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Submitted for publication November 21, 2012.

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Abbreviations and Acronyms

α -SMA = alpha-smooth muscle actin

ECM = extracellular matrix

FA = focal adhesion

FAK = focal adhesion kinase

LAP = latency-associated peptide

NAC = N-acetylcysteine

NOX = nicotinamide adenine dinucleotide phosphate oxidase

ROS = reactive oxygen species

TGF- β = transforming growth factor beta

Integrin β 1: A Mechanosignaling Sensor Essential for Connective Tissue Deposition by Fibroblasts

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Significance: There is no effective drug treatment for fibrosis (*i.e.*, pathological scarring). Identifying the fundamental mechanisms responsible for normal and pathological connective tissue deposition is likely to yield novel insights into how to control fibrotic conditions.

Recent Advances: An increasing body of evidence suggests a link between mechanical tension and the development of scar tissue. Integrins are the cell surface receptors that mediate interactions between the cell and the surrounding extracellular matrix (ECM). Recent evidence has suggested that, in fibroblasts, the integrin β ₁-subunit plays an essential role in mechanosignaling and in dermal homeostasis, repair, and fibrosis. The mechanism underlying these activities of integrin β ₁ appears to involve its ability to (1) mediate activation of latent transforming growth factor beta-1 via ECM contraction and (2) modulate collagen production via a focal adhesion kinase/rac1/nicotinamide adenine dinucleotide phosphate oxidase (NOX)/reactive oxygen species (ROS) pathway. Moreover, the integrin β ₁-binding protein CCN2, a secreted matricellular protein located within the cellular microenvironment, is required for dermal fibrogenesis.

Critical Issues: Mechanical tension is a key feature underlying the development of scar tissue. The mechanosignaling sensor integrin β ₁ is an essential, central mediator of dermal fibrosis, wound healing, and homeostasis.

Future Directions: Drugs targeting the molecular mechanism underlying integrin β ₁-mediated signaling may represent a novel therapeutic approach for treating fibroproliferative disorders. Clinical trials directly testing this hypothesis are warranted.

SCOPE AND SIGNIFICANCE

FIBROPROLIFERATIVE DISORDERS, caused by excessive deposition of scar tissue by fibroblasts resident within connective tissue, are characterized by progressive deterioration of the normal structure and function of organs. Chronic fibrotic diseases can result in organ failure and, in the worst cases, death. Affected organs include the liver, kidney, lung, heart, pancreas, and skin. Fibroproliferative disorders of the skin include systemic sclerosis, Dupuytren's contracture, hypertrophic

scars, and keloids. Mechanobiology, namely, how organisms sense mechanical signals and transduce them into biological responses, is being increasingly appreciated to play a critical role in fibroproliferative disorders.^{1,2} For example, keloids tend to appear on areas where the body suffers local mechanical tension, such as on the shoulders and upper body.¹ Thus, mechanotransductive signals present in the local microenvironment are expected to play a major role in the pathogenesis of fibrotic disorders.

Not only are certain areas of the body more subject to increased local mechanical forces, but the skin also becomes under increased local mechanical tension, for example, in response to tissue injury, or within scar tissue. Indeed, a shared feature of scars is their markedly elevated stiffness compared with the relatively compliant tissue texture of healthy tissue. For example, the force present in muscle has been estimated to be ~ 10 kPa; in tissues that are macroscopically soft, such as the liver, fat, and brain, forces have been estimated to range between 0.1 and 1 kPa, whereas the dermis of healthy human skin is estimated to range between 1 and 5 kPa.³ In contrast, fibrotic tissue is substantially less compliant and exhibits substantially greater forces of 20–100 kPa, similar to that of collagen-dense tendon.³

