EXPERIMENTAL CELL RESEARCH 316 (2010) 2390–2401

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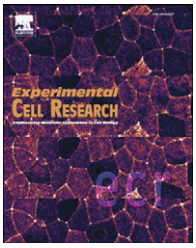

Review

Regulation of myofibroblast activities: Calcium pulls some strings behind the scene

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ARTICLE INFORMATION

Article Chronology:
Received 8 March 2010
Revised version received 27 April 2010
Accepted 30 April 2010
Available online 6 May 2010

Keywords:
Fibrosis
Rho kinase
Wound healing
Contraction
α-Smooth muscle actin
Stress fiber
Collagen
Mechanotransduction

ABSTRACT

Myofibroblast-induced remodeling of collagenous extracellular matrix is a key component of our body's strategy to rapidly and efficiently repair damaged tissues; thus myofibroblast activity is considered crucial in assuring the mechanical integrity of vital organs and tissues after injury. Typical examples of beneficial myofibroblast activities are scarring after myocardial infarct and repair of damaged connective tissues including dermis, tendon, bone, and cartilage. However, deregulation of myofibroblast contraction causes the tissue deformities that characterize hypertrophic scars as well as organ fibrosis that ultimately leads to heart, lung, liver and kidney failure. The phenotypic features of the myofibroblast, within a spectrum going from the fibroblast to the smooth muscle cell, raise the question as to whether it regulates contraction in a fibroblast- or muscle-like fashion. In this review, we attempt to elucidate this point with a particular focus on the role of calcium signaling. We suggest that calcium plays a central role in myofibroblast biological activity not only in regulating contraction but also in mediating intracellular and extracellular mechanical signals, structurally organizing the contractile actin–myosin cytoskeleton, and establishing lines of intercellular communication.

Contents

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Abbreviations: α-SMA, α-smooth muscle actin; $[Ca^{2+}]_i$, intracellular free cytoplasmic Ca^{2+} concentration; AJ, adherens junction; AT-II, angiotensin-II; ECM, extracellular matrix; ER, endoplasmic reticulum; ET-1, endothelin-1; FN, fibronectin; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PDGF, platelet-derived growth factor; ROCK, Rho-associated kinase; SMC, smooth muscle cell; TGFβ1, transforming growth factor β1; TRPC, transient receptor potential channel

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doi:10.1016/j.yexcr.2010.04.033

Introduction

The myofibroblast is a specialized cell for connective tissue remodeling. Myofibroblasts are critical for normal wound healing but deregulation of their remodeling functions is associated with fibrosis and pathological contracture. *A priori*, myofibroblasts contribute to the physiological repair of injured connective tissues such as skin, tendon, bone and cartilage. They ensure tissue integrity by forming a mechanically resistant scar, such as what occurs after a myocardial infarction [1]. Myofibroblasts form scars through two activities: enhanced production of a collagen-rich extracellular matrix (ECM) and organization of this matrix into a mechanically supportive structure, which is accomplished by the application of high contractile forces [1,2].

Towards the end of physiological wound healing, myofibroblasts normally disappear by apoptosis [3]. When myofibroblasts persist, the same activities that are beneficial for wound healing can lead to tissue deformities and loss of organ function [4]. This phenomenon relates to several important conditions that affect human health such as the hypertrophic scarring of skin seen in burn healing [5], systemic sclerosis [6,7], and Dupuytren's disease [8]. In organ fibrosis, myofibroblast-generated contractures lead to reduced organ function and failure of the liver [9–11], heart [12], lung [13–15], and kidney [16,17]. In novel therapeutic approaches that are currently being applied in regenerative medicine, there is a risk of tissue-delivered mesenchymal stem cells differentiating into myofibroblasts, which could lead to loss of regenerative potential and deterioration of fibrotic conditions [18,19]. Myofibroblasts that are activated by engrafted biomaterials [20] such as at breast implant sites, produce tissue contractures around the implant surface similar to those seen in hypertrophic scars, frequently leading to implant malfunction and ultimately failure. Further, myofibroblasts play a crucial role in stromal reactions to epithelial tumors by providing a chemical and mechanical environment that promotes tumor progression [21,22]. Clearly, identifying the molecules that regulate myofibroblast function would be beneficial for therapeutic control of wound healing, particularly if detrimental side-effects could be avoided. Previously we have reviewed the chemical and mechanical factors that govern the formation of myofibroblasts from different types of precursor cells [23–25]. Here, we focus on the mechanisms that regulate their contractile activity, a critical function that contributes to tissue remodeling and pathological tissue contracture.

A short history and characterization of the myofibroblast

The first description of myofibroblasts in granulation tissue and fibrotic lesions was based on morphological techniques [26]. Subsequently it was determined that tissues containing these cells could contract in a similar fashion as smooth muscle, which facilitated determination of their role in wound healing and development of fibrosis. Contractility was tested using organ baths containing tissue strips, similar to those used in classical experiments of muscle contraction [27–29]. The results from these experiments confirmed the contractile ability of experimental [27,28] and human [29] wound granulation tissue (Fig. 1A), Selye's granuloma pouch and fibrotic tissues from liver cirrhosis and nodules of Dupuytren's disease. In some instances it was possible to demonstrate the contractility of normal tissues that were under tension *in vivo* and that contained morphologically identifiable myofibroblasts (e.g. lung alveolar septa [30]). An interesting but unexplained result from these experiments is that myofibroblast-containing tissues from different origins (e.g. liver, lung) respond differently to specific agonists [28,29]. These data suggest functional heterogeneity of myofibroblasts, which has been previously described in various populations of fibroblasts [31–33]. One prominent example is myofibroblasts in the fibrotic liver that can originate from hepatic stellate cells and from portal fibroblasts, exhibiting different characteristics. In situ, the myofibroblast character of hepatic stellate cells appears to be reversible whereas myofibroblast differentiation of portal fibroblasts is suggested to be a one-way road, ending with apoptosis [9]. In standard culture, hepatic stellate cells almost instantaneously develop into myofibroblasts and cease to proliferate whereas portal fibroblasts continue to multiply [34,35]. It is likely, yet not studied, that the contraction physiology will also differ between myofibroblasts coming from such different backgrounds.

