# MYOFIBROBLASTS AND MECHANO-REGULATION OF CONNECTIVE TISSUE REMODELLING

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During the past 20 years, it has become generally accepted that the modulation of fibroblastic cells towards the myofibroblastic phenotype, with acquisition of specialized contractile features, is essential for connective-tissue remodelling during normal and pathological wound healing. Yet the myofibroblast still remains one of the most enigmatic of cells, not least owing to its transient appearance in association with connective-tissue injury and to the difficulties in establishing its role in the production of tissue contracture. It is clear that our understanding of the myofibroblast — its origins, functions and molecular regulation — will have a profound influence on the future effectiveness not only of tissue engineering but also of regenerative medicine generally.

CONNECTIVE TISSUES
Tissues that form the architectural framework of the vertebrate body. In these tissues, the extracellular matrix is plentiful, and cells are sparsely distributed within it.

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Almost all CONNECTIVE TISSUES seem to be under some sort of mechanical tension, even at rest. With the exception of individuals of advancing years and those who have inherited defects, our soft connective tissues do not sag, even around relaxed musculature or during sleep. Indeed, the resting tension that is built into the anatomy of these tissues is most obvious from the fact that important blood vessels and nerves, when dissected free from an arm or a leg, have a resting length that is 25–30% less than their *in situ* length. Establishment of this situation during childhood growth could be explained if soft-tissue growth is activated by a 'threshold external tension'. This apparently basic analysis clearly raises many key questions; in particular, what is the cell-molecular basis of such tensional homeostasis in tissues, how is it maintained during adult life and, crucially, how is it re-established in adults after tissue repair? Another example of tension development in connective tissue is represented by wound contraction, a process that occurs during the healing of an open wound. Finally, pathologies that involve the mechano-regulation of connective tissue include burn-scar contracture, breast-implant failure, abdominal adhesion strictures and Dupuytren's disease (BOX 1) — a lesion of the palmar fascia that results in the immobilization of one or more fingers. Yet tissue tension is an essential regulator of tissue function, such as in lung alveoli, kidney capsule, GRANULATION TISSUE contraction and UTERINE INVOLUTION.

Although most tissues exist under a mechanical tension, the same is not necessarily true of their resident cells. They are protected from the relatively massive external loads by the mechanical properties of the surrounding matrix. In engineering terms, such cells are 'stress-shielded' by the matrix that they deposit and remodel[1,2]. Stress-shielding is well known in engineering where one element (A) of a heterogeneous structure is mechanically loaded in preference to others (B and C); for example, as a result of its shape, orientation or compliance. Consequently, components B and C are shielded from applied stresses by element A. As examples, submariners are stress-shielded by the submarine hull (compressive loading), or windows and walls function to shield against the forces of wind. Similarly, resident cells of a mature connective tissue are under minimal loading (where only the matrix is under significant load) in a way that is not necessarily true for growing or damaged tissues. The close interdependence of matrix remodelling and mechanical stress-shielding is well recognized in bone biology, but its importance for soft tissues is only recently becoming appreciated[3,4].

Box 1 | **Normal and pathological wound healing**

**Skin wound healing involves two main phenomena. First, re-epithelialization, which involves the replication and movement of epidermal cells to reconstitute tissue continuity; and second, *de novo* formation and contraction of granulation tissue that is essentially composed of small vessels, fibroblasts, myofibroblasts and variable amounts of inflammatory cells (for a review, see REF. 34). When re-epithelialization is complete, an important decrease in the number of cellular elements, and in particular of myofibroblasts, takes place in granulation tissue, owing to apoptotic phenomena; then granulation tissue evolves into a poorly cellularized scar[128]. In many instances — due to mechanisms not yet understood — scarring does not occur even despite epithelialization, and granulation tissue evolves into a hypertrophic scar. This contains many myofibroblasts and shows inappropriate production of extracellular matrix, a process called FIBROSIS, which results in the deformation of the surrounding PARENCHYMA or connective tissue[132]. It has been proposed that the onset of hypertrophic-scar formation coincides with a lack of apoptotic phenomena in the final phase of wound healing[132]. This general scheme of fibrosis formation applies to many organs after different types of injury (for example, kidney, lung, liver and heart). Again, deformation and malfunction is the pathological issue.**

GRANULATION TISSUE
Tissue that is formed as part of the initial response to a wound during the healing and repair process, so-called because its surface has a granular appearance due to sprouting of neo-capillaries. It also contains abundant fibroblasts and variable numbers of inflammatory cells.

UTERINE INVOLUTION
The degenerative and regenerative process that the tissues that constitute the uterus and oviducts undergo on their return to a non-pregnant state.

FIBROSIS
The production of fibrous connective tissue as a consequence of chronic inflammation or healing. Its function is to replace lost parenchymal tissue.

During the past 20 years, it has become generally accepted that the myofibroblast — a specialized contractile fibroblast — has an important role in establishing tension during wound healing and pathological contracture[5,6], although its formation and function remain to be explained. Part of this difficulty has been the limited account taken of the inescapable mechanical laws that are integral to the regulation of myofibroblasts by growth factors, matrix attachments and other cell signals. Recent studies using various model systems have begun to explain the interplay between EXTRACELLULAR-MATRIX (ECM) organization, cytomechanical stress and growth-factor signals that regulate the transition from fibroblast to differentiated myofibroblast[5,7].

## The myofibroblast

The notion that granulation tissue contraction depends on force that is generated in this tissue is very old[8]. The traditional view has been that the mechanism of contraction was mainly due to collagen shortening[9]. However, several observations had indicated that granulation-tissue cells could be important for force generation[10]. Modified fibroblasts with smooth-muscle (SM)-like features (myofibroblasts) were first observed in granulation tissue of healing wounds, which led to the suggestion that these cells have a role in the production of the contractile force that is involved in this process[11]. The presence of myofibroblasts in most fibro-contractive diseases (for example, Dupuytren's disease) and even in developing[12] and specialized normal tissues (for example, lung septa) has extended this possibility to several situations (TABLE 1). It has subsequently been shown that most myofibroblasts express α-SM actin (the actin isoform typically found in vascular SM cells)[13,14] and that the expression of α-SM actin and collagen type I in these cells is coordinately regulated by transforming growth factor β1 (TGF-β1)[15]. All these observations indicate that the myofibroblast has a role in the synthesis of ECM and in force generation, which results in ECM reorganization and wound contraction[5].

Morphologically, myofibroblasts are characterized by the presence of a contractile apparatus that contains bundles of actin microfilaments with associated contractile proteins such as non-muscle myosin, and which is analogous to STRESS FIBRES that have been described in cultured fibroblasts[5,16,17] (FIG. 1). These actin bundles terminate at the myofibroblast surface in the fibronexus — a specialized adhesion complex that uses transmembrane integrins to link intracellular actin with extracellular FIBRONECTIN FIBRILS[16,18,19]. Functionally, this provides a mechano-transduction system, so that the force that is generated by stress fibres can be transmitted to the surrounding ECM[16]. In addition, extracellular mechanical signals can be transduced into intracellular signals through this mechano-transduction system[16,20,21]. It should be emphasized that fibroblasts in connective tissues *in vivo* lack the contractile microfilamentous apparatus, or stress fibres, that are observed in myofibroblasts; rather, actin microfilaments are organized predominantly into a cortical meshwork. Similarly, highly organized fibronexus adhesion complexes are lacking at the surfaces of fibroblasts in connective tissues *in vivo*. So, in connective tissues *in vivo*, myofibroblasts are both morphologically and functionally different from fibroblasts.

Another characteristic of myofibroblasts is that, similar to SM cells, they are connected directly to each other through GAP JUNCTIONS. Functional gap junctions have been identified morphologically between myofibroblasts in dermal wounds[22], as well as between myofibroblasts that are derived from CORNEAL fibroblasts[23]. This indicates that myofibroblasts might form multicellular contractile units during granulation tissue contraction. Gap junctions are composed of several hemichannels in the plasma membrane of one cell that are joined in mirror symmetry with the same number of hemichannels in the opposing cell membrane. Hemichannels contain distinct but functionally related proteins called CONNEXINS. Not much is known about myofibroblast gap junctions; however, connexin 43 is expressed by myofibroblasts in breast-cancer

Table 1 | **Fibroblastic cells of normal organs with myofibroblastic features**

| Localization | Reference |
|---|---|
| Uterine submucosa | 139 |
| Reticular cells of lymph nodes and spleen | 140 |
| Intestinal pericryptal cells | 141 |
| Intestinal villous core | 142 |
| Testicular stroma | 14 |
| *Theca externa* of the ovary | 143 |
| Periodontal ligament | 144 |
| Adrenal-gland capsule | 145 |
| Hepatic perisinusoidal cells | 146 |
| Lung septa | 147 |
| Bone-marrow stroma | 148 |
| Capillary and venular pericytes | 33 |

PARENCHYMA
The essential or functional elements of an organ as distinguished from its stroma or framework.

