression of this isoform also in SMC (Mykkanen *et al.*, 2001). Hence, at present no cytoskeletal protein allows to reliably discriminate between myofibroblasts and SMC; however, recent advances in proteomics and gene array analysis may lead to the identification of such a unique marker of the myofibroblast, provided that it exists (Chambers *et al.*, 2003; Malmstrom *et al.*, 2004).

The myofibroblast surface. Of particular interest not only for the purpose of

identification but also for the delivery of specific drugs are proteins specifically expressed on the plasma membrane of myofibroblasts. The membrane protein Thy-1 is frequently used for the inverse approach as it is expressed by fibroblasts but absent from differentiated myofibroblasts (Koumas *et al.*, 2003). Interestingly, Thy-1-negative cells fail to respond to fibrogenic stimuli suggesting that Thy-1 expression characterizes a specific subpopulation of fibroblasts that may not be able to undergo myofibroblast differentiation (Zhou *et al.*, 2004; Hagood *et al.*, 2005). A promising new strategy to target and identify myofibroblasts is the expression of specific cadherins (Hinz and Gabbiani, 2003a), transmembrane cell–cell adhesion proteins that are intracellularly linked to the actin cytoskeleton (Gumbiner, 2005). In contrast to virtually cadherin-negative fibroblasts of normal dermis, protomyofibroblasts of early granulation tissue and in culture express N-cadherin (cadherin-2, A-cell adhesion molecule (A-CAM)) (Hinz *et al.*, 2004). In conjunction with the appearance of α-SMA after TGFβ1-treatment in culture or in differentiated myofibroblasts of contractile wound granulation tissue, N-cadherin becomes gradually replaced by OB-cadherin (cadherin-11). OB- but not N-cadherin seems to play a functional role in coordinating myofibroblast contraction in populations (Hinz *et al.*, 2004). A similar shift from N- to OB-cadherin expression has been described in stroma fibroblasts reacting against human prostate cancer progression (Tomita *et al.*, 2000). OB-cadherin is absent from SMC but it is expressed on a variety of cell types of mesenchymal origin; hence, it cannot be considered as unique myofibroblast surface marker.

The ECM of myofibroblast-populated tissue. In addition to producing tension for remodeling, enhanced ECM synthesizing and processing activity significantly contributes to tissue remodeling. Myofibroblasts produce several components of the ECM that can potentially be used as molecular markers, depending on the context of myofibroblast appearance; the most prominent myofibroblast ECM products are collagens of types I, III, IV, and V (Schurch *et al.*, 2006) which, however, are produced by a variety of cells. More recently, collagen type VI attracted attention as it is upregulated together with myofibroblast differentiation in human renal fibrosis (Groma, 1998), in myocardial interstitial fibrosis (Kitamura *et al.*, 2001), and in the stroma of hepatocellular carcinoma (Faouzi *et al.*, 1999). Interestingly, culturing cardiac fibroblasts on collagen type VI substrates induces myofibroblast differentiation but not culture on collagen types I and III (Naugle *et al.*, 2006). At present, the most reliable marker of the myofibroblast ECM is the FN splice variant ED-A FN (Serini *et al.*, 1998). ED-A FN is also expressed in low amounts by fibroblastic cells in culture (Dugina *et al.*, 2001; Hinz *et al.*, 2001a) and by vascular SMC *in vivo* and *in vitro* (Glukhova *et al.*, 1989). Another component of the myofibroblast ECM, glycoprotein tenascin-C is associated with tissue repair phenomena (Chiquet-Ehrismann and Chiquet, 2003). Tenascin-C appears to attract fibroblasts and to promote their differentiation into myofibroblast in injured tissue (Tamaoki *et al.*, 2005) and at the tumor invasion front of cancers (De Wever *et al.*, 2004). However, the shared expression in SMC limits its usage as myofibroblast marker (Chiquet-Ehrismann and Chiquet, 2003).

In view of the expression profile of cytoskeletal and ECM proteins, the differentiated myofibroblast appears to be situated in a continuous spectrum existing between fibroblasts and SMC. It may be necessary to combine common markers of the contractile cell phenotype with the exclusive surface expression of proteins like OB-cadherin and Thy-1 to unmistakably discriminate between fibroblasts, myofibroblasts, and SMC.

Chemical factors inducing myofibroblast formation

Apart from being important diagnostic tools, myofibroblast-specific proteins are likewise potential targets to modulate myofibroblast differentiation and/or contractile function; this has been shown by interfering with α-SMA (Hinz *et al.*, 2002) and with ED-A FN (Serini *et al.*, 1998). Alternative antifibrosis strategies aim to prevent development of tissue contractures by affecting physiological cytokines that control myofibroblast differentiation. TGFβ1 is considered the major growth factor directly promoting myofibroblast development by inducing expression of α-SMA (Desmouliere *et al.*, 1993; Ronnov-Jessen and Petersen, 1993), ECM proteins (Werner and Grose, 2003), and a number of cytoskeletal proteins that construct the myofibroblast contractile apparatus (Malmstrom *et al.*, 2004). TGFβ1 effects are ''fine-tuned'' by cooperative or antagonistic growth factors and some recent studies report myofibroblast differentiation in the absence of TGFβ1 signaling.