The capacity of myofibroblasts to develop high tension is closely related to their development of a muscular phenotype, which occurs in two main steps. In the first step, which is governed by the development of tension and occurs shortly after wound injury, proto-myofibroblasts are formed. These cells exhibit contractile stress fibers but do not express α-smooth muscle actin (α-SMA). It is important to state that proto-myofibroblasts are efficient in reorganizing the ECM of early wound granulation tissue and collagen gels under low mechanical stress; part of this reorganization has been contributed to the traction forces developed during cell migration [2,24,36]. In the second step, mechanical tension, transforming growth factor beta 1 (TGFβ1) and the extra domain-A (ED-A) portion of cellular fibronectin collaborate to enhance the formation of differentiated myofibroblasts. These cells are characterized by the *de novo* expression of α-SMA [2]. Notably, expression of α-SMA enhances the contractile forces generated by cells, probably because of a qualitative change in the organization and function of stress fibers instead of simply higher levels of total cellular actin content [37–39]. Indeed, over-expression of α-SMA enhances the contraction of 3T3 fibroblasts significantly more than the similar over-expression of α-cardiac, γ-, and β-cytoplasmic actin isoforms. This increased contraction occurs without changes in the expression of other contractile proteins, such as non-muscle or smooth muscle myosin [37].

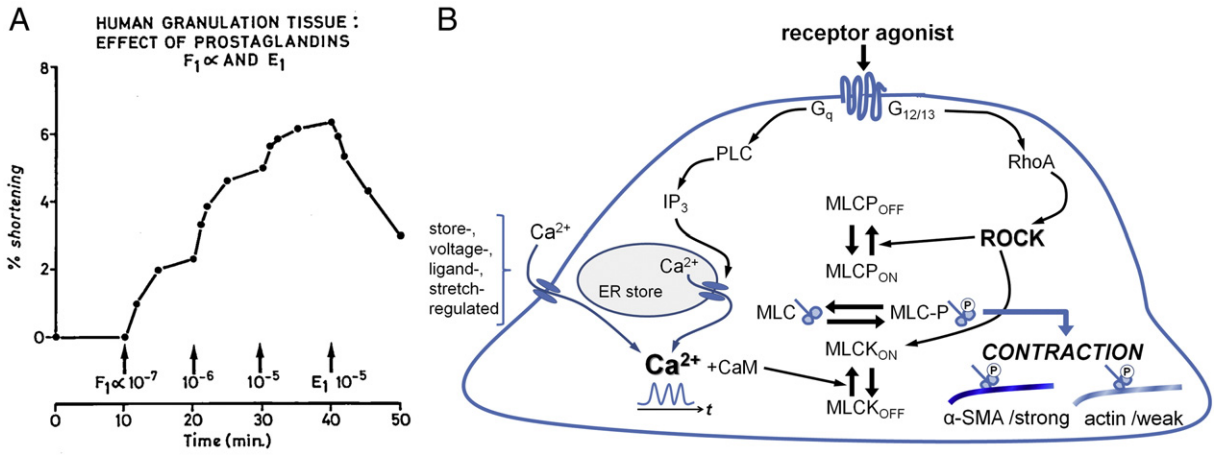

Fig. 1 – (A) Pioneer data on the contractile ability of human wound granulation tissue. Strips of granulation tissue behave similarly to smooth muscle in that they contract upon treatment with SMC contraction agonists (e.g. prostaglandin $F_1\alpha$) and relax with antagonists (e.g. prostaglandin E1) in an organ bath (drug concentrations in g/ml). Figure reprinted with permission from Ryan et al. [29]. (B) The two-pathway regulation of cell contraction. Smooth muscle and non-muscle cell contraction is promoted by phosphorylation of MLC (MLCP), which allows the interaction of myosin II with actin stress fibers. MLC phosphorylation can be achieved by two distinct pathways, Ca^{2+}/MLCK and RhoA/ROCK. As $[Ca^{2+}]_i$ increases, binding of Ca^{2+} to calmodulin (CaM) leads to the activation of MLCK which triggers MLC phosphorylation, leading to rapid and transient contraction. Conversely, activation of RhoA/ROCK leads to MLCP inactivation which maintains phosphorylation of the MLC, resulting in a long-lasting contraction. ROCK can also directly phosphorylate MLC. PLC, phospholipase C.

The molecular basis for the higher contractility exhibited by cells expressing α-SMA remains elusive. However, myofibroblasts have been shown to express higher levels of myosin light chain (MLC) kinase (MLCK) than fibroblasts from the same origin. MLCK may play a role in regulating the expression and contraction of α-SMA in stress fibers [40,41]. The contractile forces developed by stress fibers are efficiently transmitted to the surrounding environment at sites of well-developed connections with the ECM in the form of "mature" and "super-mature" focal adhesions [42,43]. Further, myofibroblasts develop intercellular connections (gap and adherens junctions, AJs) that appear to contribute to the coordination of myofibroblast populations during matrix remodeling [44].

When considering cell classification, myofibroblasts are situated at the interface between fibroblastic cells and smooth muscle cells (SMCs). Typically, fibroblasts express collagen type I, vimentin and non-muscle myosins whereas SMCs express α-SMA and can generate high levels of contractile activity. Depending on their origin and the specific *in vivo* or *in vitro* conditions, myofibroblasts may exhibit features that are closer to one or the other end of the fibroblast ↔ SMC spectrum. Closely related to this problem of cellular identity is the question of how myofibroblast contraction is controlled. An improved understanding of the mechanisms that control tension development by myofibroblasts has important therapeutic implications that will be discussed in detail below.