EXTRACELLULAR MATRIX (ECM). A complex, three-dimensional network of very large macromolecules that provides contextual information and an architectural scaffold for cellular adhesion and migration.

STRESS FIBRES
Intracellular axial bundles of filamentous-actin and actin-associated proteins.

FIBRONECTIN FIBRILS
Extracellular fibrils that are composed of fibronectin, and are found primarily in tissues that undergo active morphogenesis, such as embryonic tissue and granulation tissue.

GAP JUNCTION
A junction between two cells that consists of pores that allow passage of molecules (up to 9 kDa).

CORNEA
The transparent, curved part of the front of the eyeball. It is made up of many layers (in particular a thick stromal layer), and refracts incident light onto the lens.

CONNEXIN
The main protein component of a connexon, the structural subunit of a gap junction. Six connexins make up one connexon.

EPIRETINAL MEMBRANE
A membrane that can sometimes form on the surface of the central portion of the retina when the vitreous gel that fills the central eye cavity shrinks away from the retina.

DIFFERENTIATION
The elaboration of particular characteristics that are expressed by an end-stage cell type or by a cell *en route* to becoming an end-stage cell. This term is not synonymous with commitment, but differentiation features are used to determine when a cell is committed.

FIBROCONTRACTIVE DISEASES
Connective tissue disorders, such as Dupuytren's disease, that involve both tissue contracture and fibrosis.

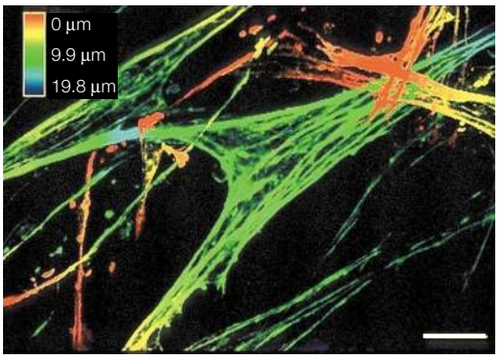


Figure 1 | **Myofibroblasts in epiretinal membrane.** Three-dimensional reconstruction by means of laser-scan confocal microscopy of a differentiated myofibroblast that is present in an EPIRETINAL MEMBRANE from a patient suffering from proliferative vitreous retinopathy. The membrane recovered at surgery has been stained *in toto* by means of immunofluorescence with an antibody against α-smooth muscle actin. The colours change as a function of the depth of the section. Differentiated myofibroblasts show typical stress fibres. Scale bar, 10 µm. Reprinted with permission from REF. 5 © 1999 Academic Press.

stroma[24] and by myofibroblasts that are derived from corneal fibroblasts[23].

Although myofibroblasts are clearly present in granulation tissue during wound closure and in pathological contracture tissues, questions have arisen as to whether they are essential for collagen/granulation tissue contraction. It is clear that tractional forces that are generated by fibroblasts as they migrate on a compliant substratum can reorganize collagen matrices[25,26]. In fact, in free-floating collagen lattices the resulting reduction in lattice diameter is due entirely to fibroblast tractional forces[27,28]. It has been proposed that tractional forces that are generated by fibroblasts migrating into granulation tissue are sufficient to result in wound closure, which makes the involvement of a specialized contractile cell unnecessary[28]. Although tractional forces that are generated by fibroblasts might be sufficient to initiate wound closure, the subsequent appearance of resistance in the surrounding tissue induces the DIFFERENTIATION of myofibroblasts[29], and only then can contraction be considered to start. Other studies have questioned whether granulation tissue, and thereby myofibroblasts, are even necessary for wound closure in animals that have relatively immobile skin, such as pigs and humans[30,31]. The ability of some models of immobile skin to contract without granulation tissue might reflect traction and contraction processes that can compensate for each other[30]. It now seems likely that both traction and contraction are important at different stages in wound healing[26] (discussed below), and questions now should focus on the detailed mechanisms.

Myofibroblasts, in addition to expressing the β- and γ-cytoplasmic actins that are found in fibroblasts, may express α-SM actin, which is similar to that expressed by SM cells[13,14]. This finding has led to the misconception that for a cell to be classified as a myofibroblast it must express α-SM actin. There are, however, several locations *in vivo* in which cells show the morphological characteristics of myofibroblasts, such as stress fibres, but do not express α-SM actin — for example, in the lung alveolar septa and the early phases of granulation-tissue formation[29,32]. Therefore, this shows that there are two types of myofibroblasts (TABLE 2): those that do not express α-SM actin, which we propose should be termed 'proto-myofibroblasts'; and those that do express α-SM actin, for which we propose the term 'differentiated myofibroblast' (FIG. 2). The distinction between fibroblast and proto-myofibroblast is particularly clear *in vivo*, whereas, when cultured on a plastic substrate in the presence of foetal calf serum, practically all fibroblasts acquire proto-myofibroblastic features. The presence of proto-myofibroblasts in certain adult tissues shows that they can persist and function as an independent cell type; however, proto-myofibroblasts can be induced to express α-SM actin and form differentiated myofibroblasts in response to specific factors, as occurs in certain adult tissues, granulation tissue and FIBROCONTRACTIVE DISEASES. The following two sections address what is known so far about the formation of proto-myofibroblasts and differentiated myofibroblasts.

### The formation of proto-myofibroblasts

The signals that are responsible for the formation of proto-myofibroblasts *in vivo* are just beginning to be understood. One crucial signal for the formation of the contractile features of the proto-myofibroblast is

Table 2 | **Fibroblast–myofibroblast features in different situations**

| Cell | *In vivo* | Closest *in vitro* model |
|---|---|---|
| Fibroblast | Normal connective tissue | Low stiffness substrates ± TGF-β<br>Free-floating collagen gels ± TGF-β |
| Proto-myofibroblast (stress fibres that express cytoplasmic actins) | Specialized normal connective tissue (for example, alveolar septum)<br>Early granulation tissue (for example, open wound) | Medium stiffness substrates |
| Differentiated myofibroblast (stress fibres that express α-smooth muscle actin) | Specialized normal connective tissue (for example, bone marrow stroma, liver capsule) | Medium stiffness substrates + TGF-β |
| | Late contracting granulation tissue (for example, open wound) | High stiffness substrates ± TGF-β |
| | Fibrocontractive diseases | Attached collagen gels ± TGF-β |

TGF-β, transforming growth factor β.