Control of α-SMA transcription by TGFβ1 signaling. The major pathway through which TGFβ1 regulates expression of α-SMA in fibroblastic cells appears to involve Smad signaling (Figure 1). Binding of active TGFβ1 to the TGFβ receptor type II leads to the phosphorylation and recruitment of TGFβ receptor type I into a heteromeric receptor complex. The serine/threonine kinase activity of the activated complex phosphorylates Smad2 and Smad3, that each bind to Smad4 and translocate into the nucleus to enhance gene transcription by cooperating with DNA transcription factors (Feng and Derynck, 2005; Massague *et al.*, 2005). Transcription of α-SMA in lung myofibroblasts and in myofibroblast-like activated hepatic stellate cells is predominantly mediated by the binding of Smad3 to the Smad-binding element 1 upstream of the α-SMA core promoter sequence; the role of Smad3 binding to a second Smad-binding element (Smad-binding element 2) in the core region is unclear at present (Hu *et al.*, 2003; Uemura *et al.*, 2005). In contrast, another study demonstrated that overexpression of Smad2 but not of Smad3 induces myofibroblast differentiation in lung fibroblasts (Evans *et al.*, 2003); this discrepancy may be due to different roles of Smad2 and Smad3 depending on the level of myofibroblast differentiation (Liu *et al.*, 2003). Moreover, rather than being essential factors for α-