Fibroblast → myofibroblast → SMC: one cell type, one mode of contraction?

Importantly, the cellular basis for global tissue remodeling is the contraction of single myofibroblasts and their subsequent stabilization of tissues by secreted collagens and other ECM molecules. These processes result in irreversible retractile rather than reversible contractile phenomena [2]. The retractile nature of connective tissue remodeling implies that myofibroblasts exert a contractile activity which is somehow different from the classical Ca^{2+}-dependent contraction of SMCs. There are two principal pathways that act through various stimuli and agonists in both non-muscle cells and SMCs. These two pathways ultimately regulate phosphorylation of the MLC, which promotes actin–myosin contraction (Fig. 1B). In the first pathway, increased intracellular free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) results in Ca^{2+}/calmodulin-mediated activation of MLCK that in turn phosphorylates the MLC. Because phosphate is continuously removed from the MLC by the MLC phosphatase (MLCP), this type of contraction is considered rapid and transient. In the second pathway, activation of the small GTPase RhoA leads to activation of its downstream target Rho-(associated) kinase (ROCK or ROK). ROCK inactivates MLCP by phosphorylating its myosin-binding site [45]. Further, ROCK directly phosphorylates MLC, although less effectively than MLCK [46] (Fig. 1B). The net result of these activities is continued phosphorylation of MLC and persistent actin–myosin contraction. In the context of contractility, is it possible to attribute a distinct Ca^{2+} regulatory pathway to SMCs, fibroblasts, and myofibroblasts?

SMC contraction is generally described as being rapid, transient and mainly regulated by Ca^{2+}/calmodulin-mediated MLC phosphorylation via MLCK [47]. However, RhoA/ROCK-mediated inhibition of MLCP can generate a basal level of contractile tone in SMCs that tunes the $[Ca^{2+}]_i$ sensitivity of the contractile apparatus [48]. Deregulation of the RhoA/ROCK pathway in SMCs is associated with various cardiovascular disorders such as atherosclerosis, restenosis, arterial and pulmonary hypertension, and cardiac hypertrophy [49]. In fibroblasts, RhoA/ROCK-mediated inhibition of MLCP is considered to be the predominant pathway regulating contraction. This view is influenced largely

by the seminal works of Fujiwara and co-workers, who demonstrated contraction of isolated stress fibers after RhoA/ROCK stimulation in the absence of Ca^{2+} [50]. However, in a subsequent study, the same group demonstrated that contraction of a subpopulation of fibroblast stress fibers depends on Ca^{2+}/MLCK [51].

The regulation of contraction in myofibroblasts is controversial. Intuitively, the persistent contractures that characterize tissue repair and fibrosis are likely to involve sustained contractions by myofibroblasts. This notion suggests that regulation is mediated predominantly by RhoA/ROCK and by inactivation of MLCP [2]. Indeed, inhibition of MLCP with calyculin alone induces sustained contraction of wound granulation tissue strips whereas direct elevation of $[Ca^{2+}]_i$ by membrane depolarization and Ca^{2+} ionophore promotes only a low level of tension development [52]. However, the same study also showed that addition of angiotensin-II (AT-II) and serotonin stimulates rapid and transient granulation tissue contraction [52]. This finding confirms earlier studies that demonstrated contraction of myofibroblast-populated tissue strips in response to increased $[Ca^{2+}]_i$, which was mediated by several agonists including AT-II, endothelin-1 (ET-1), serotonin, vasopressin, and bradykinin [27,28,53,54]. For contraction of granulation tissue, the most potent contractile agonist is apparently ET-1 followed by AT-II [55].

Experiments using granulation tissue strips provide important information on the acute development of tension by myofibroblasts, particularly after pharmacological stimulation and/or inhibition. These types of contractions are generally reversible (Fig. 1A). Subsequent inhibition of myofibroblast contraction leads to measurable tissue relaxation (e.g. treatment with the N-terminal peptide Ac-EEED of α-SMA [38]). In contrast, the long-term contractile behavior of fibroblasts and myofibroblasts that leads to irreversible tissue contracture is usually not recapitulated by the granulation tissue strip model. Only one study has examined the long-term effect of MLCK modulation on wound contraction. In this report, application of calmodulin inhibitors delayed closure of full-thickness rat skin wounds [56]. However, cell contraction was not directly assessed and it is unknown whether the broad-spectrum inhibitors that were used affected (proto-myo)fibroblast migration/traction in the early phase of wound retraction or myofibroblast contraction in the contractile granulation tissue, or both. In addition, other cell types including inflammatory cells and vascular SMCs may be affected. In contrast to the visualization of $[Ca^{2+}]_i$ in SMCs of contracting blood vessels [57], $[Ca^{2+}]_i$ variations in single myofibroblasts have not yet been correlated with cell and tissue contraction *in vivo*.

Ca^{2+} signaling

Ca^{2+} signaling and its relationship to cell contractility have been intensively investigated in SMCs but there is much less data on Ca^{2+} signaling in fibroblastic cells *in vivo*. Several *in vitro* studies have demonstrated the importance of Ca^{2+} signaling in cultured fibroblasts. Intracellular Ca^{2+} is a universal second messenger in fibroblastic cells that affects proliferation, cell division [58,59], gene expression [60,61], cell differentiation [62] and collagen synthesis [63]. Some of the most intensively studied elements of Ca^{2+} signaling relate to its regulation of the organization of the cytoskeleton, including the formation of intercellular junctions [64], the formation and disassembly of cell–matrix adhesions [65,66], cell migration [67,68], and cell contraction and mechanosensing which are described below.

