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The tension mounts: Stress fibers as force-generating mechanotransducers

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Stress fibers (SFs) are often the most prominent cytoskeletal structures in cells growing in tissue culture. Composed of actin filaments, myosin II, and many other proteins, SFs are force-generating and tension-bearing structures that respond to the surrounding physical environment. New work is shedding light on the mechanosensitive properties of SFs, including that these structures can respond to mechanical tension by rapid reinforcement and that there are mechanisms to repair strain-induced damage. Although SFs are superficially similar in organization to the sarcomeres of striated muscle, there are intriguing differences in their organization and behavior, indicating that much still needs to be learned about these structures.

Introduction

Shortly after the development of techniques for growing cells in culture, "tension striae" or "stress fibers" (SFs) were detected by light microscopy as dark lines or fibrils crossing many types of cells growing in culture (Lewis and Lewis, 1924). After some initial interest, these structures were neglected for many years and were even considered to be possible fixation artifacts. The application of electron microscopy to analyze cellular cytoplasmic organization renewed interest in SFs, as they were seen to be prominent structures in many cells in tissue culture (Buckley and Porter, 1967; Abercrombie et al., 1971; McNutt et al., 1971; Perdue, 1973). The speculation that SFs relate to striated muscle thin filaments composed of actin was confirmed by "decoration" with heavy meromyosin (Perdue, 1973). Research into SFs accelerated with the application of immunofluorescence microscopy, which made it possible to compare the organization of SFs in thousands of cells (Lazarides and Weber, 1974; Goldman et al., 1975; Lazarides and Burridge, 1975). In the nearly 100 years since they were first discovered, SFs continue to command attention. Not only are they often the most conspicuous cytoskeletal arrays in cultured cells (and as such are highly photogenic), but their ease of visualization has made them models for studying cytoskeletal assembly, organization, dynamics, and repair. SFs are load-bearing, tension-generating mechanosensitive structures. Their presence reflects a cell's response to the external environment, not only the soluble factors that drive assembly and disassembly but also the physical properties of the environment, such as its rigidity or compliance. In this review, we will discuss SF organization, how they assemble and disassemble, their dynamics, and their relationship to mechanical force as well as unresolved questions in the field.

Definitions

Initially, SFs were defined simply from their appearance as large bundles of actin filaments extending across much of a cell's diameter. Further analysis revealed that most SFs are anchored at one or both ends by focal adhesions and that the filaments are cross-linked by a periodic distribution of α-actinin that alternates with myosin II (together generating a sarcomeric appearance). Containing myosin, SFs were interpreted to be contractile, but shortening was rarely seen, leading to the interpretation that they are usually under isometric tension because of strong focal adhesion attachments preventing shortening (Burridge, 1981). Several types of SFs have been distinguished (Fig. 1; Small et al., 1998). The most commonly observed are ventral SFs; these are anchored at each end by focal adhesions. Ventral SFs are frequently many micrometers long and may extend most of the length of a cell. Dorsal SFs (also known as radial SFs) are usually shorter and anchored at just one end to a focal adhesion or focal complex. They are often precursors to ventral SFs and form just behind the leading edge of migrating cells, extending back toward the cell nucleus. In many migrating cells, as well as in spreading cells, contractile bundles of actin filaments develop at the base of lamellipodia. These "transverse arcs" display a convex shape and move away from the leading edge (Soranno and Bell, 1982; Heath and Holifield, 1993). Because they are not directly anchored at adhesions, they were not originally classified as SFs, although now they frequently are and

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Abbreviations used in this paper: GEF, guanine nucleotide exchange factor; ROCK, Rho kinase; SF, stress fiber; VASP, vasodilator-stimulated phosphoprotein.