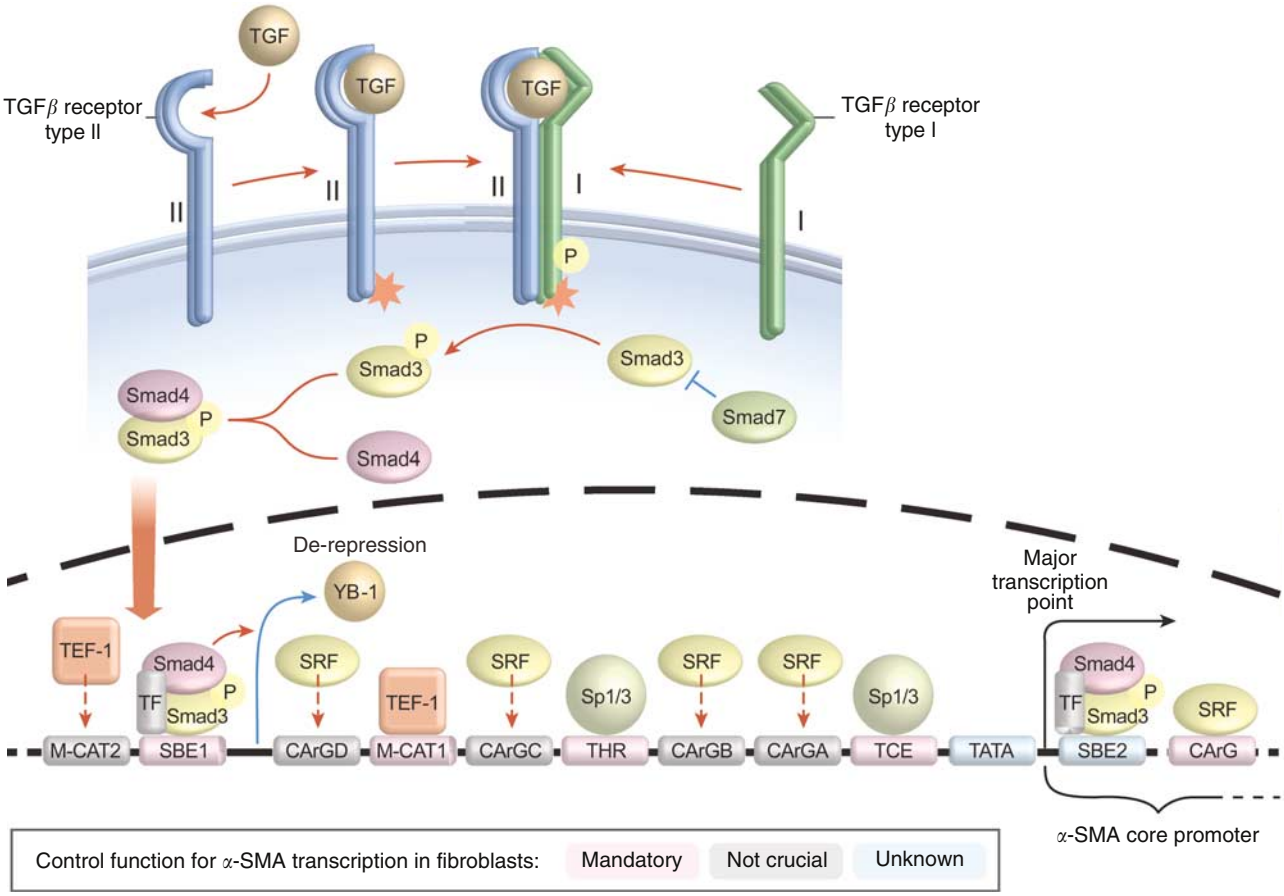

Figure 1. Regulation of α-SMA transcription in myofibroblasts. The major pathway of TGFβ1-induced α-SMA expression in myofibroblasts is mediated via Smad3 activation by the TGFβ1 receptor complex, leading to Smad3 association with Smad4 and translocation into the nucleus. Smad3 binding to Smad-binding elements in the promoter region regulates α-SMA transcription in conjunction with a variety of transcription factors (TF). Some TGFβ1-antagonizing factors like IFN-γ operate via activation of YB-1 that inhibits Smad3-enhanced gene transcription and upregulates expression of the inhibitory Smad7. TGFβ1-induced, Smad-independent transcription of α-SMA is enhanced by binding of the krüppel-like factors Sp1/3 to the TGFβ1 control element and the TGFβ1 hypersensitivity region. Moreover, binding of the TEF-1 to the MCAT-1 element is crucial for α-SMA expression in myofibroblasts and SMCs. In contrast to SMC, interaction of the serum-response factor with CArG elements A, B, C, and D is not mandatory for α-SMA transcription; however, the intronic CArG element regulates α-SMA transcription in both cell types. Please refer to text for details and references.

SMA transcription, Smad 2/3 should be considered as modulators of the activity of other transcription factors. This may explain different results obtained from acute removal of Smad, for example, by using an antisense approach compared with chronic Smad deficiency in knockout animals. In the first setting, cells may not be able to compensate for the instant loss of the important modulating activity of Smads; adaptation and compensation may, however, occur in the knockout system.

Alternatively, TGFβ1 can regulate fibroblast gene expression independently from Smad signaling (Derynck and Zhang, 2003; Moustakas and Heldin, 2005) and expression of α-SMA has been documented during the activation of Smad3-null hepatic stellate cells in culture (Schnabl *et al.*, 2001). One pathway of Smad-independent TGFβ1 induction of the myofibroblastic phenotype has been revealed in Smad3-knockout fibroblasts, transfected with dominant-negative Smad2 and appears to function via activating phosphatidylinositol 3-kinase and the downstream effector p21-activated kinase-2; expression of α-SMA has not been assessed in this study (Wilkes *et al.*, 2005). Smad-independent transcription of α-SMA in fibroblasts is regulated via the TGFβ1 control element (Roy *et al.*, 2001; Tomasek *et al.*, 2005) that together with the TGFβ1 hypersensitivity region serves as binding site for the transcriptional activator proteins Sp1/2, members of the family of krüppel-like factors (Cogan *et al.*, 2002; Subramanian *et al.*, 2004). Another mandatory element for α-SMA transcription in myofibroblasts and SMC is the MCAT-1 site that is enhanced by binding of the transcription enhancer factor-1 which apparently competes with MCAT-suppressor proteins; the presence of a second MCAT element further upstream of the promoter appears not crucial (Swartz *et al.*, 1998; Carlini *et al.*, 2002).

Importantly, transcription of α-SMA in SMC differs from fibroblasts by being additionally regulated via serum-response factor binding to CC(A/T)6GG-like sequence motifs (CArG elements) in the promoter region (Hautmann *et al.*, 1997); these elements seem not

to be required for α-SMA transcription in myofibroblasts (Roy *et al.*, 2001). In contrast, the CArG element in the first intron promoter region has recently been demonstrated to regulate α-SMA transcription in myofibroblasts similar to SMC (Tomasek *et al.*, 2005). It remains to be shown whether upregulated serum-response factor in response to mechanical stimulation (Wang *et al.*, 2006) acts predominantly through this intronic CArG element to enhance α-SMA expression. Deciphering the distinct upstream events in each cell type may provide future gene targeting strategies to specifically inhibit myofibroblast formation without affecting SMC function.

Soluble factors cooperating with TGFβ1 in myofibroblast differentiation. One of the best studied cytokines induced by TGFβ1 is connective tissue growth factor that potentiates the profibrotic action of TGFβ1 and at the same time induces fibroblast proliferation (Leask *et al.*, 2002). These pleiotrophic effects have been attributed to distinct connective tissue growth factor subdomains and to the mutually exclusive cooperation with other cytokines like EGF and IGF-2 (Grotendorst and Duncan, 2005). Connective tissue growth factor cooperates with TGFβ1 but does not substitute for its action, as α-SMA expression remains unchanged after administration of recombinant connective tissue growth factor to cultures of corneal fibroblasts (Folger *et al.*, 2001). A novel cooperative factor of TGFβ1 seems to be the cell-surface protein galectin-3. Galectin-3 is mandatory for TGFβ1-induced myofibroblast differentiation of hepatic stellate cells in culture and is upregulated in experimental liver, kidney, and lung fibrosis (Henderson *et al.*, 2006). Knockout of galectin-3 significantly reduces hepatic fibrosis at unchanged levels of TGFβ1 and inhibits myofibroblast activation of cultured stellate cells in the presence of TGFβ1; addition of recombinant galectin-3 rescues myofibroblast differentiation. Binding of galectin-3 to growth factor receptors has been demonstrated but its cooperation with TGFβ1 is not yet understood (Henderson *et al.*, 2006).