In eukaryotic cells $[Ca^{2+}]_i$ is controlled by Ca^{2+} entry across the plasma membrane and by release/uptake of Ca^{2+} from intracellular stores, such as the endoplasmic reticulum (ER) and mitochondria. Ca^{2+} flux across the plasma membrane is enabled in part by ATP-dependent pumps, Na^+/Ca^{2+} exchangers and by cation-permeable channels that can be regulated by mechanical stress, alterations of membrane potential, ligand binding to plasma membrane receptors or feedback from intracellular stores (Ca^{2+}-activated Ca^{2+} release). Ca^{2+} exchange between the cytosol and intracellular stores relies on uptake via ATP-dependent pumps and by ER channels that are gated by inositol trisphosphate and ryanodine [69].

Mechanical stimuli and contractile forces applied to cell membrane receptors induce Ca^{2+} entry across the plasma membrane and/or Ca^{2+} release from the ER in many cell types including osteoblasts [70], osteocytes [71], chondrocytes [72,73], mast cells [74], cardiac myocytes [75], endothelial cells [76], vascular SMCs [77,78] and fibroblasts [79]. Application of forces to certain cell types promotes intracellular Ca^{2+} release [80] that may rely on a direct physical connection between the ER and plasma membrane adhesion receptors [81]. The exact molecular identity of mechanically sensitive, cation-permeable channels in the plasma membrane is currently not defined. One candidate mechanosensor is the family of transient receptor potential channel (TRPC) proteins [82]. Notably, the mechanosensing properties of TRPC6 channels suggest a central role in regulating myogenic tone in SMCs in vascular tissue [83] and possibly in the regulation of cell contractility in fibroblasts. The mechanosensitivity of cation-permeable channels is highly dependent on the extent of polymerized actin in fibroblasts [84] and on the extent of cell spreading. While cell shape, which is very much affected by cell spreading, may have some effect on responsiveness to mechanical stretch, the rigidity of the cell membrane mediated by the extensive cortical actin network appears to be a central determinant in the regulation of stretch-induced calcium signals [85].

Integration of Ca^{2+} signaling between cells

The ability of fibroblasts to sense and respond to mechanical stimuli is important for cell contractility and for the translation of various types of mechanical stimuli into appropriate physiological outcomes. A coherent contractile response in whole tissues is very dependent on integration of cellular activities, which likely depends on the functional connectivity between populations of cells in the tissue. For example, sub-epithelial fibroblasts in the intestine may sense mechanical loading and, as a result of their formation of interconnected cells into a network, may contribute to the control of SMC contractions that are evident in peristalsis. Conceivably, the functional connectivity could be mediated by ATP released by mechanical stimulation. ATP stimulates the propagation of Ca^{2+} waves through the cell network via purinergic activation to regulate the peristaltic motility of the smooth muscle [86]. Mechanical stimuli can also promote release of ATP from cells that in turn acts on purinergic receptors to discharge Ca^{2+} from ER stores and induce Ca^{2+} wave propagation [87,88]. Other mechanisms by which networks of fibroblasts may be functionally

integrated in tissues involve the intercellular propagation of Ca^{2+} waves and the diffusion of inositol trisphosphate [89] and the formation of electrically coupled cells into an excitable syncytium [90] that generate oscillatory signals that could regulate contractile events.

An alternative, direct type of functional integration may involve the formation of AJs between adjacent fibroblasts. Cells in mechanically active environments form extensive, cadherin-mediated intercellular junctions that may be important in tissue remodeling, cell differentiation [91] and possible contractility. To examine the role of intercellular adhesions in mechanotransduction, experiments were conducted using N-cadherin antibody-coated beads attached to fibroblasts; cells were then subjected to mechanical tension. The data showed that N-cadherin mediates intercellular mechanotransduction by activating Ca^{2+}-permeable, stretch-sensitive channels [92]. Consequently, AJs may coordinate multi-cellular functions in generation and adaptations to mechanical forces. This role has been directly demonstrated in myofibroblasts: during the course of wound healing and in culture, fibroblasts and myofibroblasts couple their stress fibers between adjacent cells via cadherin-containing AJs [93,94]. Inhibition of OB-cadherin, expressed in myofibroblasts, reduces the contraction of attached/released collagen gels [93], suggesting a role of AJs in transmitting contractile forces between myofibroblasts. It was recently reported that subcutaneous myofibroblasts utilize mechanical AJ coupling to communicate between each other [95]. These findings implicate intercellular coordination of $[Ca^{2+}]_i$ oscillations, mechanosensitive channels and cell contraction [95] that was summarized in a model of intercellular mechanical coupling (Fig. 2). The Ca^{2+}-induced contraction of stress fibers in one myofibroblast is transmitted to the contacting cell at the sites of AJs. This leads to the opening of mechanosensitive channels in the plasma membrane, triggering a $[Ca^{2+}]_i$ increase in the second cell. The resulting contractile event can feed back to the first cell to maintain coordination and recruit other cells to form a cluster of coordinated cells. AJ coupling of stress fibers may coordinate tension distribution within the tissue and propagate intracellular mechanical signals, therefore improving overall connective tissue remodeling. In the following two sections, we will address the phenomenon of Ca^{2+} oscillations and how transient $[Ca^{2+}]_i$ changes can regulate fibroblast and myofibroblast contraction.