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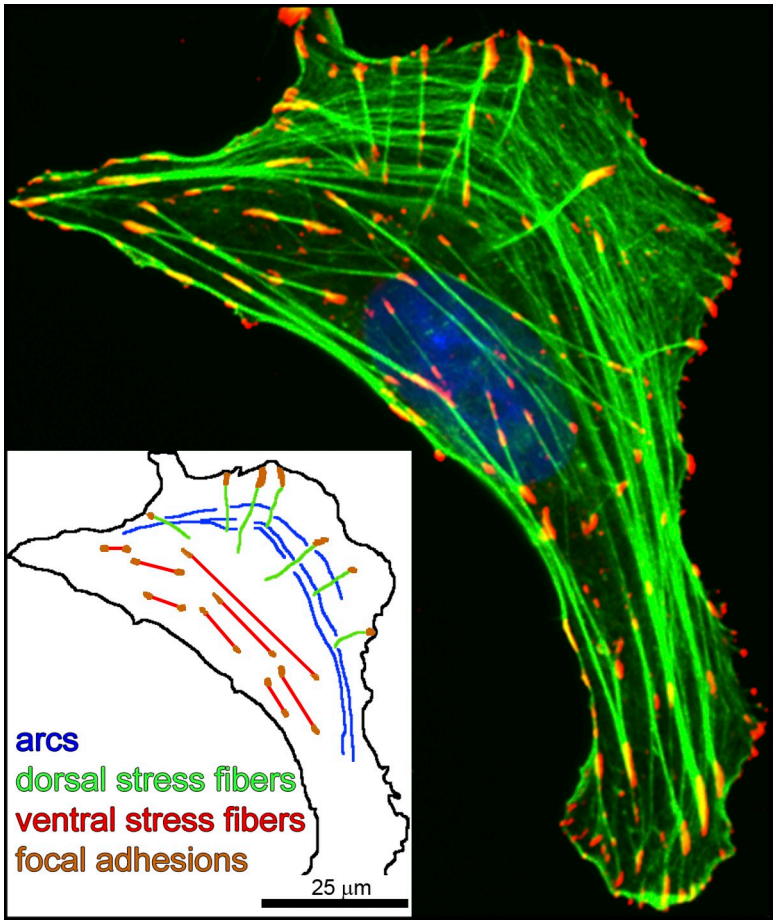

Figure 1. **Three types of actin SFs.** U2OS human osteosarcoma cells were plated on 10 µg/ml fibronectin-coated coverslips and allowed to attach and spread for 4 h before fixation (Hotulainen and Lappalainen, 2006). In the immunofluorescence image, antiphosphotyrosine was used as a marker for focal adhesions (red), phalloidin was used for F-actin SFs (green), and the nucleus (blue) was detected by DAPI. This single cell exhibits the three main types of actin SFs: (transverse) arcs, dorsal SFs, and ventral SFs. (inset) Schematic drawing depicting the SF subtypes.

will be here because, together with dorsal SFs, they give rise to ventral SFs (Hotulainen and Lappalainen, 2006).