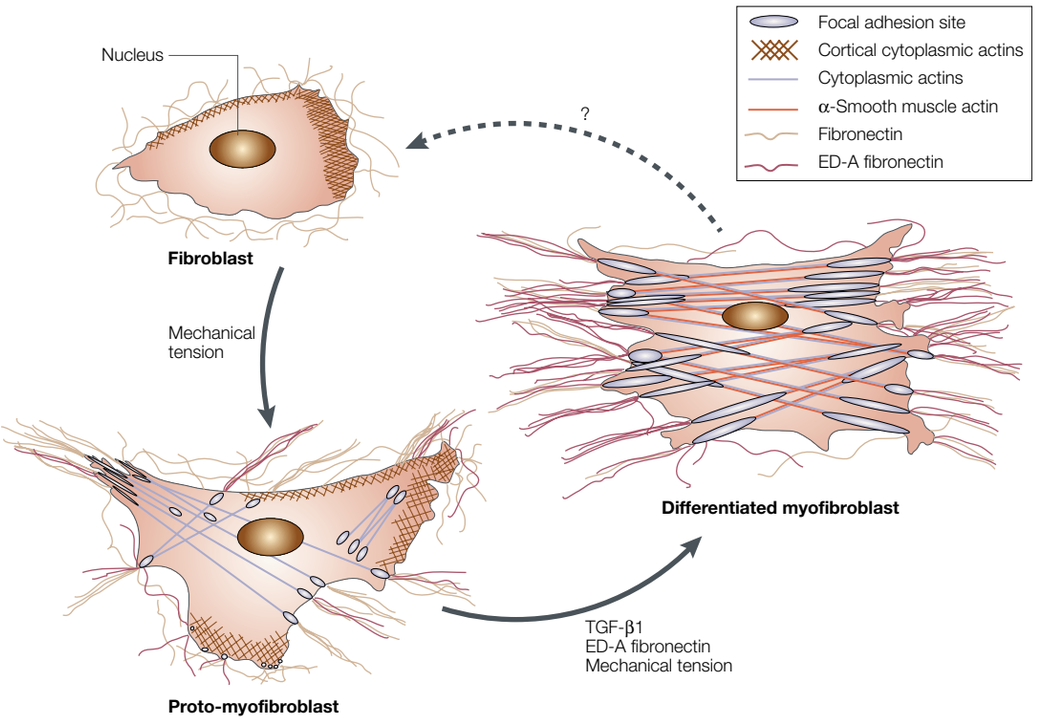


Figure 2 | **The two-stage model of myofibroblast differentiation.** *In vivo*, fibroblasts might contain actin in their cortex but they neither show stress fibres nor do they form adhesion complexes with the extracellular matrix. Under mechanical stress, fibroblasts will differentiate into proto-myofibroblasts, which form cytoplasmic actin-containing stress fibres that terminate in fibronexus adhesion complexes. Proto-myofibroblasts also express and organize cellular fibronectin — including the ED-A splice variant — at the cell surface. Functionally, these cells can generate contractile force. Transforming growth factor β1 (TGF-β1) increases the expression of ED-A fibronectin. Both factors, in the presence of mechanical stress, promote the modulation of proto-myofibroblasts into differentiated myofibroblasts that are characterized by the *de novo* expression of α-smooth muscle actin in more extensively developed stress fibres and by large fibronexus adhesion complexes (*in vivo*) or supermature focal adhesions (*in vitro*). Functionally, differentiated myofibroblasts generate greater contractile force than proto-myofibroblasts, which is reflected by a higher organization of extracellular fibronectin into fibrils.

GRANULOMA POUCH
A fluid-filled pouch that is created by subcutaneous injection of air and a necrotizing agent, such as croton oil, which later fills with fluid and becomes surrounded by granulation tissue.

PERICYTES
Support cells of capillaries (referred to as smooth muscle cells in larger vessels).

FOCAL ADHESIONS
Cellular structures that link the extracellular matrix on the outside of the cell, through integrin receptors, to the actin cytoskeleton inside the cell.

DERMIS
The innermost layer of the vertebrate skin that lies beneath the epidermis and is responsible for the tensile strength of skin. It comprises loose connective tissue that contains blood capillaries, smooth-muscle fibres, sweat glands and sebaceous glands with their ducts, hair follicles and sensory nerve endings.

mechanical tension. Fibroblasts populating the granulation tissue of a wound that has been mechanically stressed by splinting with a plastic frame form stress fibres — and, therefore, proto-myofibroblasts — earlier than normally healing wounds. Conversely, release of tension in wound granulation tissue and in the GRANULOMA POUCH leads to a gradual loss of stress fibres[29]. Similarly, in normal tissues, proto-myofibroblasts are always present where there is the need to generate mechanical tension[5]. In addition to mechanical tension, platelet-derived growth factor (PDGF) seems to be important in the formation of the proto-myofibroblast during embryonic development (for a review, see REF. 33) and wound healing in the adult (for a review, see REF. 34). In particular, capillary PERICYTES and alveolar proto-myofibroblasts are lost in *PDGF*-null mice[35,36]. Interestingly, although PDGF seems to be important in the formation of the proto-myofibroblast, it does not induce the expression of α-SM actin or the formation of the differentiated myofibroblast either *in vitro* or *in vivo*[37,38].

During normal wound healing in adult animals, fibroblasts initially migrate into the wound area and, as they do so, lay down a collagen- and cellular fibronectin-rich ECM[11,29]. With the initial closure of the wound, presumably owing to tractional forces that are developed by migrating cells, the collagen fibres and fibroblasts become oriented parallel to the wound bed and along expected lines of stress[11,29]. These fibroblasts acquire the proto-myofibroblast phenotype with stress fibres, FOCAL ADHESIONS and extracellular fibronectin fibrils[29]. In addition to increased levels of fibronectin production, fibroblasts also change the repertoire of fibronectin transcripts to resemble that found during early embryogenesis. In normal DERMIS, fibroblasts produce fibronectin messenger RNA, but these transcripts lack the two splice segments ED-A and ED-B[39]. However, during wound healing, these segments are included in the fibronectin mRNA, and lead to *de novo* expression of ED-A fibronectin in the granulation tissue, which is important to promote further myofibroblast differentiation[40].

Most of our understanding of proto-myofibroblast formation comes from *in vitro* studies. Fibroblasts that are isolated from various connective tissues or organs and placed into culture on plastic tissue-culture dishes rapidly acquire stress fibres, focal adhesions and fibronectin fibrils — that is, they acquire a proto-myofibroblast phenotype (TABLE 2). As the cell attempts

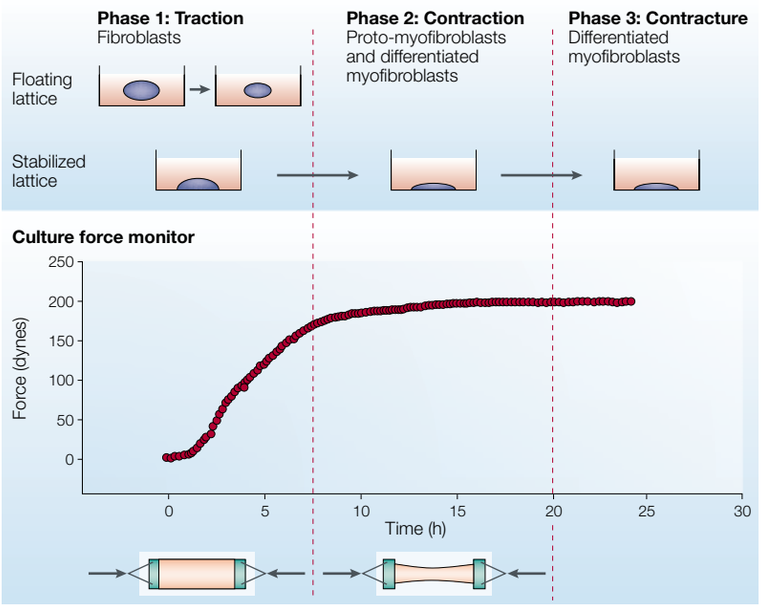


Figure 3 | **Three-dimensional collagen-lattice model.** Free-floating, fibroblast-populated collagen lattices that are untethered from the plastic culture dish are placed in culture media. As fibroblasts attach to the collagen fibrils, spread and migrate, they exert tractional forces (phase 1), which results in the slow compaction of the lattice. Mechanical tension cannot develop in untethered lattices, and fibroblasts do not acquire the proto-myofibroblast phenotype; free-floating lattices do not enter the contraction phase. Stabilized, fibroblast-populated collagen lattices are tethered to the underlying plastic tissue-culture dish. Fibroblasts reorganize the collagen fibrils through tractional forces that are parallel to the underlying plastic dish (phase 1). Mechanical stress begins to develop and cells align along lines of stress. The mechanical stress induces fibroblasts to become proto-myofibroblasts and acquire their characteristic features, including stress fibres, fibronexus adhesion complexes and fibronectin fibres (phase 2). If transforming growth factor β1 (TGF-β1) is present, proto-myofibroblasts can form differentiated myofibroblasts. Proto- and differentiated- myofibroblasts can exert contractile force on the extracellular matrix, which defines the contraction phase. The culture force monitor allows force measurement of fibroblast populations in uniaxially tethered collagen lattices. The force–time plot indicates how force increases linearly in phase 1 as fibroblasts exert tractional forces on the collagen matrix. As mechanical stress develops at the beginning of phase 2, fibroblasts become proto-myofibroblasts and differentiated myofibroblasts. These cells generate contractile force that is maintained relatively constant. The lower diagrams illustrate collagen lattices (tan) connected to floating plastic bars (green). One bar is fixed and the other is connected to a strain gauge to measure force. As force develops, the collagen lattice acquires an hourglass shape.

to pull against points of tight adhesion to the rigid substrate, tension develops and this causes the alignment of individual microfilaments and the recruitment of more filaments to the bundles, as well as formation of focal adhesions[16,18,26,41]. High extracellular tension is a key stimulus for fibroblasts that are grown on plastic to differentiate into proto-myofibroblasts, as fibroblasts cultured on compliant planar polymer substrates do not form stress fibres[42].

On rigid planar substrates, fibroblasts produce an ECM that contains cellular fibronectin that includes the ED-A splice variant[18]. It is noteworthy that during the early steps of wound healing (3–5 days after wounding), the fibroblasts that accumulate in granulation tissue also contain stress fibres that are composed of cytoplasmic actins, and produce ED-A fibronectin[29]. These observations indicate that the presence of ED-A fibronectin might contribute to the formation of proto-myofibroblasts, but again this is also linked to an altered cytomechanical environment.