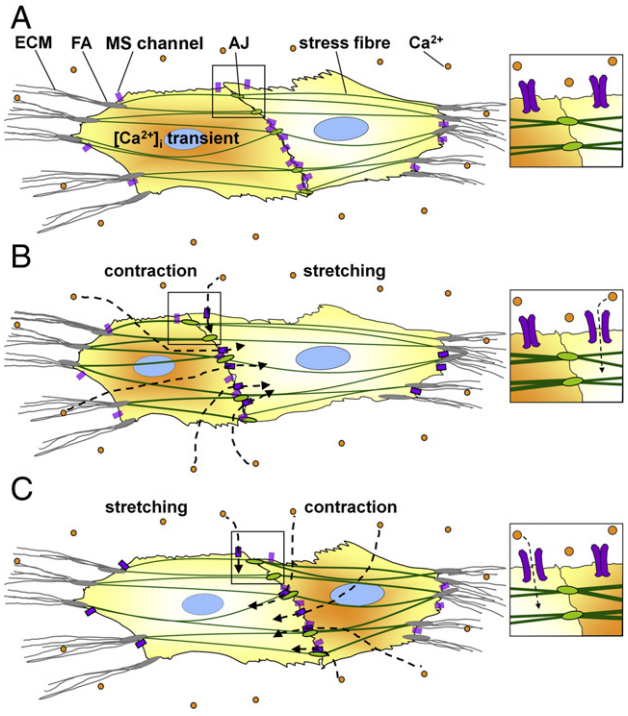

Fig. 2 – Model of mechanical communication between contacting myofibroblasts. (A) The α-SMA-positive stress fibers (green) of contacting myofibroblasts are connected to the ECM at sites of focal adhesions (FAs, grey) and intercellularly at sites of OB-cadherin-type AJs (light green). Mechanosensitive channels (MS channels, purple) in the plasma membrane are closed in relaxed cells (inset A) and do not permit entrance of Ca^{2+} ions (orange). Cytoskeleton-mediated or extracellular signaling events trigger a $[Ca^{2+}]_i$ transient (indicated by orange cytoplasm). (B) This rise in $[Ca^{2+}]_i$ leads to stress fiber contraction of the left cell (indicated by thicker fibers and cell shortening) that is transmitted to the right myofibroblast at sites of AJs. The induced stretch leads to the opening of MS channels (inset B). (C) The resulting influx of Ca^{2+} through open MS channels then triggers a contractile event in the right cell that again feeds back to the left myofibroblast. At this point, the cycle can start again.
Figure reprinted with permission from Follonier et al. [95].

Ca^{2+} oscillations

Actomyosin-based cortical contractility is a common feature of many types of eukaryotic cells including fibroblasts. Recent modeling based on oscillatory levels of $[Ca^{2+}]_i$ and control of contractile behavior [96] is generally consistent with the most current data [97]. Oscillatory contractile behavior may occur because of the antagonistic effects of Ca^{2+}-induced contractility and stretch-activated Ca^{2+} channels [84], which may lead to high amplitude $[Ca^{2+}]_i$ oscillations [96]. In the context of Ca^{2+} signaling leading to contractile events, it is notable that waves of intracellular Ca^{2+} can be triggered experimentally by mechanical stimulation of the plasma membrane in cultured rat sub-epithelial fibroblasts [86], ligament and tendon fibroblasts [98,99], and in rat subcutaneous fibroblasts and myofibroblasts [95]. Ca^{2+} signaling in response to mechanical stimulation can also be manifested in periodic $[Ca^{2+}]_i$ oscillations that have been observed after stretching human gingival fibroblasts [84] and by depolymerizing microtubules in 3T3 fibroblasts [100]. Periodic $[Ca^{2+}]_i$ oscillations with frequencies of 2–30 mHz also occur spontaneously in fibroblasts cultured from human heart ventricles [101], rat kidney [90], sub-urothelium [102] and subcutis [95], as well as in myofibroblasts cultured from rat subcutis and heart ventricles [95,97]. Spontaneous but irregular $[Ca^{2+}]_i$ oscillations have been observed in human gingival fibroblasts [103] and in myofibroblasts from human intestine [104] and heart valves [105]. Conceivably, spontaneous $[Ca^{2+}]_i$ oscillations are initiated by growth factors present in the culture medium, which has been demonstrated for platelet-derived growth factor-BB (PDGF-BB) in 3T3 cells and in human foreskin fibroblasts [61,106] and for

fibroblast growth factor-2 (FGF-2) in mouse cardiac fibroblasts [60].

Other potent inducers of $[Ca^{2+}]_i$ oscillations in fibroblasts are agonists of SMC contraction, including vasopressin in depolarized rat embryonic fibroblasts [107], prostaglandin F2α in rat kidney fibroblasts [108], bombesin in 3T3 fibroblasts [109], bradykinin in human foreskin fibroblasts [110], and ET-1 in rat sub-epithelial fibroblasts [111]. Similarly, ET-1 stimulates $[Ca^{2+}]_i$ oscillations in granulation tissue myofibroblasts [53], and histamine in human valvular myofibroblasts [112]. Although most of these studies did not systematically assess the functional consequence of $[Ca^{2+}]_i$ oscillations in terms of contraction, the generation of $[Ca^{2+}]_i$ oscillations by contraction agonists suggests a potential role in regulation of cell tension.

As myofibroblasts develop higher levels of tension than do α-SMA-negative fibroblasts, contraction-related Ca^{2+} signaling may be different between these cell types. This difference is difficult to define because current data are obscured by the fact that virtually all fibroblastic cells develop contractile stress fibers (i.e. features of proto-myofibroblasts) in standard culture conditions. Further, fibroblasts spontaneously differentiate into varying percentages of α-SMA-positive myofibroblasts depending on culture conditions, which is a rarely controlled yet important parameter. The few studies that systematically compared Ca^{2+} signaling between myofibroblasts and fibroblasts demonstrated distinct differences of Ca^{2+} regulation. Mechanical stimulation provokes a Ca^{2+} response in ET-1-induced sub-epithelial myofibroblasts but not in cells which are α-SMA-negative [86]. Similarly, activated hepatic stellate cells that acquire a myofibroblast phenotype on tissue culture plastic appear to be sensitive to PDGF-BB and not to ET-1 whereas quiescent hepatic stellate cells grown on Matrigel are responsive to ET-1 only [62]. Comparisons of the spontaneous $[Ca^{2+}]_i$ oscillations exhibited by fibroblasts and myofibroblasts from rat subcutis showed significantly higher frequencies in myofibroblasts [95]. Very recent works indicate that this difference of $[Ca^{2+}]_i$ oscillatory behavior may be related to different contractile activities, as described below.