Another type of SF is found in endothelial cells. These SFs are essentially identical to ventral SFs except that, rather than inserting into focal adhesions, they insert into the adherens junctions, linking endothelial cells together (Millán et al., 2010). In most resting endothelial cells, F-actin is predominantly found as a circumferential belt, similar to the apical belt in epithelial cells that associates with tight junctions and adherens junctions (Fig. 2 A). These cortical actin bundles generate centrifugal (outwardly directed) tension (Prasain and Stevens, 2009) that opposes the contractile forces exerted by SFs that form when, for example, RhoA is activated. Association of actin filaments with adherens and tight junctions in endothelia is critical for the regulation of barrier function (Lai et al., 2005). Junction-associated actin morphology is dynamic, thus allowing rapid permeability adjustments, transendothelial migration of immune cells during the inflammatory response (Adamson et al., 1999), and adaptation to mechanical forces, such as shear flow and vascular stretch (Noria et al., 1999; Birukov et al., 2002; Birukov et al., 2003; Wojciak-Stothard and Ridley, 2003). Barrier-enhancing agents, such as sphingosine-1-phosphate, cAMP analogues, and certain oxidized phospholipids, promote the formation of cortical F-actin structures, usually in a Rac1 and/or Rap1 GTPase-dependent fashion (Garcia et al., 2001; Birukov et al., 2004; Cullere et al., 2005). In contrast, RhoA signaling triggers junctional disruption. One mechanism for junctional disruption is the Rho kinase (ROCK)–dependent phosphorylation of endothelial tight junction proteins, such as occludin and claudin 5, which leads to blood–brain barrier breakdown (Yamamoto et al., 2008). Another is RhoA-associated myosin-driven contractility and SF formation; the tension transmitted to the junctions is an easily envisioned mechanism for enhanced permeability. There can often appear to be a physical connection between SFs of neighboring endothelial cells (Fig. 2 B). Because these SFs are anchored at adherens junctions, the junctions have been termed focal adherens junctions (Huveneers et al., 2012). Junctional SFs are increased in response to permeability-enhancing factors, such as TNF (Millán et al., 2010; Huveneers et al., 2012). It is noteworthy that, in highly polarized epithelia characterized by very tight junctions, cortical actin belts, not SFs, are the predominant apical junction–associated F-actin structures, indicative of their more static barrier properties. The interplay between actin filaments and cadherin-based junctions is reviewed in detail by Ratheesh and Yap (2012).

Myths and fallacies

The prominence of SFs in cells grown in tissue culture compared with their rarity in intact tissues has raised the question of whether they are in vitro artifacts. Under specific conditions, however, SFs are seen in tissues. For example, they were observed in wound granulation tissues (Gabbiani et al., 1972; Tomasek et al., 2002), in which they have been implicated in the contraction of wounds and scar tissue. They have also been noted in

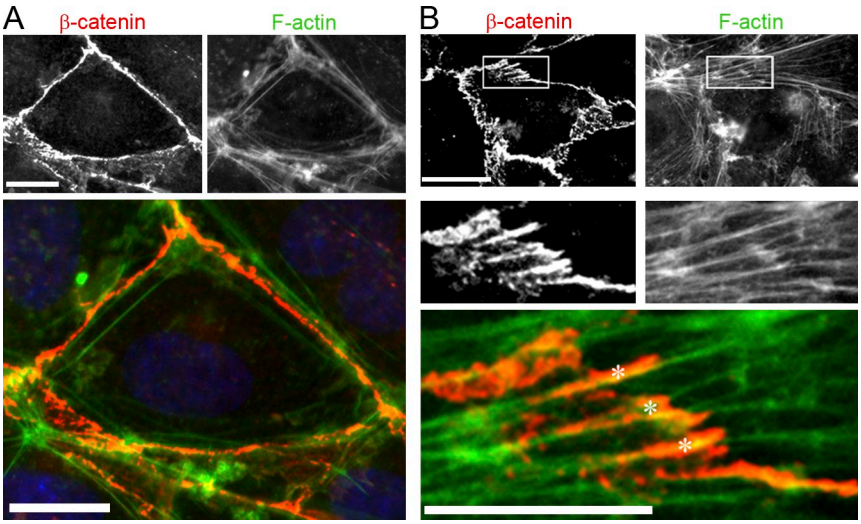

Figure 2. **Endothelial cell junctional F-actin structures.** Human umbilical vein endothelial cells grown as confluent monolayers were fixed and stained for β-catenin to identify cell junctions and phalloidin to label F-actin. (A) Example of cortical actin belts. This cell demonstrates a strong cortical enrichment of F-actin, arranged parallel to cell junctions. Bars, 15 µm. (B) Example of inserted junctional SFs. Another cell exhibits several discontinuous junctions (indicated by asterisks in enlarged merged image), where the insertion of SF ends can be observed. These junctions appear to physically connect SFs between two adjacent endothelial cells. Boxed areas show the area enlarged below. Bars, 25 µm.