What happens to a fibroblast when it is placed into a three-dimensional ECM that provides an environment with very different mechanical properties to a non-deformable, planar substratum? At present, three-dimensional hydrated collagen lattices provide the most potent model for examining *in vitro* the reciprocal mechanical interactions that occur between cells and the ECM, and how these can influence the formation and function of the myofibroblast[3,26,43]. In the earliest, and still the most widely used, version of this model, the cells are embedded within the collagen matrix, which floats freely in tissue-culture media[26,27,44] (FIG. 3). The diameter of these lattices is reduced over time (hours to days) by the small tractional forces that are generated by fibroblasts during attachment, spreading and migration on collagen fibres. However, such unrestrained gels are poor models for mechanically regulated processes, because in a living organism almost all tissues are tethered in such a way that cell contraction will inevitably increase stress in the surrounding matrix. This feedback mechanical signal is missing in floating collagen lattices, and resident fibroblasts therefore fail to acquire a proto-myofibroblast phenotype[26,45–48].

Endogenous tension (also called pre-stress) can be generated in the collagen matrix by tethering the lattice[26,43,45]. Under these conditions, tractional forces begin to compact the collagen fibres; however, as the collagen fibres are tethered, tension develops, which reflects the *in vivo* situation. Direct force measurements by various methods have shown that there is a linear rise in the force generated by fibroblasts in stabilized collagen lattices that closely correlates with cell attachment, extension and spreading of processes, and cell motility[49–52] (FIG. 3; phase 1). As tension develops, the collagen fibres and the cells begin to align parallel to the principal strain in the collagen lattice[4]. Once this pre-stress is established, it is maintained at a relatively constant level over long periods of time (FIG. 3; phase 2). As phase 2 is reached, the fibroblasts acquire the proto-myofibroblast phenotype and form stress fibres, adhesion complexes and fibronectin fibrils that are composed of both plasma fibronectin from the culture serum and secreted cellular fibronectin[26,43,45].

The maintenance of the proto-myofibroblast phenotype requires a continuous interaction between cell-generated stress and the reaction of a substratum that is sufficiently stiff to resist this force. If the collagen lattice is released from its points of attachment — such that stress in the matrix is lost — the cells rapidly contract and subsequently lose their stress fibres and fibronexus adhesion complexes[43,45]. Similarly, stress fibre organization is lost when fibroblasts that are cultured on a stiff planar substratum and show the proto-myofibroblast phenotype are treated with inhibitors of actin-myosin force generation[53].

### Formation of differentiated myofibroblasts

Once proto-myofibroblasts have developed in response to mechanical stress, they can be stimulated to develop into differentiated myofibroblasts.

Box 2 | **Corneal wound healing**

**Similar to excisional cutaneous wounds, α-smooth muscle (SM)-actin-containing differentiated myofibroblasts form in corneal wounds, where they generate contractile forces that result in wound closure[133,134]. As the cornea is an avascular tissue, these force-generating cells probably form from the surrounding differentiated corneal stromal fibroblasts, which clearly shows a non-SM origin for these cells in this tissue[135]. However, on the basis of new knowledge of adult MESENCHYMAL progenitor (or stem) cells, we cannot exclude other origins based on plasticity of such uncommitted cells *in vivo*. In addition, proto-myofibroblasts are present adjacent to the wound in the undamaged corneal stroma[134]. Like differentiated myofibroblasts from other tissues, these corneal myofibroblasts have large vinculin-containing focal adhesions and express high levels of the fibronectin receptor $\alpha_5\beta_1$ (REF. 136). The differentiation of myofibroblasts from corneal fibroblasts seems to require transforming growth factor β1 (TGF-β1) and the assembly of cellular fibronectin and its interaction with the cell surface[137], which is similar to what has been described for dermal fibroblasts[5]. Interestingly, cell density is also important for corneal fibroblast differentiation in response to TGF-β1 (REF. 138).**

Differentiated myofibroblasts can be distinguished from proto-myofibroblasts by the *de novo* expression of α-SM actin and the increased expression of ED-A fibronectin, as well as increased assembly of stress fibres and focal adhesions that become more and more complex. Many experiments and clinical observations[54,55] have shown that TGF-β1 has a key role in stimulating all of these characteristics of the differentiated myofibroblast both *in vitro* and *in vivo* (see BOX 2 for a discussion of TGF-β1-stimulated myofibroblast differentiation in the cornea). TGF-β1 in damaged tissue could be derived from PLATELETS, white blood cells (particularly MACROPHAGES) or parenchymal cells[54–59]. Autocrine production of TGF-β1 by fibroblasts is of great importance in preserving the fibrogenic activity once the inflammatory stimulus has ceased[60,61]. Injured epithelial cells can also produce TGF-β1, and thereby contribute to paracrine fibroblast stimulation[62]. TGF-β1 has long been known to induce collagen synthesis by fibroblastic cells[63]; it has also been shown to enhance the production of plasminogen activator inhibitor-1 (PAI-1)[64], the expression of cellular fibronectin — particularly the ED-A splice variant[65] — and, more recently, the expression of α-SM actin[15,54,55].

Although several studies have been carried out to investigate the signalling events that are elicited by these single stimuli, it is not known which molecular mechanism integrates them to promote myofibroblast differentiation. TGF-β1 is generally released in a biologically latent form (L-TGF-β1), which is stored within the ECM through the L-TGF-β1-binding factor (LTF)[66]. After cleavage from LTF, TGF-β1 is converted to its biologically active form[67]. The binding of active TGF-β1 to the transmembrane TGF-β-receptors type I and II leads to the assembly of a receptor complex with protein serine/threonine kinase activity (for reviews, see REFS 68,69). The type I subunit of this complex then binds and activates a series of transcription factor proteins called SMADS[68]. These subsequently form a transcriptional complex with co-Smads to translocate to the nucleus and activate the transcription of target genes. TGF-β1 activation of the Smad signalling pathway is responsible for increased expression of PAI-1 (REF. 64). Maximum expression of collagen type I in TGF-β1-activated mouse HEPATIC STELLATE CELLS (differentiated myofibroblasts) requires Smad3; however, Smad3 is not necessary for TGF-β1-induced increased expression of α-SM actin in activated mouse hepatic stellate cells either *in vitro* or *in vivo*[70]. Similarly, increased expression of α-SM actin in response to TGF-β1 can occur in cultured mouse dermal fibroblasts that lack Smad3 (REF. 71). A TGF-β1 response element that has a sequence different to that known for Smad binding has been identified in the α-SM actin promoter and seems to be essential for expression of α-SM actin in both SM cells[72] and myofibroblasts[73]. How TGF-β1 activates expression of α-SM actin through this TGF-β1 control element is, as yet, unknown.

Inhibiting the interaction between the ED-A segment of fibronectin and the cell surface blocks TGF-β1-induced myofibroblast differentiation[40]. The molecular mechanism that underlies this action is not known, but the identification of the integrins $\alpha_4\beta_1$ and $\alpha_9\beta_1$ as specific receptors of the ED-A domain (L. van de Water, personal communication) might lead to further insights. ED-A fibronectin alone in the absence of TGF-β1 does not induce α-SM actin expression[40], so whether and how the downstream pathways that are activated by TGF-β1 and ED-A-fibronectin signalling converge remains unknown at present. Even in the presence of both factors, the myofibroblast phenotype is lost when mechanical tension is removed, as shown by tension-release of granuloma pouches and splinted-wound-granulation tissue[29]. However, increased mechanical tension in splinted wounds increases ED-A fibronectin and α-SM actin expression (FIG. 4) without changing the expression levels of TGF-β1. Although the effect of mechanical tension is apparently not transmitted by changes in the levels of TGF-β1, blocking

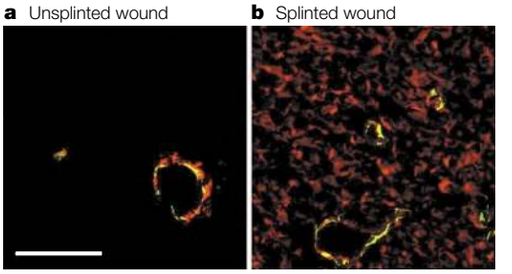


Figure 4 | **Mechanical tension induces myofibroblast differentiation in wound granulation tissue.** Protein expression in sections of 6-day-old granulation tissue from unsplinted wounds (**a**) is compared to that in splinted wounds (**b**). α-smooth muscle (SM) actin (red) is constitutively expressed by SM cells, and co-localizes (yellow) with desmin (green) in vessels. Enhanced mechanical tension by splinting wounds with a plastic frame results in the expression of α-SM actin by differentiated myofibroblasts in 6-day-old granulation tissue, which are absent in unsplinted wound tissue. Scale bar, 50 μm. Reprinted with permission from REF. 29 from the American Society for Investigative Pathology via the Copyright Clearance Center.