The role of Ca^{2+} in regulating fibroblast and myofibroblast contraction

Progress in dissecting the mechanisms regulating myofibroblast contractile activity at the cellular and subcellular levels has been advanced by the development of two experimental approaches that have been successfully employed by several laboratories: 1) two-dimensional fibroblast culture on deformable substrates, and 2) three-dimensional collagen gel contraction. Culture of cells on deformable substrates, which is based on the elegant silicone "wrinkling" substrates developed by Harris et al. [113], permits direct observation of contraction and relaxation on a single myofibroblast. Refinements to this method, usually referred to as "traction force microscopy" using silicone elastomers or polyacrylamide gels, have been recently reviewed [114]. Stimulations with ET-1 and prostaglandin F2α [115], and also with AT-II and thrombin [116], trigger $[Ca^{2+}]_i$ transients and promote transient contraction of liver myofibroblasts on wrinkling substrates, suggesting Ca^{2+}-dependent contractility. These elastic substrates are particularly suitable for visualizing quasi-isometric tension development but cannot be used to assess cell-mediated remodeling because they are not biodegradable.

In contrast, collagen gels display a fibrillar ECM that is more likely to be remodeled by embedded cells. Collagen gels are also relevant models for exploring different phases of tissue repair, ranging from the low levels of tension seen in early stages of healing (simulated by floating gels) to later stages of healing in which scar tissue is more stressed and stiff (simulated by mechanically restrained gels) [117–120]. The mechanical state of the gel (i.e. relaxed or restrained) influences the mechanisms of remodeling by fibroblast migration, motility and contraction, and activates different signaling pathways [121,122]. Notably, the myofibroblast phenotype only develops in mechanically restrained gels [43]. Several studies using pre-stressed and then released collagen gels demonstrate that the Ca^{2+}-dependent pathway contributes at least partly to the regulation of myofibroblast contraction. Rat ventricular myofibroblasts contract collagen lattices after depolarization with KCl and after stimulation with TGFβ1 [123] or PDGF-BB [124]. Contraction induced by PDGF-BB is Ca^{2+}-dependent and involves MLCK, the Na^+/Ca^{2+} exchanger and L-type Ca^{2+} channels [124]. Further, ET-1-promoted contraction of attached/released gels by human colon myofibroblasts appears to be mediated by both Ca^{2+} and RhoA/ROCK [125]. Ca^{2+}/MLCK has been suggested to be the main pathway regulating collagen gel contraction by activated (myofibroblastic) hepatic stellate cells whereas Rho/ROCK contributes to Ca^{2+} sensitization [126]. Other studies indicate that Rho/ROCK/MLCP plays a leading role in controlling fibroblast collagen contraction [127–129]. The contraction of attached/released myofibroblast-populated gels is increased after stimulation of Rho/ROCK and is completely blocked by inhibition of Rho/ROCK. The intentional increase of $[Ca^{2+}]_i$ with ionophores does not augment gel contraction but inhibition of MLCK can block LPA-stimulated gel contraction [130]. This observation led to the hypothesis that activation of Ca^{2+}-dependent MLCK is required but is not sufficient for efficient myofibroblast contraction [2].

Assessing global collagen contraction after the release of pre-stressed collagen gels, as well as monitoring tension development in the whole cell population over time [127–129], highlights critical steps in contraction. However, this approach cannot be used to assess the contractile events that lead to persistent remodeling of the collagen matrix. Landmark studies from the Grinnell and Petroll laboratories described collagen transport with subcellular resolution, together with global collagen gel contraction. Local remodeling was assessed by tracking the movement of focal adhesions [131], collagen fibril translocation [132] and collagen-bound micro-beads [131,133]. Stress fiber contraction was assessed with GFP-tagged α-actinin [132]. Both groups demonstrated that fibroblasts undergo rapid, local changes in the subcellular pattern of force generation, which extended from the center to the periphery of the cell and which enabled local remodeling of collagen by repetitive centripetal translocations. As opposed to global remodeling, local collagen reorganization appears to be independent of Rho/ROCK in these models [133,134], suggesting a possible switch between ROCK-dependent and ROCK-independent contractile activity of stress fibers. The role of $[Ca^{2+}]_i$ was not directly assessed in these studies.

With a two-dimensional culture system it was recently shown that distinct elements of myofibroblast contraction are separately regulated by $[Ca^{2+}]_i$ and Rho/ROCK in the same cell [97]. By

simultaneously assessing: 1) $[Ca^{2+}]_i$ changes using Ca^{2+}-sensitive dyes; 2) subcellular stress fiber contraction by tracking linked ECM-coated micro-beads, and 3) whole cell contraction by recording wrinkling of deformable silicone substrates, it was revealed that contractions of bead-engaged stress fibers are mediated by variations of $[Ca^{2+}]_i$ whereas overall isometric cell tension is independently maintained through ROCK. By relating measurements of $[Ca^{2+}]_i$ to contractility (with atomic force microscopy) $[Ca^{2+}]_i$ oscillations were demonstrated to occur with a regular period of ~100 s in ventricular myofibroblasts and to control ~400 nm small and ~100 pN weak contractile events [97]. Based on these results, the earlier [2] proposed lock-step model for long-term tissue contracture was refined (Fig. 3): Rho/ROCK mediates strong isometric cell contractions, which promote slack in individual collagen fibrils. These fibrils, which are relaxed following release of tension, are gradually pulled towards the cell center by periodic $[Ca^{2+}]_i$-dependent micro-contractions until higher levels of tension in the fibrils are restored. The shorter ECM fibril configuration is stabilized by yet unexplored mechanisms and can again sustain mechanical loads. Stabilization and reorganization may include local collagen digestion, deposition of new collagen fibrils and cross-linking with the existent matrix. At this point myofibroblast isometric tension is no longer needed and cells are able to re-spread. Hypothetically, repetitions of this cycle will result in incremental and irreversible tissue contracture.