Within the connective tissue of wounded or fibrotic skin is a specialized form of fibroblasts termed myofibroblasts, which share characteristics of smooth muscle cells, as they contain highly contractile stress fibers consisting of the protein α -smooth muscle actin (α -SMA).⁴ The presence of the myofibroblast is what distinguished clinically defined scar tissue from neighboring healthy tissue.^{5,6} The myofibroblasts themselves inherently exhibit enhanced mechanotransductive abilities as evidenced by their elevated abilities to adhere to and contract the extracellular matrix (ECM), and hence directly contribute to the enhanced tensile strength of scar tissue.^{6–8} Intriguingly, the appearance of the myofibroblasts within connective tissue seems to depend entirely on mechanical loading. For example, the potent fibrogenic cytokine transforming growth factor beta-1 (TGF- β 1) can only upregulate α -SMA in fibroblasts grown on stiff, but not on compliant, collagen.⁹ Furthermore, TGF- β 1 cannot induce myofibroblast formation in focal adhesion kinase (FAK)-deficient fibroblasts, or in fibroblasts treated with the FAK/src inhibitor PP2.¹⁰ Finally, α -SMA is only capable of being incorporated into stress fibers in cells subjected to significant mechanical loading.^{11,12} Collectively, these data strongly suggest that extracellular mechanical forces regulate how dermal fibroblasts contribute to connective tissue deposition and remodeling in skin homeostasis, wound repair, and fibrosis. This review discusses recent data suggesting that integrin β ₁ plays an essential role in these processes.

TRANSLATIONAL AND CLINICAL RELEVANCE

The overall concept that local variances in mechanotransduction are important for dermal homeostasis and fibroproliferative conditions is

supported by recent clinical data obtained using humans. For example, although it is generally considered that resting fibroblasts in healthy connective tissue are stress shielded (due to the highly compliant surrounding ECM), a recent report has indicated that keloids tend to occur on highly mobile sites in the body that inherently possess elevated skin stretching and hence are normally subject to comparatively high, local tension compared to the surrounding tissue.¹³ As a result, understanding how fibroblasts sense and transduce local mechanical signals from the microenvironment is likely to lead to novel therapeutic approaches that prevent, reduce, or even reverse the development and/or progression of these conditions and is therefore likely to be of future clinical benefit.

BASIC SCIENCE PERSPECTIVE

Cells sense extracellular mechanical forces through the cytoskeleton, which is connected to the surrounding ECM through cell surface structures known as focal adhesions. Thus, distant molecules within the ECM, cytoplasm, and nucleus are mechanically coupled. In the case of myofibroblasts, the connective tissue cells responsible for wound repair and fibrosis, α -SMA-containing stress fibers, span the length of the cell and are connected to cell surface ECM receptors called integrins, which are localized to focal adhesions.^{3,4} Integrins, responsible for sensing mechanical stress, are heterodimers comprised of α - and β -subunits; the main integrins that actively participate in fibroblast proliferation, collagen contraction, and myofibroblast differentiation include $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_{11}\beta_1$.^{14–16} Although other integrins, notably $\alpha_v\beta_5$, appear to also participate in myofibroblast differentiation,^{17,18} these observations suggest that, in fibroblasts, integrin β_1 may play a central role in mechanotransduction and fibrogenic responses in the dermis. This review discusses the data underlying this hypothesis.

DISCUSSION OF FINDINGS AND RELEVANT LITERATURE

Integrin β_1 is an essential mechanotransduction receptor in dermal fibroblasts

As discussed above, the key distinguishing feature of fibrotic tissue is the presence of the myofibroblasts, which contains α -SMA stress fibers. These stress fibers are attached to the ECM through specialized cell surface structures called focal adhesions, which contain clusters of cell surface ECM receptors called integrins. The