Soluble factors that antagonize TGFβ1-induced myofibroblast differentiation. Attractive with respect to therapeutic treatment of fibrosis are growth factors that antagonize TGFβ1 and thereby reduce myofibroblast differentiation. The inflammatory mediator IL-1 efficiently inhibits TGFβ1-induced α-SMA expression in cultured dermal fibroblasts when added as recombinant protein (Shephard *et al.*, 2004b); overexpression of the intracellular IL-1 receptor antagonist (IL-1ra) has similar effects (Kanangat *et al.*, 2006). Co-culture experiments with fibroblasts suggest that time-controlled secretion of IL-1 by keratinocytes may be one mechanism during cutaneous wound healing to suppressing fibroblast-to-myofibroblast transition in early wounds (Shephard *et al.*, 2004b). IFN-γ, a cytokine produced by T cells also reduces α-SMA expression in cultured fibroblasts. This effect appears to be mediated by activation of the repressor protein YB-1, which translocates to the nucleus and interferes with Smad3-mediated transcription of TGFβ1-induced genes (Higashi *et al.*, 2003) (Figure 1). In addition, YB-1 enhances the transcription of Smad-7, a negative regulator of Smad3 signaling (Dooley *et al.*, 2006).

Myofibroblast differentiation without TGFβ1?. Only few factors have been identified that potentially induce myofibroblast differentiation independently from TGFβ1. IL-6 has been suggested to be such a factor as IL-6-null mice exhibit impaired cutaneous wound healing and reduced myofibroblast differentiation at unchanged levels of TGFβ1. Moreover, recombinant IL-6 increases α-SMA transcription in fibroblast cultures from IL-6-null mice in the absence of exogenous TGFβ1 (Gallucci *et al.*, 2006). Similarly, nerve growth factor enhances α-SMA expression in cultured skin and lung fibroblasts (Micera *et al.*, 2001). However, as myofibroblast differentiation in response to IL-6 and nerve growth factor was not tested with simultaneous inhibition of TGFβ1 signaling, cooperation with autocrine TGFβ1 cannot be ruled out. Very recently, Fizz1 (found in inflammatory zone), a novel protein secreted by alveolar epithelial cells, has been demonstrated to undergo dramatic upregulation in an animal model of lung fibrosis. When added to cultured fibroblasts, recombinant Fizz1 induces expression of α-SMA in the absence of TGFβ1; the molecular mechanism of Fizz1 action remains elusive (Liu *et al.*, 2004). In addition to proinflammatory cytokines, agonists of myofibroblast contraction, like angiotensin-II (Swaney *et al.*, 2005), endothelin-1 (Shi-Wen *et al.*, 2004), and thrombin (Bogatkevich *et al.*, 2003) induce expression of α-SMA expression *in vitro*; however, it is unclear whether endogenous TGFβ1 contributes to this effect. It is also conceivable that contraction agonists enhance α-SMA expression by augmenting the level of intracellular stress (Shephard *et al.*, 2004a).

Myofibroblasts are controlled by the mechanical microenvironment

Despite high levels of active TGFβ1 in early wounds (Yang *et al.*, 1999), expression of α-SMA and enhanced myofibroblast contraction are restricted to the later phases of dermal wound healing. Earlier myofibroblast appearance may be suppressed by antagonistic factors released by inflammatory cells and keratinocytes. Moreover, it becomes increasingly clear that myofibroblast development is profoundly influenced by the mechanical microenvironment, in particular, by the organization and stiffness of the ECM (Hinz and Gabbiani, 2003a). The level of α-SMA expression in cultured differentiated myofibroblasts increases with increasing matrix rigidity as demonstrated using collagen and polyacrylamide gel substrates of varying stiffness (Arora *et al.*, 1999; Hinz *et al.*, 2003). Mechanically preventing wound closure by splinting the edges of experimental wounds accelerates expression of α-SMA compared with normally healing wounds; stress release by removing the splint leads to reduced α-SMA expression (Hinz *et al.*, 2001b). TGFβ1 is always present in these conditions; however, the mechanical characteristics of the environment appear to modulate its action to induce either migration or contraction (Grin-

nell and Ho, 2002). In addition to wound healing and fibrosis, altered matrix stiffness is increasingly considered as major factor activating stroma myofibroblasts in the vicinity of ''stiff'' tumors and thus feeding back on tumor progression (Paszek *et al.*, 2005).