One important observation arising from this study is the apparent independence of contraction between different subsets of stress fibers, which is consistent with previous data of stress fibers in fibroblastic cells [135,136]. Assembly and contraction of stress fibers are differentially regulated in the central/ventral and peripheral/dorsal parts of the cell. ROCK maintains stress fibers in the center of cells whereas MLCK drives stress fiber assembly in the periphery. Notably, peripheral stress fibers contract more quickly after stimulation than do centrally located stress fibers [51,137]. One question that arises from these data is what determines whether a stress fiber contracts in a Ca^{2+}-dependent or independent manner? One determining factor may be the level of extracellular applied stress. Conceivably, fibers under low tension are particularly sensitive to variations of $[Ca^{2+}]_i$ whereas fibers under high levels of tension are more likely to contract under the influence of Rho/ROCK.

This hypothesis predicts that the localization of proteins involved in the regulation of contraction is stress-dependent. In this context, myosin, α-SMA, zyxin, and α-actinin have all been demonstrated to localize in stress fibers according to the level of applied mechanical load [138–141]. Interestingly, the distribution of myosin isoforms in spreading fibroblasts is spatially controlled; myosin IIA plays a major role in force generation, focal adhesion and stress fiber development in the whole cell, whereas myosin IIB controls static maintenance of tension and local force generation [142]. Currently it is unknown whether this distribution is stress-guided.

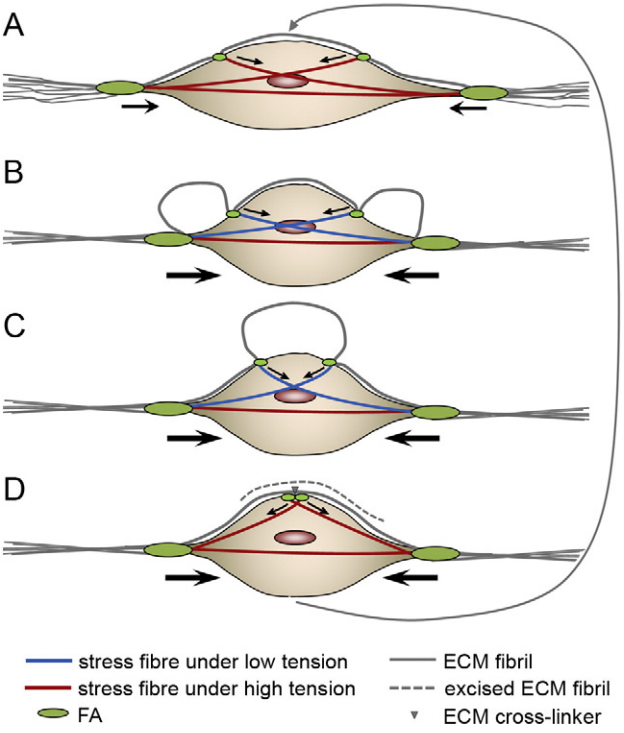

Fig. 3 – Lock-step model of ECM remodeling. (A) In a three-dimensional environment, stress fibers are connected to ECM fibrils (grey) through focal adhesions (FAs, green) and keep the partially remodeled ECM under tension. (B) ROCK-mediated global cell contraction (red stress fibers) shortens the bulk ECM and generates slack in individual ECM fibrils (grey loops). Such locally relaxed fibrils are now free to be contracted in a $[Ca^{2+}]_i$-dependent manner by low tension stress fibers (blue). (C) Incremental pulling events gradually shorten and stress the ECM fibrils. (D) Local proteolytic processing and stabilization of the fibrils by new ECM material and/or cross-linking (grey triangle) mechanically stabilize the remodeled ECM. Then, the cell can spread again to start a new cycle, while the ECM remains shortened. Figure adapted and reproduced with permission from Follonier Castella et al. [97].

Ca^{2+} and the regulation of the actin cytoskeleton

Cell contractility in fibroblasts is based on the function of actin–myosin systems, which in turn are highly dependent on the appropriate organization of actin filaments into arrays that will enable delivery of contractile forces through ECM adhesions to collagen fibrils. The turnover of actin filaments in response to environmental signals (including exogenous and endogenously generated mechanical stimuli) underlies a large group of other fundamental cellular processes such as mitosis, phagocytosis and cell motility. The bulk turnover of actin monomers is >100 times faster in cells than in pure actin solutions, which indicates a complex level of regulation *in vivo*. Much of this regulation is governed by actin-binding proteins, which play central roles in determining cell shape and stiffness [143], phagocytosis [144], matrix remodeling, and impact on several important medical conditions [145]. Actin-binding proteins determine actin-filament turnover [146] through the regulation of the relative sizes of actin monomer and filament pools, and the arrangement of filaments into higher order structures such as contractile stress fibers.