precise origin of myofibroblasts in skin connective tissue is unclear; however, it is likely that myofibroblasts arise, at least in part, via differentiation of local, resident fibroblasts in response to growth factors and mechanical tension.⁴ Normally, fibroblasts resident in connective tissue are shielded from stress by the surrounding, compliant ECM; yet, upon injury, this stress shielding is lost, and a series of events is initiated in which fibroblasts possess progressively stronger attachments to the ECM.¹⁹ This increase in mechanical loading results in activation of latent TGF- β through a complex mechanism involving integrins, focal contacts, and actin-based ECM contraction.²⁰ In resting tissue, TGF- β , together with its latency-associated peptide (LAP), is bound to latent TGF- β 1-binding protein-1, which is a part of the ECM.²⁰ Cell force, via integrins (at a force estimated to be ~ 40 pN), is exerted on LAP, leading to the unfolding of two LAP domains: the α 1 helix and the latency lasso that normally keep TGF- β 1 associated with LAP.²⁰ Integrin β 1 knockout dermal fibroblasts are less able to adhere to and contract the ECM.²¹ In mice harboring a fibroblast-specific deletion of integrin β 1, activation of latent TGF- β is impaired; delayed wound healing results, which is rescued by addition of active TGF- β .²¹

Not only does integrin β 1 appear to be involved with activation of latent TGF- β during the wound healing process in mice, but integrin β 1 also appears to directly mediate collagen and α -SMA production in skin. Fibroblast-specific integrin β 1 knockout mice not only are resistant to bleomycin-induced skin fibrosis, but also possess a progressive thinning of the dermis.^{22,23} Relative to wild-type control dermal fibroblasts, integrin β 1-deficient dermal fibroblasts express reduced levels of collagen type I and α -SMA mRNAs.²³ Integrin β 1 knockout dermal fibroblasts also show diminished rac1 activation and reactive oxygen species (ROS) generation.²³ Reintroduction of active rac1 into integrin β 1 knockout fibroblasts restores ROS generation, and addition of H₂O₂ into integrin β 1 knockout fibroblasts restores collagen type I and α -SMA mRNA expression.²³ rac1 is known to promote ROS generation via NADPH oxidases (NOXes); NOX inhibition reduces collagen type I and α -SMA mRNA expression in wild-type dermal fibroblasts.²³ Phosphorylation of FAK is reduced in integrin β 1 knockout fibroblasts and in wild-type fibroblasts treated with a neutralizing anti-integrin β 1 antibody.²⁴ Moreover, FAK is required for the expression of collagen type I and α -SMA mRNAs.²⁴ Collectively, these data suggest that integrin β 1 can directly control the expression of

ECM genes in fibroblasts via a FAK/rac1/NOX/ROS-dependent mechanism (Fig. 1).

Specific preclinical *in vivo* data illustrating the role of the signaling pathway operating downstream of integrin β 1 in tissue repair and fibrosis have recently been generated. For example, mice harboring fibroblast-specific deletions for FAK and rac1 are resistant to bleomycin-induced skin fibrosis.^{25,26} Moreover, mice harboring a fibroblast-specific knockout for rac1 show delayed wound closure related to a relative failure to generate ROS.²⁷ Dermal fibroblasts taken from lesional (scarred) areas of patients with the autoimmune connective tissue disease scleroderma show a persistent fibrotic phenotype, including the overproduction of type I collagen and the enhanced ability to contract the ECM. However, when these cells are treated with either FAK or rac inhibitors, this fibrotic phenotype is reversed.^{24,28} It is important to note that both FAK and rac seem to be important for the expression of type I collagen in normal fibroblasts, as well as the behavior of normal fibroblasts.^{24,28} Similarly, the antioxidant epigallocatechin-3-gallate suppresses collagen production and contraction by normal and fibrotic sclerodermal fibroblasts.²⁹ These mechanistic observations are interesting as the involvement of NOX/ROS in fibrosis has been recently recognized (for reviews, see

Figure 1. Mechancosignaling pathways mediating dermal connective tissue homeostasis, repair, and fibrosis. In differentiating myofibroblasts, ECM contraction via FA mediated by α -SMA results in the liberation of active TGF β from LAP. In fibroblasts, integrin β 1 contributes to basal collagen and α -SMA expression via a ROS-dependent mechanism. α -SMA, alpha-smooth muscle actin; ECM, extracellular matrix; FA, focal adhesion; FAK, focal adhesion kinase; LAP, latency-associated peptide; NOX, nicotinamide adenine dinucleotide phosphate oxidase; ROS, reactive oxygen species; TGF- β , transforming growth factor beta.