MESENCHYMAL STEM CELL
A cell from immature connective tissue — tissue that consists of cells embedded in extracellular matrix — that has the potential to divide and reproduce a replica cell as well as a differentiated progeny.

PLATELETS
The smallest blood cells, which are important in haemostasis and blood coagulation.

MACROPHAGES
Any cells of the mononuclear phagocyte system that are characterized by their ability to phagocytose foreign particulate and colloidal material.

SMADS
A family of transcription factors that mediate transforming factor β (TGF-β) signals. The term SMAD is derived from the founding members of this family, the *Drosophila* protein MAD (Mothers Against Decapentaplegic) and the *Caenorhabditis elegans* protein SMA (small body size).

HEPATIC STELLATE CELLS
Also known as perisinusoidal cells, these cells are normally quiescent, but when they are stimulated by several toxins, they differentiate into myofibroblasts, which results in liver fibrosis.

CArG BOX MOTIF
A CArG box motif — CC(A+T-rich)$_6$GG — is a DNA element that is required for muscle-specific gene transcription.

TGF-β1 in a stressed environment inhibits myofibroblast formation[29]. So, it seems that TGF-β1 is the central regulator of myofibroblast differentiation through its capacity to promote accumulation of intracellular contractile proteins, high collagen density and the ED-A fibronectin splice variant[40,54,63,65]. Interestingly, studies of wound healing during development have shown that foetal wounds heal without scarring. One reason for this might be the small amount of TGF-β1 in embryo and foetus compared with adult stages, with no apparent conversion of fibroblasts into myofibroblasts until after birth[74].

In addition to increasing the expression of α-SM actin, TGF-β1 dramatically enhances the assembly of stress fibres and the formation of the fibronexus adhesion complexes. This has been observed on both planar substrates[18] and in tethered three-dimensional collagen lattices[54]. A detailed study using fibroblasts that were plated on a planar substrate has shown that TGF-β1 administration induces, in addition to α-SM actin-rich stress fibres, the formation of mature and 'supermature' focal adhesions[18]. Compared with immature focal adhesions that are predominant in proto-myofibroblasts, both mature and supermature focal adhesions in differentiated myofibroblasts show *de novo* appearance of tensin and focal adhesion kinase (FAK), and an important increase in vinculin and paxillin content[18].

Therefore, myofibroblast differentiation depends both on the mechanical stress that develops within a given tissue and on the local expression of growth factors such as TGF-β1. In combination, these agents induce the gradual accumulation of α-SM actin-containing stress fibres as well as the other characteristics of the differentiated myofibroblast. We postulate that their concurrent action results in a positive feedback loop, in which tension facilitates TGF-β1 production and/or activation of α-SM actin expression, which, in turn, increases force production and tension development. These reciprocal interactions are probably important for the continued formation and sustained function of myofibroblasts.

## Myofibroblasts and smooth muscle cells

The expression of α-SM actin is regulated differently in differentiated myofibroblasts and SM cells. As discussed previously, the α-SM actin promoter contains a TGF-β response element that seems to be essential for the expression of α-SM actin in both myofibroblasts and SM cells[72,73]. The α-SM actin promoter also contains CArG response elements that seem to regulate the expression of α-SM actin differently in myofibroblasts and SM cells. Using transgenic mice, it has been shown that CArGs A and B are required for SM cells to express a transgene construct from –2600 to the first intron of the α-SM actin gene[75]. By contrast, CArGs A and B did not seem to be necessary for the increased expression of α-SM actin in cultured myofibroblasts, as analysed using transient transfection of 'decoy' oligonucleotides that were homologous to CArG A or B (REF. 73). In addition, the MCAT A and B response elements seem to repress α-SM-actin expression in SM cells, while enhancing expression in non-muscle cells[73,76]. Future studies should shed light on how these regulatory elements function differently in SM cells and myofibroblasts.

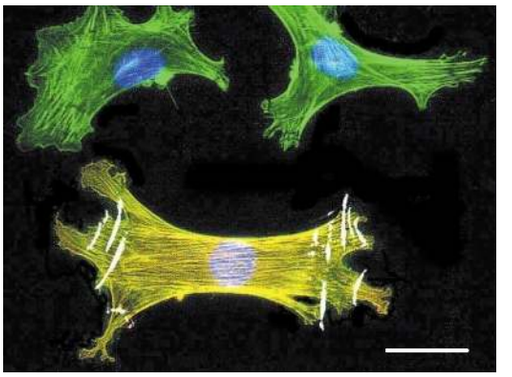

Figure 5 | **α-smooth muscle (SM) actin expression correlates with wrinkling on silicone substrates.** Rat lung myofibroblasts were cultured on a deformable silicone substrate of high stiffness and stained for α-SM actin (red) and filamentous-actin (green). Overlay with the phase-contrast image shows that α-SM-actin-negative proto-myofibroblasts (green) do not form wrinkles, which are restricted to α-SM-actin expressing differentiated myofibroblasts (yellow). Scale bar, 50 μm. Reprinted with permission from REF. 86 from the American Society for Cell Biology via the Copyright Clearance Center.

The expression of α-SM actin is at present considered to be the most reliable marker of the differentiated myofibroblast[5,6] (TABLE 2). In addition, differentiated myofibroblasts might express other markers of SM cells that correlate with more advanced differentiation, such as SM myosin heavy chains, caldesmon or desmin[5]. Expression of these markers varies depending on the context. For example, differentiated myofibroblasts in granulation tissue express only α-SM actin and no other SM-specific proteins, whereas a proportion of differentiated myofibroblasts in Dupuytren's disease may also express desmin and/or SM myosin[77]. Differentiated myofibroblasts do not express smoothelin[78,79], a recently described late differentiation marker of both vascular and parenchymal SM cells[80]. The expression of smoothelin is at present the most reliable criterion by which to distinguish between the differentiated myofibroblast, as defined here, and the mature SM cell[79]. During the evolution of vascular diseases, such as atheromathosis or restenotic phenomena, SM cells might lose differentiation markers, such as smoothelin, and become similar to myofibroblasts[79]. At present, it is unclear whether myofibroblasts and SM cells are distinctly different cell types or whether the myofibroblast represents a step of a continuous differentiation spectrum that exists between the fibroblast and the SM cell[7,78–80]. The existence of such a spectrum could explain vasculogenesis processes in which media SM cells can differentiate from local fibroblasts, as seen during pulmonary hypertension[81].

## Force generation and transmission

Granulation tissue can generate contractile force. This was first directly shown[82] by connecting a force

ISOMETRIC TENSION
A condition in which contraction of muscle, non-muscle cells or the actomyosin network is opposed by an equal load that prevents net shortening, even though tension increases.

ISOTONIC CONTRACTION
A condition in which contraction results in a shortening of muscle, nonmuscle, or the actomyosin network in order that tension remains constant.

transducer to the granulation tissue that formed in an excisional wound on the dorsum of a rabbit and measuring generation of ISOMETRIC TENSION. Moreover, studies have shown[29,82–85] that, contrary to connective tissue strips, granulation tissue strips placed into tissue baths contract on stimulation with SM cell agonists such as endothelin, angiotensin II and prostaglandin F1. Not surprisingly, granulation-tissue strips isolated from early wounds that contain only proto-myofibroblasts generate less force than do strips from later wounds that contain differentiated myofibroblasts[29].

When cultured in tethered collagen lattices, fibroblasts can acquire the proto-myofibroblast phenotype and undergo isometric contraction that is transmitted to the surrounding collagen matrix to maintain a relatively constant level of tension over long periods of time[50–52] (FIG. 3). If the collagen matrix is released from its points of attachment, the cells rapidly undergo ISOTONIC CONTRACTION and pull in the surrounding collagen fibres[43,45]. Treatment of proto-myofibroblasts in collagen lattices with TGF-β1 induces differentiated myofibroblasts to form, and dramatically enhances force generation that is correlated with an increase in expression of α-SM actin[36,54,86,87]. More and more evidence is accumulating in support of the hypothesis that increased expression of α-SM actin is sufficient to increase the capacity of myofibroblasts to generate force both *in vitro* and *in vivo*. Myofibroblasts that contain α-SM actin exert a stronger traction on stiff silicone substrates (FIG. 5) or on collagen gels compared with myofibroblasts that express only cytoplasmic actins[54,86,88]. Recent experiments[86] have shown that 3T3 fibroblasts that are stably transfected with α-SM actin cDNA contract collagen lattices significantly more than wild-type fibroblasts or fibroblasts that are transfected with α-cardiac or cytoplasmic actins. This contractile activity takes place in the absence of SM myosin-heavy-chain expression and without increasing the content of non-muscle myosin heavy chain. The cellular mechanism by which increased levels of α-SM actin might elicit increased force generation is not known. However, it has been shown that intracellular application of α-SM actin antibody or treatment with antisense oligonucleotides that target α-SM actin mRNA increase the motility of SM cells or myofibroblasts[89]. Moreover, the α-SM actin-specific antibody stimulates the polymerization of this isoform[90], and the introduction of the amino-terminal peptide sequence Ac-EEED (which comprises the epitope of this antibody) reduces spontaneous and induced contraction of myofibroblasts in culture, as well as granulation-tissue contraction *in vivo*[91]. These observations provide new information on the mechanism of tension production by the myofibroblast and represent a new strategy in preventing inappropriate wound contraction.