Collagen binding to cells and the processes of cell contraction involve the binding to integrins, which is often accompanied by a

transient increase in $[Ca^{2+}]_i$ [147]. Ca^{2+} signals may directly induce alterations of cytoskeletal structures. In addition, Ca^{2+} signaling may be modulated by other upstream signals including GTPases, protein kinases and proteins that form ion channels. The net effect of increased Ca^{2+} on the actin cytoskeleton is to depolymerize actin filaments and to promote the assembly of non-muscle myosin II filaments, contraction and change of cell shape [148].

Relatively little is known about the *in vivo* regulation of actin-binding proteins but a large body of *in vitro* data have implicated $[Ca^{2+}]_i$ as a critical determinant [148]. Here we will review just one structurally related class of actin-binding proteins that regulates actin-filament length by severing pre-existing filaments, capping the fast-growing end, or both [149], and which is regulated by Ca^{2+}. This family of actin severing proteins includes gelsolin, which is highly abundant in fibroblasts, widely expressed in mammalian tissues and is involved in cancer progression [150]. Notably, initial ligation of β1 integrins can activate Ca^{2+} permeable channels [151,152], which can lead to gelsolin-induced severing of actin filaments and enhanced β1 integrin clustering in response to collagen binding [153]. In gelsolin knock-out mice, fibroblast function is severely impaired [154], motility and wound healing are abnormal, and the mice display marked gingival fibrosis [155]. Cultured gelsolin null cells exhibit abnormally abundant stress fibers [156] and marked inhibition of phagocytic function [153]. Thus gelsolin is evidently important for the maintenance of normal connective tissue integrity but its role in mediating cell contractility is not yet defined.

Gelsolin is an 88 kDa, 6 segment (S1–S6) capping, nucleating and severing protein that binds two Ca^{2+} and has three binding motifs for actin: two recognize actin monomers (S1 and S4) and one attaches to the sides of filaments (S2) [157,158]. The minimal actin severing domain is S1–S2 but full nucleating activity requires S1–S6. Activation of gelsolin by physiological levels of Ca^{2+} occurs via Ca^{2+} binding to S6, which removes structural constraints that maintain the inaccessibility of the actin-binding sites [157,158]. Among the actin-modifying functions exhibited by gelsolin, filament severing has the most stringent Ca^{2+} requirement [159]. Half-maximal gelsolin severing and nucleation occur at 1–10 μM $[Ca^{2+}]_i$, indicating that gelsolin is activated in regions of high $[Ca^{2+}]_i$ such as near the channels of intracellular stores or subjacent to the cell membrane [148]. Gelsolin–actin complexes form transiently during Ca^{2+} and spreading-induced cell activation [160,161]. Activation is associated with the shuttling of gelsolin between membrane-bound and cytoplasmic sites [162] but virtually nothing is known regarding gelsolin shuttling or restriction to contractile stress fibers.

Conclusions

ECM remodeling by myofibroblast contraction is generally considered beneficial for wound healing but may be detrimental in fibrocontractive diseases. Conceivably, focal interference of Ca^{2+} signaling could be developed as a therapeutic strategy to control the function of myofibroblasts. By acting as a second messenger, as a "string puller" in the cytoskeleton or as a multi-cellular choreographer, Ca^{2+} evidently plays multiple roles in myofibroblasts that are not mutually exclusive. Recent studies combining observations of subcellular $[Ca^{2+}]_i$ dynamics with cell mechanics reveal a dual function of stress fiber contractions that follow periodic $[Ca^{2+}]_i$ oscillations in cultured myofibroblasts. Weak and transient contractions produce small but steady ECM movements that are mediated through ECM adhesions, and may also mediate mechanotransduction in neighboring cells via intercellular AJs. Moreover, Ca^{2+} locally reinforces the actin cortex at sites of force sensing and transmission in a mechanically controlled fashion. All of these activities appear to culminate in enhanced myofibroblastic contraction at the single cell level but also possibly at the whole cell population level. In spite of this progress there are many missing parts to the puzzle before envisioning a therapeutic strategy involving interference with Ca^{2+} signaling in myofibroblasts. Virtually no animal experimental data or clinical data are available on the subcellular regulation of Ca^{2+} in tissue myofibroblasts, despite the promising *in vitro* findings. Moreover, any Ca^{2+}-modulation strategy should be specific for myofibroblasts and target functions that are distinct from fibroblasts, SMCs and other cell types populating wounded and fibrotic tissues. Finally, although we here focused on the role of intracellular Ca^{2+} in regulating cell contraction and cytoskeletal features, other effects and targets of Ca^{2+} can profoundly influence the fibroblast–myofibroblast status. ATP-induced Ca^{2+}-waves were shown to induce the expression of pro-fibrotic genes, such as TGFβ1, collagen and fibronectin in cultured human pulmonary fibroblasts [163]. This is consistent with earlier findings of Ca^{2+} playing a role to regulate gene transcription in fibroblasts [60,61]. A similar regulatory function of Ca^{2+} has been reported for the expression of SMC-specific genes in vascular SMCs, including α-SMA and smooth muscle myosin heavy chain. This phenomenon has been termed "excitation–transcription coupling" and appears to be deregulated in certain vascular diseases [164]. It remains to be shown whether Ca^{2+} signaling directly regulates fibroblast-to-myofibroblast differentiation and whether its deregulation contributes to the development of connective tissue diseases. Compared to the large extant research on Ca^{2+} signaling in striated and smooth muscle contraction, the role of Ca^{2+} in fibroblasts/myofibroblasts is still a relatively unexplored field; new discoveries are keenly anticipated in the near future.

Acknowledgments

The work of BH and LFC is supported by grants from the Swiss National Science Foundation (SNF) (#3100A0-113733/1), the Novartis Science Foundation, the Connaught Funding Program, the Ontario Heart and Stroke Foundation (NA-7086), and the Canadian Institutes of Health Research (CIHR) (#210820). The work of CMcC is funded by the Ontario Heart and Stroke Foundation (NA-6736).

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