Samarkoon *et al.* and Hecker *et al.*).^{30,31} The individual *NOX* genes responsible for skin tissue repair and fibrosis are unclear. However, *NOX4* is upregulated in patients with idiopathic pulmonary fibrosis, and *NOX4*-produced ROS are critical for models of lung injury and the ability of TGF- β to induce lung myofibroblast differentiation.^{32,33} These results are consistent with the general hypothesis that mechanosignaling via the integrin β_1 subunit is required for dermal homeostasis, wound healing, and fibrotic responses *in vivo*.

The matricellular integrin β_1 -binding protein CCN2: a more specific antifibrotic target?

It is clear that, however, the loss of integrin β_1 or *rac* expression and FAK or ROS inhibition affect normal physiology in that basal expression of type I collagen (for example) in healthy fibroblasts is impaired by loss of integrin β_1 expression or FAK inhibition (as discussed above).^{23,24,26–28} This notion leads to the hypothesis that identifying a more selective target for antifibrotic drug intervention that is nonetheless involved with the profibrotic action of integrin β_1 would be important.

In this regard, the emerging awareness of the involvement of matricellular proteins in mechanosignaling may be especially useful. The term matricellular protein was first introduced by Bornstein in 1995 to describe a group of ECM proteins that plays minimal roles in matrix structural integrity, but regulates a multitude of cellular responses.^{34–36} Matricellular proteins are secreted, nonstructural ECM proteins with regulatory functions exerted through different mechanisms, including as the direct binding to other matrix proteins, triggering of their specific surface receptors, and binding to growth factors and cytokines modulating their activity. Abundantly expressed during development, their level is generally low in steady-state condition in adult tissues, but they are readily upregulated in wound healing and tissue remodeling.^{37–39} The overall function of matricellular proteins is to act in the cellular microenvironment to modulate cellular processes such as cell adhesion and migration, ECM deposition, cell survival, and proliferation. However, the actual *in vivo* role of each matricellular protein appears to vary depending based on the particular context and the microenvironment that accompanies tissue homeostasis or repair activities. As such, they appear to be ideally suited to contribute to tissue plasticity by acting as adaptor molecules

TAKE-HOME MESSAGES

- Mechanotransduction plays an essential role in dermal homeostasis, wound healing, and fibrosis.
- Integrins are heterodimers, and in fibroblasts, the integrin β_1 subunit is a essential mechanosensory receptor necessary for fibroblasts to maximally sense mechanical tension and acts to promote connective tissue maintenance and deposition via a FAK/*rac*/NADPH oxidase/ROS-dependent mechanism and through the activation of latent TGF- β .
- Clinical studies in which drugs targeting the mechanism underlying integrin β_1 -mediated mechanotransduction are warranted in conditions of dermal scarring and connective tissue disease.

within the cellular microenvironment enabling a rapid response to changing conditions.