The presence of focal adhesions seems instrumental for force transmission[18,19,54]. Tissue fibroblasts and fibroblasts that are cultured on compliant substrates[42] or in the presence of contraction inhibitors[53] develop no, or only small (1 μm length), focal adhesion sites that are generally described as focal complexes[92] and that are analogous to the recently termed 'immature' focal adhesions[18]. These small, or immature, focal adhesions do not transmit measurable forces to the substrate[93] and are, together with mature focal adhesions, characteristic of proto-myofibroblasts. The size of mature focal adhesions (2–6 μm) and their protein composition correspond to the features of focal adhesions of fibroblasts that are cultured on planar stiff substrates, and which were recently summarized by Geiger and co-workers[94]. They are mainly situated in the cell periphery and typically contain β- and γ-cytoplasmic actins, (frequently also α-SM actin), $\alpha_v$ integrin, vinculin, paxillin, α-actinin, talin, FAK and tyrosine-phosphorylated proteins[18,95]. Such (mature) focal adhesions were recently shown to transmit an average force of 5.5 nN $\mu m^{-2}$ to a deformable substrate[93]. Treatment with TFG-β1 increases the number of mature focal adhesions and induces 'supermature' focal adhesions[18]. Supermature focal adhesions are typical of differentiated myofibroblasts; they are considerably longer (6–30 μm) than mature focal adhesions and always contain α-SM actin and tensin. They extend as continuous structures from the cell periphery into the cell centre and thereby differ morphologically from fragmented fibrillar adhesions that are usually defined on the basis of tensin expression[94]. In fact, supermature focal adhesions of cultured differentiated myofibroblasts resemble in many ways the recently described three-dimensional matrix adhesions of fibroblasts that were grown in tissue-derived three-dimensional matrices[96].

So far, the amount of force that acts through a single supermature contact has not been measured. However, as force transmission seems to be almost linearly proportional to the adhesion area[93], supermature adhesions might transmit considerably high forces, simply because they are larger. Moreover, different ratios between the constituents of mature and supermature focal adhesions[18] might lead to different structural and signalling properties. It has yet to be investigated whether supermature adhesions contain a specialized subset of integrins or other components. This is conceivable, as TGF-β1 — the main promoter of myofibroblast differentiation and maturation of focal adhesions — influences the integrin expression pattern of human fibroblasts[97–99].

## Regulation of myofibroblast contraction

Myofibroblasts can sustain a contractile force over long periods of time. This force is generated by contractile stress fibres — which are composed of bundles of actin microfilaments with associated non-muscle myosin and other actin-binding proteins[5,16,43] — and is regulated by myosin light-chain (MLC) phosphorylation, which is similar to that which occurs in SM cells[51,53]. Two kinase systems seem to regulate this phosphorylation: the calcium ($Ca^{2+}$)-dependent myosin-light-chain (MLC) kinase (MLCK) and the Rho-kinase systems[16].

RHO FAMILY GTPASES
Ras-related GTPases that are involved in controlling the polymerization of actin.

LYSOPHOSPHATIDIC ACID
Any phosphatidic acid that is deacylated at positions 1 or 2. It binds to a G-protein-coupled receptor, which results in the activation of the small GTP-binding protein Rho and the induction of stress fibres.

Increased concentrations of intracellular $Ca^{2+}$ will activate the MLCK pathway, which results in increased levels of MLC phosphorylation and contraction[16,100]. This contraction tends to be rapid and short-lived, as the increase in $Ca^{2+}$ is transient and active myosin phosphatase terminates the reaction by removing the phosphate group from MLC. In the Rho kinase pathway, RhoA — a small RHO FAMILY GTPASE — becomes activated in response to an appropriate agonist and can activate one of its target proteins, Rho kinase/ROKα/ROCK II (REFS 101,102). Activated Rho kinase can increase MLC phosphorylation by one of two mechanisms: first, by direct phosphorylation of MLC — however, the catalytic activity of Rho kinase is significantly lower than that of MLCK with regard to phosphorylation of MLC[103–105] — or second, by inactivating myosin phosphatase by phosphorylating the myosin-binding subunit of this enzyme complex[106,107]. The inhibition of myosin phosphatase by activated Rho kinase has been shown to promote the contraction of isolated stress fibres[108]. This pathway would result in sustained contraction, as the state of phosphorylated MLC would be maintained by inhibiting myosin phosphatase. Therefore, the two regulatory systems of MLC phosphorylation seem to be responsible for the two different modes of contraction: $Ca^{2+}$-dependent MLCK would be used to generate rapid contraction, whereas Rho kinase is important in maintaining sustained contraction.

The regulation of contraction of myofibroblasts is different to that described for SM cells. In SM cells, elevation of intracellular $Ca^{2+}$ is the main process through which an immediate and rapid increase in force is created. Inhibition of myosin phosphatase by Rho–Rho kinase sensitizes the cell to intracellular $Ca^{2+}$ levels[109]. In contrast to SM cells, the Rho–Rho-kinase pathway seems to be the predominant pathway that regulates myofibroblast contraction through regulated activity of myosin phosphatase, whereas elevation of intracellular $Ca^{2+}$ does not promote contraction of myofibroblasts within collagen lattices or granulation tissue[110,111]. Inhibition of Rho or of Rho kinase dramatically reduces the amount of force that is generated in response to LYSOPHOSPHATIDIC ACID by both proto- and differentiated myofibroblasts that are cultured within three-dimensional collagen lattices, whereas the phosphatase inhibitor calyculin — used to inhibit myosin phosphatase — can promote force generation[111]. Similarly, differentiated myofibroblasts in wound granulation tissue or in granuloma pouch do not contract in response to an agonist when treated with the Rho kinase inhibitor Y27632 (REF. 110).

The apparent fundamental difference between the regulation of SM-cell- and myofibroblast contraction might relate to their functional differences. SM cells need to contract and relax in response to environmental cues, whereas myofibroblasts need to continuously generate force over long periods of time. Maintenance of sustained contraction in myofibroblasts would be difficult to regulate by a $Ca^{2+}$-dependent system alone, as this would require $Ca^{2+}$ concentrations inside the cell to be finely tuned for long periods of time. In addition, myosin-phosphatase activity would be continuously removing the phosphate group from the MLC, so that high intracellular $Ca^{2+}$ concentrations would be required to maintain MLCK activity. By contrast, regulation of contraction by inhibition of myosin phosphatase through the Rho–Rho-kinase pathway provides an energetically more favourable mechanism for maintaining sustained isometric tension, as observed in myofibroblasts.

### Contraction and contracture

Although it is clear that myofibroblasts can generate and maintain contractile force, the question arises as to how this translates into the tissue shortening that is observed during wound healing and pathological contractures. In this context, it is essential to distinguish between connective-tissue contracture and muscle-based contraction. The terms are often used synonymously and their outward, gross anatomical appearances can seem identical; however, they are very different processes. Muscle contraction is a rapid and reversible shortening of tissues, which is dominated by intracellular events and energy expenditure. By contrast, connective-tissue contracture is a slow, (semi) permanent, low-energy, shortening process, which involves matrix-dispersed cells and is dominated by extracellular events such as matrix remodelling[112,113]. Viewing connective-tissue contracture simply as a result of muscle-like contraction of myofibroblasts is an oversimplification, and leads to the misconception that myofibroblast function is simple and muscle-like.