What mechanical properties display the ECM in a physiological tissue context and how do they change during pathological contracture? During embryonic development, soft tissues are established with an elastic modulus of 100–20,000 Pa, depending on their location and function in the adult organism (Fung, 1993; Discher *et al.*, 2005); fibroblasts residing in intact tissues are generally ''stress-shielded'' by this ECM (Tomasek *et al.*, 2002). When the protective matrix structure is lost by injury, fibroblasts experience a dramatically changed mechanical microenvironment and endeavor to re-establish ECM tension by increasing remodeling and synthesizing activity (Eckes and Krieg, 2004; Marenzana *et al.*, 2006). The stiffness of the provisional ECM of early wounds is comparable with the elastic modulus of ~10–100 Pa of newly polymerized collagen gels (Kaufman *et al.*, 2005), frequently used *in vitro* models of tissue repair (Grinnell, 2003; Carlson and Longaker, 2004). Fibroblasts initiated in such gels are devoid of contractile fibers and organize actin filaments in the cortex of the cell body and of dendritic extensions (Tamariz and Grinnell, 2002). In mechanically restrained gels, tension is gradually increasing and first induces the formation of α-SMA-negative stress fibers (Tamariz and Grinnell, 2002; Marenzana *et al.*, 2006); this is similar to the *de novo* appearance of proto-myofibroblast in 5- to 6-day-old rat wound granulation tissue (Hinz *et al.*, 2001b). Gel and tissue stiffness at this stage have not been measured but neoformation of stress fibers in fibroblasts grown on soft two-dimensional culture substrates occurs at an elastic modulus of ~3,000–6,000 Pa (Discher *et al.*, 2005; Yeung *et al.*, 2005).

Progression to the differentiated myofibroblast requires significantly higher stiffness that begins to develop in anchored collagen gels after 2–3 days of remodeling by proto-myofibroblasts (Hinz, 2006) and after 8–9 days in experimental rat wounds (Hinz *et al.*, 2001b). The threshold stiffness for *de novo* expression of α-SMA in stress fibers ranges around 20,000 Pa as demonstrated for contractile wound granulation tissue and for myofibroblasts cultured on elastic substrates (Goffin *et al.*, 2006). A comparable matrix stiffness of ~15,000 Pa activates hepatic stellate cells into α-SMA-positive myofibroblasts *in vitro* and during development of liver fibrosis *in vivo* (Wells, 2005). In other fibrotic tissues and in granulation tissue toward the end of wound healing, a matrix stiffness of greater than 50,000 Pa has been measured (Goffin *et al.*, 2006); this significant tissue rigidification by fibrogenic stroma cells is used to localize tumor metastases *in situ* with improving imaging methods (Greenleaf *et al.*, 2003).

The mechanisms and intracellular pathways through which tension potentially controls α-SMA transcription have recently been reviewed (Wang *et al.*, 2006). In addition, α-SMA itself is beginning to be considered as mechano-sensitive protein that only localizes to stress fibers under significant mechanical load; this provides a rapid mechanism to control myofibroblast contractile function (Goffin *et al.*, 2006; Hinz, 2006). Reducing stress fiber tension by reducing substrate stiffness and by inhibiting myosin contraction leads to the disassembly of α-SMA from stress fibers that persist as β-cytoplasmic actin filament bundles. Stress fiber-derived α-SMA accumulates in cytosolic aggregates that resemble short rods (Goffin *et al.*, 2006). Similar rod-like aggregates of α-SMA have been demonstrated during physiological spreading of suspended myofibroblasts (Clement *et al.*, 2005), which is characterized by gradually increasing intracellular tension and *de novo* formation of stress fibers (Hinz *et al.*, 2003). From these studies, it has been suggested that construction of α-SMA-positive stress fibers requires pre-formation of β-cytoplasmic actin bundles; absence of such an organization template seems to leads to cytosolic accumulation of α-SMA. It is conceivable that the α-SMA-specific N-terminal sequence AcEEED contributes to α-SMA mechano-sensitivity as cytoplasmic delivery of this sequence as a peptide similarly induces α-SMA rod formation (Clement *et al.*, 2005) and selectively removes α-SMA from stress fibers (Chaponnier *et al.*, 1995; Hinz *et al.*, 2002).

How myofibroblasts feel stress

The question remains how myofibroblasts sense the gradually increasing change in matrix stiffness that accompanies tissue remodeling. Three major mechanisms of mechano-sensing are conceivable: (1) mechano-sensitive ion channels in the plasma membrane (Martinac, 2004), (2) integrin-mediated stress perception (Katsumi *et al.*, 2004), and (3) geometry changes that reveal cryptic signaling domains in proteins of the ECM (Vogel and Sheetz, 2006). Cultured fibroblasts modulate the level of α-SMA expression after locally stretching the plasma membrane with the use of twisting magnetite beads; reaction to these rapid stimuli is thought to depend at least in part on the opening of mechano-sensitive ion channels and influx of Ca^{2+} over the plasma membrane (Ko *et al.*, 2001). However, myofibroblasts *in vivo* differentiate in response to matrix changes that occur sometimes over days and months, which demands a ''slow'' mechanosensor. Moreover, fibroblast-to-myofibroblast transition can be driven by subtle forces as produced by very slow interstitial flow that accompanies inflammation and tissue regeneration (Ng *et al.*, 2005); these physiological mechano-stimuli likely exclude membrane channels as force detection mechanism for myofibroblast differentiation.