In this scenario, the matricellular protein CCN2 (formerly known as connective tissue growth factor [CTGF]) is largely absent from normal skin, but is selectively upregulated in the dermis during wound healing and fibrosis.³⁷ As such, CCN2 has long been hypothesized to play an essential, specific role in these processes. CCN2 is a member of the CCN family of matricellular proteins, which contains six members.³⁷ The CCN acronym is derived from the first three members of the family identified, namely, CYR61, CTGF, and nephroblastoma overexpressed (NOV); mammalian members of the family have been renamed CCN1–6 of their discovery to reflect their role as matricellular proteins, as opposed to growth factors.⁴⁰ The CCN proteins possess a common modular structure, with an N-terminal secretory peptide preceding four conserved domains with sequence homologies to insulin-like growth factor-binding protein, von Willebrand factor type C repeat, thrombospondin type I repeat, and a carboxyl-terminal domain that contains a cysteine knot motif.^{37,40} The activities of the CCN proteins are largely attributed to the ability of members of the family to bind specific integrins and heparan sulfate proteoglycans in a cell type- and context-dependent manner. For example, in the context of fibroblasts, CCN2 supports cell adhesion by binding integrin subunits containing integrin β_1 .^{41,42} CCN2 also promotes integrin β_1 -mediated adhesion in other systems.⁴³ In cutaneous wound healing and fibrosis, *Ccn2* is highly expressed in myofibroblasts of the granulation tissue.^{44,45} *In vitro* and *in vivo*, CCN2 enhances and alters profibrotic, adhesive signaling responses to ECM components, and growth factors.^{42,46,47} Although dispensable for development of connective tissue in skin, CCN2 is required for bleomycin-induced skin fibrogenesis.^{48,49} CCN2 appears to be required for the appearance of myofibroblasts in conditions of fibrogenesis.⁴⁹ CCN2 appears to act not on affecting

myofibroblast differentiation of resident fibroblasts, but may act to recruit myofibroblasts (possibly pericytes or mesenchymal precursor cells).⁴⁹ CCN2 appears not to mediate normal tissue repair (Liu and Leask, unpublished observations). Thus, CCN2 may prove in the future to be an exquisitely suitable target to affect mechanotransduction in the fibrotic milieu.

CAUTION, CLINICAL REMARKS, AND RECOMMENDATIONS

Integrin β_1 is an essential sensor of mechanotransduction, and signaling pathways through which integrin β_1 promotes this activity have been elucidated. The basic principles that mechanotransduction appears to be important for fibrosis in humans, and that integrin β_1 is an essential mechanotransduction signaling mediator have been suggested by preclinical observations using patients and cells derived from patients. Accordingly, pilot studies using compounds targeting the mechanism through which integrin β_1 in patients are warranted. Since integrin β_1 has been shown to act via FAK, rac, NADPH oxidase, and ROS, studies using inhibitors of these pathways might be used in patients to see if fibrosis can be attenuated or reversed. Drugs that block these pathways that might be evaluated in clinical trials to block scarring include clinical-stage FAK inhibitors VS-6063 (formerly PF-04554878; Verastem) or PF-00562271 (Pfizer), NADPH oxidase inhibitors such as GKT137831 (Genkyotex) or antioxidants such as N-acetylcysteine (NAC; an over-the-counter herbal supplement), the selective rac GTPase inhibitor, PD3766 (P2D Bioscience), and the Akt inhibitor GSK690693 (Glaxo Smith Kline). Most of

these drugs are being evaluated in, or being considered for, clinical trials for cancer;^{50–55} however, some of these, notably NAC, have also been tested in lung fibrosis and have shown to have positive effects.^{56,57} Moreover, it should be noted that the dual NOX1/NOX4 inhibitor GKT137831 reduced liver fibrosis in mice, does not exert toxic effects in animal models, and is also well tolerated in phase I clinical trials.^{58,59} The similarity between fibrosis and cancers is perhaps not surprising, since the oncogenic capacity derives communication between cells in the stroma as well as from the tumor cells themselves, mediated by alterations in ECM-mediated tensegrity.^{60,61} Indeed, in both fibrosis and cancers, it is believed that the ECM microenvironment may be responsible for regulating cellular behavior, and that altering the microenvironment can orchestrate cellular plasticity offering a way reprogramming diseased cells back to a healthy fate.^{1,61,62} Similarly, anti-CCN strategies are being considered for cancer;⁶³ drugs targeting CCN2 action are currently being pursued in hypertrophic scars and in lung fibrosis include RXI-109 (RXi Pharmaceuticals), EXC 001 (Pfizer), and FG-3019 (Fibrogen).

AUTHOR DISCLOSURE AND GHOSTWRITING

No competing financial interests exist. The content of this article was expressly written by the author listed. No ghostwriters were used to write this article.

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