From our present understanding, it is reasonable to conclude that myofibroblasts are predominantly responsible for contracture (FIG. 6), whereas smooth (and skeletal) muscle cells act by contraction. Although some mechanical contraction is an inevitable part of the process of contracture, the reverse is not necessarily true. As Glimcher and Peabody[113] point out, gut or vascular SM cells contract connective tissues for decades without causing contractures. If myofibroblasts in scars or Dupuytren's

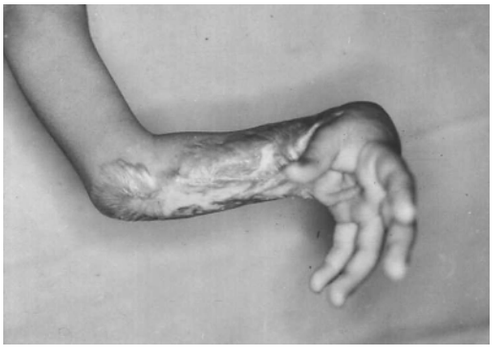

Figure 6 | **Scar contracture across the wrist following a burn injury.** Dermal scar tissue has the remarkable ability to continue to shorten a long time after healing. In this individual, the wrist is held in permanent flexion by this tissue, despite the opposing power of skeletal musculature in the arm (Courtesy of Dr. Jaysheela Mudera, University College, London).

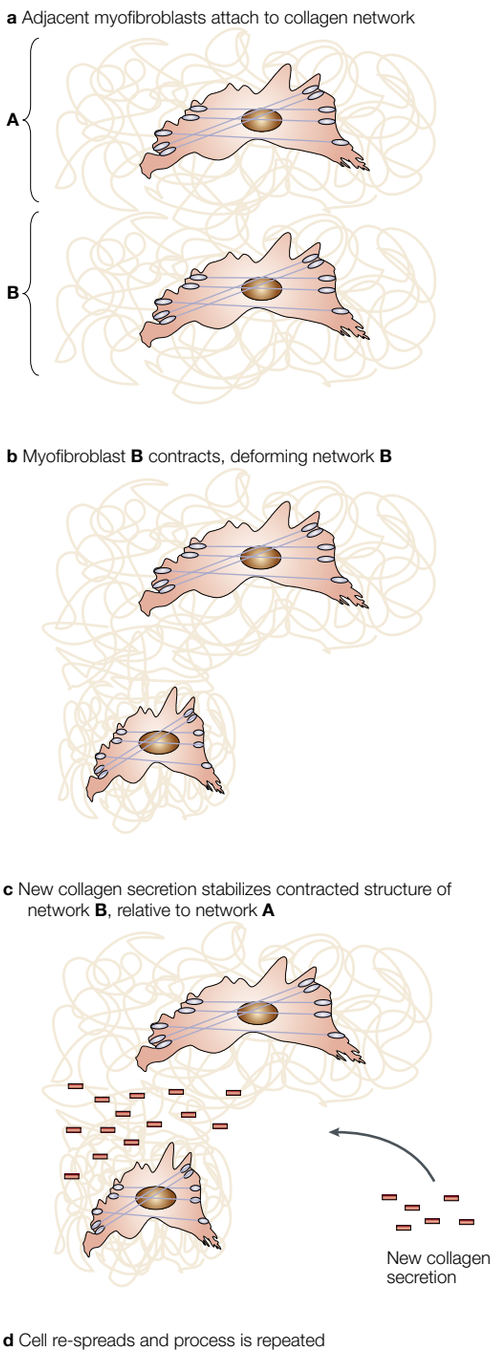


Figure 7 | **Model of extracellular-matrix-remodelling phase of matrix contracture.** Matrix contracture is mediated by myofibroblasts. How is the contraction of myofibroblasts translated into functional collagen-network shortening? The following working hypothesis serves to define some basic mechanisms. **a** | Myofibroblasts are embedded in a collagen network. These cells bind to collagen fibrils through fibronexus adhesion complexes that are linked to intracellular stress fibres. **b** | Cytoskeletal or signalling events in one of these myofibroblasts leads to stress-fibre contraction, which results in a local matrix contraction, shortening and bundling of the surrounding pericellular collagen network. As this is a local effect, its consequences are incremental, and affect at most a few surrounding cells and matrix. **c** | New matrix components are added to stabilize the new collagen organization, relative to its neighbours. The addition of collagen would potentially increase collagen density and orientation. **d** | The myofibroblast that contracted originally, as well as other surrounding myofibroblasts, can repeat the process so that this small incremental collagen-matrix remodelling can result in tissue contracture.

tissues had a similar mode of action to SM cells, their anatomical effects would mean that they would have to produce synchronous, prolonged contraction to oppose the tensile forces from massive skeletal-muscle blocks (FIG. 6). The implication that accumulations of myofibroblasts could achieve such an opposition is untenable on the simplest of energetic grounds. Instead, connective-tissue contracture involves incremental, anatomical shortening of the ECM material[114]. On an anatomical basis, it is clear that myofibroblasts do not need to generate enormous forces to temporarily deform their immediate collagen matrix, as skeletal muscles spend most time under low basal tension (for example, during sleep). Indeed, contractures of only a few tens of μm per day might occur. This argues against simultaneous or coordinated cell contraction, but rather supports an average effect of independent, local pericellular shortening events. These would be defined through cytomechanics rather than tissue mechanics. A similar misconception underlies the idea that cell cooperativity is needed to produce shortening in a particular plane. The force that is generated in tissues could be non-directional, as the pre-existing matrix shape and properties will largely dictate the resultant vectors. By analogy, the shape of a balloon is dictated by its envelope, but the inflating force is non-directional. Once achieved, such 'contracture' shortening does not require the continuing action of myofibroblasts as the shortened ECM restrains the surrounding tissues. The visible appearance of continuous tension in pathological contractures is simply a consequence of this changed anatomical relationship.

So, how is the contractile force that is generated by myofibroblasts translated into a shortened collagen matrix that no longer requires active cell contraction to maintain a tissue tension? So far, the mechanism for this has been ascribed simply to collagen crosslinking, but the permanency of this covalent bonding is irreconcilable with the dynamic nature of the process. Matrix shortening and increased stiffness indicates that the resident cells have locked a tension into the collagen structure, but in an interstitial, incremental manner[115]. This represents a 'slip and ratchet' theory for contracture (FIG. 7), but it can only realistically occur as a localized, dynamic and incremental process that is not consistent with massive breakdown, re-assembly and crosslinking. Related concepts in which collagen fibrils slip past one another locally and are then re-linked whereas adjacent fibrils are, in their turn, unbonded and slipped have been proposed[116–119]. It is noteworthy that in granulation tissue of a normal healing wound or in fibrocontractive diseases, collagen type I is replaced to a great extent by collagen type III, which is known to be present in remodelling tissues (for example, during development) or in normal tissues that are subject to mechanical stress[120] (for example, arteries).

Any remodelling process inevitably involves the removal of matrix molecules, and is largely mediated by matrix METALLOPROTEINASES (MMPs). Which MMPs are used will depend on the matrix components that

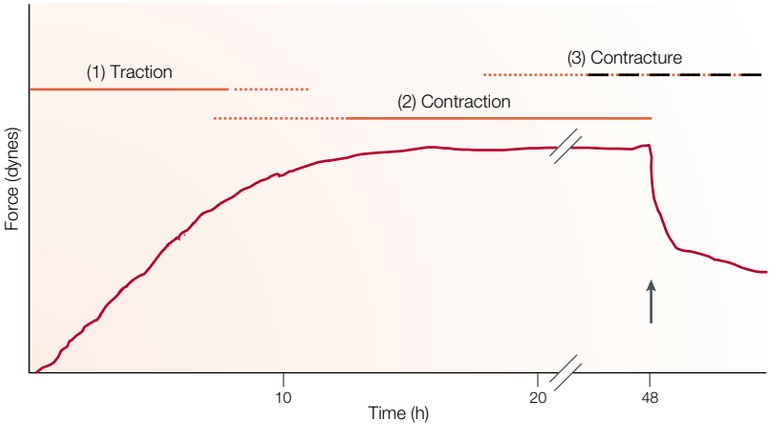


Figure 8 | **Contracture in three-dimensional collagen lattices.** The schematic force–time plot represents three phases of force generation in the culture force monitor. Uncertainty and overlap between the end of one phase and start of the next is indicated by the dotted sections of the bars. Addition of CYTOCHALASIN D shortly after the force plateau is reached in the contraction phase results in a total loss of force[51,52]. By contrast, addition of cytochalasin D much later in the putative contracture phase (arrow) results in residual tension remaining in the matrix (REF. 131; R. A. Brown and M. Marenzana, unpublished observations). This residual tension is due to irreversible remodelling-shortening of the collagen network, as would be expected with contracture.

need to be removed to allow slippage, and this is a key area of uncertainty. Several candidates have been considered in analogous models, including PROTEOGLYCANS such as decorin[121,122], collagen types V and XIV (REFS 123,124), and procollagen *N*-propeptide (itself dictated by levels of procollagen *N*-protease)[125], and non-collagenous proteins such as lumican and fibromodulin[126]. The identity of the MMPs that might be involved in the breakdown of linkage that is important in contractures is presently unknown, although combinations of, for example, MMP3, MMP2 and MMP9 would be expected to degrade proteoglycans and non-collagenous proteins. The simplest mechanism probably requires no inter-fibril linker, but relies on a collagenase, such as MMP1, to 'skim' the outermost layer of collagen, which mechanically separates two neo-fibrils. In this instance, re-fixing the fibrils after spatial rearrangement would need only an interposed layer of freshly synthesized collagen for re-crosslinking. Interestingly, this system would become dependent on the ubiquitous gelatinases (MMP2 and MMP9), as re-apposition of adjacent fibrils would require clearance of partly degraded collagen fragments from their surfaces[127].