The role of cell–matrix adhesions in myofibroblast stress perception. It is widely accepted that to perceive mechanical signals from the ECM, fibroblasts utilize integrin-based matrix adhesions, that is, the same transmembrane organelles that anchor stress fibers to the substrate (Chen *et al.*, 2004; Bershadsky *et al.*, 2006). Ultrastructural analysis of myofibroblasts in fibrotic and wound granulation tissue

has revealed extensive cell–matrix contacts, called fibronexus that are absent from normal connective tissue fibroblasts (Eyden, 2005). Analogously, cultured myofibroblasts develop specialized focal adhesions (FAs) that have been termed ''supermature FAs'' to account for their significantly longer appearance (8–30 μm) (Dugina *et al.*, 2001) compared with ''classical FAs'' (2–6 μm) of α-SMA-negative fibroblasts (Geiger *et al.*, 2001). In addition, supermature FAs exhibit a specific molecular composition by co-expressing high levels of vinculin, paxillin, and tensin and integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ (Dugina *et al.*, 2001; Hinz *et al.*, 2003). This is different from classical FAs that generally lack tensin and $\alpha 5\beta 1$ integrin and to fibrillar adhesions that contain tensin and $\alpha 5\beta 1$ integrin but not vinculin, paxillin, and $\alpha v\beta 3$ integrin (Geiger *et al.*, 2001). More comprehensive reviews of the molecular components of myofibroblast ECM adhesions have recently been published (Hinz and Gabbiani, 2003a; Hinz, 2006). Moreover, supermature FAs display highly phosphorylated focal adhesion kinase and paxillin, which may play a role in the recruitment of fibrillar adhesion components to classical FAs (Goffin *et al.*, 2006). Consistently, phosphorylated focal adhesion kinase is central in the adhesion-dependent differentiation of cultured lung (Thannickal *et al.*, 2003) and scleroderma myofibroblast (Mimura *et al.*, 2005). It remains to be shown whether and how supermature FAs assemble in focal adhesion kinase-null corneal fibroblasts that apparently acquire a constitutive myofibroblast phenotype (Greenberg *et al.*, 2006).

Establishment of supermature FAs depends both on the significant intracellular contractile activity generated by α-SMA and on a stress-resistant ECM (Hinz, 2006). Differentiated myofibroblasts convert supermature FAs into small classical FAs after administration of the AcEEED peptide (Hinz *et al.*, 2003) and when cultured on soft substrates and in newly polymerized collagen gels (Goffin *et al.*, 2006; Hinz, 2006). These recent studies suggest that fibroblasts gain information about the mechanical state of the ECM by assessing the level of tension in stress fibers,

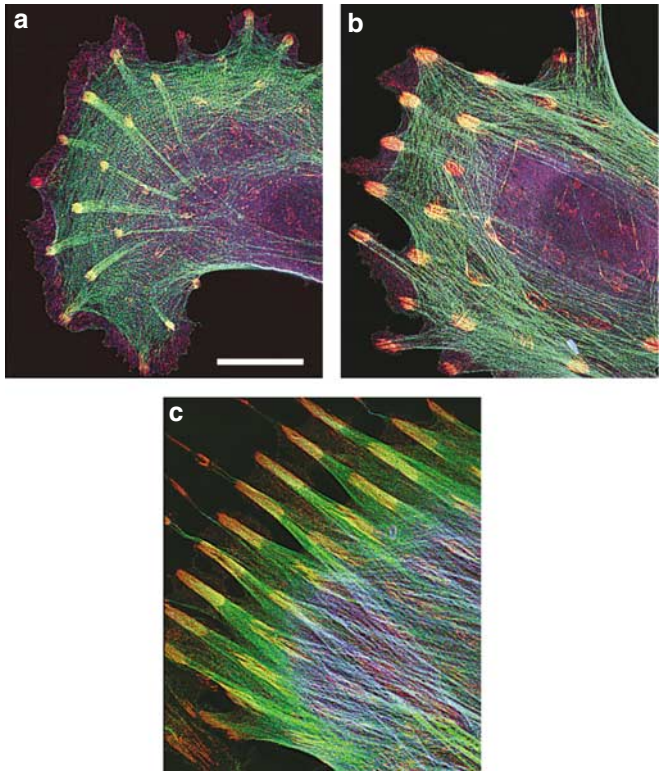

Figure 2. FA size controls recruitment of α-SMA to stress fibers. Arrays of ECM islets with a size of (**a**) 1×1.25 μm, (**b**) 4×1.25 μm, and (**c**) 10×1.25 μm were created on glass by microcontact printing; non-printed regions were passivated against cell attachment (Goffin *et al.*, 2006). After 12 hours, culture differentiated myofibroblasts were stained for vinculin (red), β-cytoplasmic actin (green), and α-SMA (blue). Bar = 20 μm. Stress fibers are formed on all substrates; however, α-SMA is only recruited to contractile fibers on islets that exceed the length of classical FAs ($\leqslant 6$ μm) and exhibit supermature FA features (> 8 μm).

which is limited by the size of their matrix anchors. Forcing myofibroblasts to grow on arrays of classical FA adhesion islets ($\leqslant 6$ μm) leads to the rapid loss of α-SMA (Figure 2); dynamically enlarging these islets on extendable membranes leads to reincorporation of α-SMA into stress fibers. Reincorporation is independent from the magnitude of the applied stretch but requires that the resulting islet size corresponds to supermature FAs (~ 8 μm). Resizing classical to supermature FAs permits exertion of $\sim$4-fold greater stress (Goffin *et al.*, 2006) and provides an exceptionally strong ECM anchor that renders myofibroblasts highly adhesive (Hinz *et al.*, 2003).