The ability of the myofibroblast to generate and maintain contractile force is essential for tissue contracture. It is during the stage of ECM remodelling that the load that is normally carried by the ECM is now carried by the contracted myofibroblast. Once a new shortened matrix is laid down, the myofibroblast is stress-shielded by the load-carrying ECM (FIG. 7). The myofibroblast can either disappear by apoptosis[128,129] or contract again to continue the process of ECM shortening. It should be emphasized that although force generation is mostly mediated by the myofibroblast, it is possible that a second cell could remodel and lay down the shortened ECM. The limited understanding of the relationship between myofibroblast contraction and ECM remodelling makes it very difficult to define how this process occurs.

Some recent insights into the process of connective-tissue contracture might be emerging from the collagen lattice model as outlined in FIG. 8. This model identifies an *in vitro* form of contracture. This supports the importance of the changing material properties of the collagen matrix on overall cell and tissue function[130,131]. Recently, it has been shown that, with time, there is a progressive increase in matrix stiffness that occurs while the matrix is under tension[131]. As this occurs, the shortening/compaction of the collagen network ceases to depend on cell-generated forces, as the increased stiffness and ability to carry load is built into the matrix material and is present, even after the loss of cell contraction[115]. In terms of cytomechanics, this increase in stress occurs a result of the process of matrix contracture (FIG. 7). This mechano-remodelling of collagen is a key component of the cycle that links cytomechanical control, altered load transmission by asymmetric matrix structures and production/revision of that structure by resident cells, of which myofibroblasts and associated cell types are the central components.

## Conclusions and perspectives

Although the model that is summarized in FIG. 9 provides a framework for understanding connective-tissue contraction and contracture, several important aspects of this complex process remain to be determined. Some of those points are discussed here.

Although it is well established that mechanical stress can promote the assembly of stress fibres[26,43,53], little is known about how this signal is transduced to induce the early actin polymerization in fibroblasts after wounding and its association with myosin and other proteins to assemble these structures. The formation of specialized adhesion sites probably has an important role in this process. Recent work has indicated that Rho-kinase activity might be important for the generation of a relatively strong and long-lasting force by stress fibres[108], but many details still remain to be discovered about the regulation of contractile activity of these structures.

Mechanical stress is necessary for the formation of the proto-myofibroblast; however, whether TGF-β1 also has a role in promoting proto-myofibroblast formation remains speculative. It is clear that both TGF-β1 and ED-A-fibronectin interaction with the cell surface are necessary for the formation of the differentiated myofibroblast but the signal pathways that are activated by these interactions remain elusive.

Furthermore, although it has been shown that the amino-terminal sequence Ac-EEED of α-SM actin is important for α-SM actin polymerization[90], nothing is known of how expression of this specific actin isoform results in increased force generation.

Collagen shortening and remodelling is the key part of tissue contracture. At present, the mechanisms by

CYTOCHALASIN D
A fungal compound that specifically interferes with actin polymerization.

METALLOPROTEINASE
A proteinase that has a metal ion at its active site.

PROTEOGLYCAN
Any glycoprotein that contains glycosaminoglycans as the carbohydrate unit.

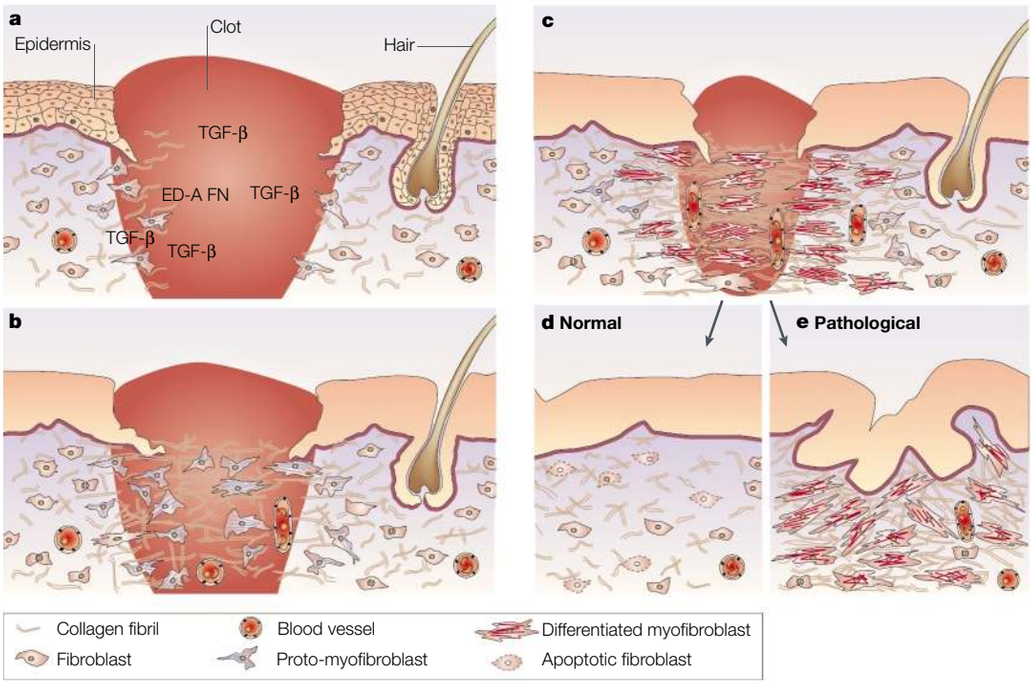


Figure 9 | **Model of the role of myofibroblasts during the healing of an open wound. a** | In normal tissues, fibroblasts experience a relatively low amount of tension owing to stress-shielding by the surrounding collagen matrix, such that the organization of a contractile cytoskeleton is not stimulated (light pink area of dermis). When a full-thickness dermal wound is filled by a fibrin clot, local growth factors stimulate fibroblasts from the adjacent intact dermis to invade this provisional matrix. These migrating fibroblasts, along with vessels, fill the wound, which results in the formation of granulation tissue. At this point, fibroblasts are already stimulated to produce ED-A fibronectin (ED-A FN). **b** | Migrating fibroblasts exert tractional forces on the collagen matrix, which results in its reorganization along lines of stress. The development of mechanical stress stimulates fibroblasts to develop stress fibres and to produce collagen, so they acquire the proto-myofibroblast phenotype. Tensional forces and growth factors stimulate proto-myofibroblasts to secrete transforming growth factor β1 (TGF-β1) and increased levels of ED-A FN. **c** | In a feedback loop, proto-myofibroblasts become differentiated myofibroblasts by synthesizing α-smooth muscle actin and generating increased contractile force. At the same time, differentiated myofibroblasts lay down collagen and other extracellular-matrix (ECM) components, and produce proteases. This complex process of remodelling results in shortening of the collagen matrix with corresponding wound closure. **d** | When a normal healing wound closes, myofibroblasts disappear by apoptosis and a scar is formed. **e** | However, in many pathological situations, such as hypertrophic scar formation, myofibroblasts persist and continue to remodel the ECM, which results in connective-tissue contracture. In conclusion, myofibroblasts, far from being a 'bad' cell type, are functionally essential cells. It is their dysregulation that is the cause of tissue dysfunction.

which a fibrillar-collagen matrix can be remodelled and shortened is unclear. Many reports have shown that proteolytic enzymes are expressed in granulation and fibrotic tissues (for a review, see REF. 34), but how their activity is coordinated with remodelling is not known. Similarly, it is unclear what effect the expression of different types of collagen that are observed in granulation tissue and scars have on collagen remodelling[34,120].

Finally, although it is clear that myofibroblast- and small-vessel disappearance from granulation tissue is mediated through apoptotic phenomena[128], again the factors that regulate these phenomena are at present unclear.

The analysis of the mechanisms that regulate the above-mentioned biological events will be important for the understanding of the biology of normal pathological wound healing, as well as for the development and the implementation of preventive and/or therapeutic strategies.


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