All these data suggest that formation of supermature FAs represents a critical control point in the mechanical feedback loop of extracellular stress and intracellular tension that regulates the state of myofibroblast differentiation (Figure 3). In physiological tissue repair, this checkpoint could ensure that enhanced myofibroblast contraction only occurs when the tissue has been sufficiently remodeled by proto-myofibroblasts for effective force exertion by differentiated myofibroblasts. It appears difficult to establish whether the primary event is represented by α-SMA-generated stress fiber tension or FA supermaturation. Nevertheless, specifically targeting supermature FAs is one potential strategy to interrupt the mechanical cycle when it has become vicious in pathological contractures.

Stress-dependent conformation of ECM proteins: where mechanical and growth factor signals may converge. The mechanical state of the ECM may directly influence myofibroblast differentiation by revealing cryptic epitopes in ECM proteins under stress. A potential candidate for such a mechanism is cellular FN, which contains cryptic

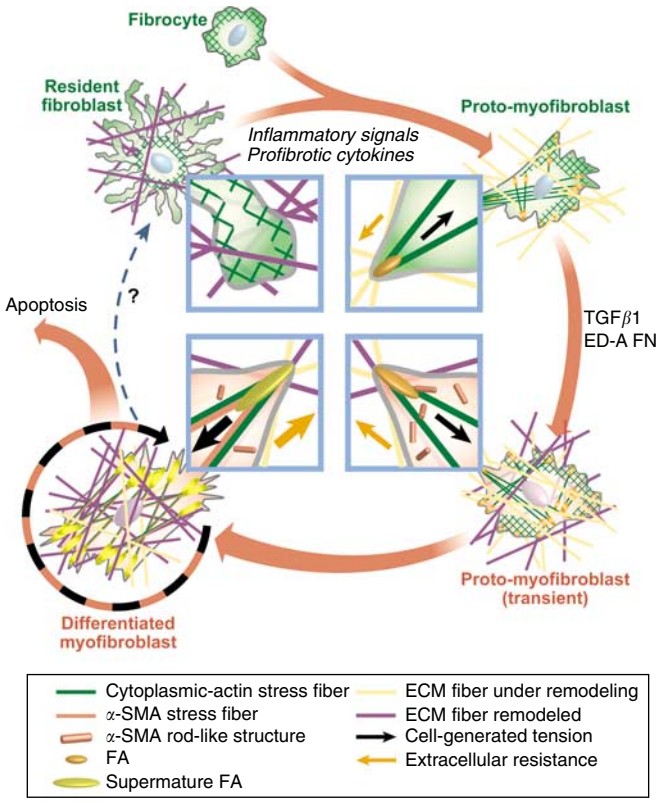

Figure 3. The mechanical feedback loop in myofibroblast development. Fibroblasts in intact tissue are stress-shielded by a functional ECM; they do not develop contractile features and cell–matrix adhesions. After injury, inflammatory signals activate fibroblasts to spread into the provisional wound matrix. Local cell remodeling activity leads to gradual increase in global matrix stiffness that counteracts cell traction forces. The resulting formation of small FAs and stress fibers that contain only cytoplasmic actins characterize the proto-myofibroblast. TGFβ1 stimulates proto-myofibroblasts to express α-SMA, which at first is not incorporated into stress fibers but organizes in cytoplasmic rod-like structures. Continuing ECM fiber alignment creates larger surfaces for adhesion formation; larger adhesions permit development of stronger stress fibers and generation of higher contractile forces. When adhesion sites grow to the size of supermature FAs, intracellular tension reaches a critical level that allows incorporation of α-SMA into pre-existing stress fibers. The force generated by α-SMA-containing stress fiber is significantly higher compared to cytoplasmic actin stress fibers leading to further FA supermaturation and ECM contraction, thereby establishing a mechanical loop. Myofibroblasts may exit this cycle when the original structure of the ECM is reconstituted and again takes over the mechanical load; stress-released myofibroblasts eventually undergo apoptosis.

domains in several type III modules that unfold upon cell traction (Vogel and Sheetz, 2006). ED-A, one of such modules is spliced into the FN molecule during tissue repair and is a prerequisite for TGFβ1-induced myofibroblast differentiation (Serini *et al.*, 1998). It remains to be shown whether cell-generated mechanical forces may change the availability of this domain for specific integrins to induce myofibroblast-promoting intracellular signals.

Another intriguing hypothesis on how increased ECM tension and/or cell traction forces enhance expression of α-SMA is by directly activating TGFβ1. Fibroblasts and myofibroblasts secrete TGFβ1 as part of a large latent complex, additionally consisting of the latency-associated protein (LAP) and the latent TGFβ1-binding protein (LTBP-1) (Mangasser-Stephan *et al.*, 2001; Koli *et al.*, 2005). LTBP-1, a member of the fibrillin family strongly links the large latent complex to the ECM via several ECM binding sites (Unsold *et al.*, 2001). Activation of TGFβ1 occurs upon its release from the large latent complex through proteolytic cleavage (e.g. by plasmin) and by the action of thrombospondin-1 (Annes *et al.*, 2003). In epithelial cells, cell-generated traction via integrins has been proposed as alternative mechanism to activate TGFβ1 from the large latent complex. Both, LAP and LTBP-1 contain RGD motifs for the binding of integrins (Keski-Oja *et al.*, 2004) and the epithelial integrin $\alpha v\beta 6$ was shown to activate TGFβ1 during development of lung fibrosis and in culture independently of protease activity (Munger *et al.*, 1999; Annes *et al.*, 2004). Fibroblasts do not express $\alpha v\beta 6$ integrin; however, integrins $\alpha v\beta 5$ and $\alpha v\beta 3$ were shown to bind to LAP-TGFβ1 (Ludbrook *et al.*, 2003) and both are involved in TGFβ1 activation by fibrogenic systemic sclerosis fibroblasts through a yet unknown mechanism (Asano *et al.*, 2005a, b). Moreover, the integrin $\alpha 8\beta 1$ was recently shown to strongly interact with LAP-TGFβ1 (Lu *et al.*, 2002) and it is upregulated in conjunction with myofibroblast differentiation during heart (Bouzeghrane *et al.*, 2004), pulmonary, and hepatic fibrosis (Levine *et al.*, 2000).

TGFβ1 activation by $\alpha v\beta 6$ integrin requires ECM binding of LTBP-1 through its N-terminal hinge region (Annes *et al.*, 2004), suggesting that matrix resistance needs to counteract cell traction in the activation process. Consistently, transformed fibroblasts expressing high levels of latent LAP-TGFβ1 do not promote fibrosis when implanted into mouse dermis (Campaner *et al.*, 2006); this may be due to the lack of association with LTBP-1 and ECM. It is tempting to speculate that the high stress transmitted at myofibroblast adhesions is particularly efficient in activating TGFβ1 from a sufficiently organized ECM. This mechanism would provide active TGFβ1 only on demand of contractile cells to uphold myofibroblast differentiation during the contraction phase of tissue repair.

When the work is done: the fate of myofibroblasts

Solving the question whether myofibroblast differentiation is terminal or reversible will help to develop strategies to specifically and ultimately inhibit myofibroblast formation. Several studies demonstrate downregulation of α-SMA in cultured differentiated myofibroblasts in response to a variety

of factors, like TGFβ1 antagonists, growth factors, cell density, and matrix compliance without obvious signs of increased cell death. *In vivo*, however, reversal of the myofibroblast has not been documented and massive apoptosis occurs after wound healing and re-epithelialization (Desmouliere *et al.*, 1995); the signals leading to myofibroblast group suicide are still unclear. Alternatively to growth factor signals, two mechanisms are conceivable to account for myofibroblast disappearance at the end of physiological tissue repair: (a) cell release from stress and (b) increased formation of specific cell–cell contacts. In properly repaired tissue, the ECM regains its original mechanical properties and again stress-shields the resident fibroblasts. Preventing the tension release of granulation tissue by splinting the wound inhibits myofibroblast apoptosis in an animal model; subsequent splint release induces cell death (Carlson *et al.*, 2003). Similarly, apoptosis is induced by relaxing fibroblast-populated attached collagen gels induces *in vitro* (Grinnell *et al.*, 1999; Niland *et al.*, 2001). The intracellular pathways linking stress release to induction of apoptosis are unclear but likely involve cell matrix junctions (Stupack and Cheresh, 2002; Reddig and Juliano, 2005), that is to say stress perception mechanisms similar to those used to control fibroblast-to-myofibroblast transition.

Furthermore, adhesion-dependent survival signals are transmitted via cell–cell contacts and cadherins (Jamora and Fuchs, 2002; Yap and Kovacs, 2003) and formation of homotypic cadherin junctions is a possible signal of the presence of myofibroblast accumulation in late granulation tissue. Consistently, corneal myofibroblasts in dense culture significantly decrease the expression of α-SMA and de-differentiate into α-SMA-negative fibroblasts; this has been attributed to contact-induced desensitization to TGFβ1 (Petridou *et al.*, 2000). It remains to be investigated whether increased formation of OB-cadherin type junctions affects myofibroblast survival differently from N-cadherin-type contacts (Hinz *et al.*, 2004). The potential involvement of both, specific cell–matrix and cell–cell contacts in regulating myofibroblast survival has the advantage of their extracellular accessibility to drugs.

Concluding remarks

Most efforts to understanding the molecular mechanism of myofibroblast differentiation and function are driven by the motivation to eliminate this cell in pathological tissue contractures. Although this may be difficult to accept from the sentimental point of view of someone who devotes his research to this cell, novel strategies and drugs are indeed needed to specifically counteract myofibroblast action. Inhibiting the pleiotrophic action of profibrotic cytokines such as TGFβ1 bears the disadvantage of affecting a multitude of cells that are also involved in tissue repair. One promising antifibrosis approach is interfering with myofibroblast mechano-perception via cell-to-matrix and cell-to-cell adhesions. Just like many human individuals, myofibroblasts work best under stress; stress release leads to instant loss of internal tension – a condition that apparently drives myofibroblasts into suicide.

CONFLICT OF INTEREST

The author states no conflict of interest.

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