endothelial cells, particularly those lining arteries exposed to high velocity flow, such as the aorta (Wong et al., 1983), and were increased in endothelial cells lining vessels from hypertensive animals (White et al., 1983). These different situations have in common that the cells are experiencing high mechanical forces. Supporting the idea that SFs are induced in response to mechanical signals, they could be experimentally induced by exposing cultured endothelial cells to high levels of sheer stress equivalent to those found in arteries but not in veins (Franke et al., 1984). Together, these results illustrate that although cells in the body rarely exhibit SFs, under appropriate conditions, many cells have the capacity to develop them.

A related question is whether SFs are a product of growing cells on two-dimensional surfaces, as it is rare to observe SFs in cells growing in a 3D matrix in vitro. For example, when fibroblasts are grown in soft collagen gels, they develop thin, elongated morphologies and lack detectable SFs. If the collagen gels are free floating, fibroblasts within them will contract the gels by as much as 90% in a few days. However, if the gels are firmly anchored to a rigid culture dish, the gels do not shrink in size even though tension is being generated. Under these conditions, the fibroblasts develop isometric tension, and now, SFs can be discerned (Mochitate et al., 1991; Tomasek et al., 1992; Grinnell, 1994; Halliday and Tomasek, 1995). Detaching the gels from their culture dishes results in rapid gel contraction that is accompanied by SF disassembly (Mochitate et al., 1991; Tomasek et al., 1992; Grinnell, 1994). These simple but elegant experiments indicate the importance of matrix compliance in determining whether cell–matrix interactions lead to isotonic or isometric contraction. Similarly, the rigidity of a two-dimensional surface together with strong adhesions to the surface can result in isometric contraction, in which SFs extending between essentially fixed focal adhesions generate tension but not shortening of their lengths (Burridge, 1981). The significance of soft versus rigid two-dimensional substrata was tested by Pelham and Wang (1999), who showed that cells developed SFs and focal adhesions on rigid surfaces but not on soft substrata. Together, these results reveal that the presence or absence of SFs reflects not whether cells are grown on two-dimensional surfaces or within three-dimensional matrices but rather whether the physical properties of the surrounding environment result in the cells generating isometric tension.

What is it about rigid substrata and the resulting development of isometric tension that leads to the assembly of SFs? A major factor is the activation of RhoA, which occurs when cells are cultured on rigid substrata (Wozniak et al., 2003; Paszek et al., 2005), and which, as discussed in the next section, drives the assembly of SFs. Integrins mediate attachment of cells to their underlying ECM, and although integrin-mediated adhesion initially depresses RhoA activity (Ren et al., 1999; Arthur et al., 2000), sustained adhesion activates several guanine nucleotide exchange factors (GEFs) to elevate RhoA activity (Dubash et al., 2007; Lim et al., 2008). Applying tension on integrins, as occurs with isometric tension, also induces RhoA activation (Zhao et al., 2007; Guilluy et al., 2011). Pursuing the mechanism has led to the identification of two signaling pathways, one involving the tyrosine kinase Fyn, resulting in activation of the GEF leukemia-associated Rho GEF, and the other involving the tyrosine kinase FAK (but not Fyn) and resulting in the activation of the Ras/MEK/ERK pathway, with ERK phosphorylation activating GEF-H1 (Guilluy et al., 2011). Heck et al. (2012) similarly identified GEF-H1 activation in cells adhering to rigid substrata but, in this situation, found that activation of GEF-H1 resulted from destabilization of microtubules and the consequent release of this GEF from these structures.

A role for SFs in cell migration has often been suggested or assumed. However, most highly migratory cells lack SFs, and the presence of SFs is correlated more with strong adhesion than rapid migration (Burridge, 1981; Herman et al., 1981). Nevertheless, in order for strongly adherent cells to move forward, it is necessary for them to break their strong adhesions in the rear. So, although there is a negative correlation between SFs and rate of migration, in situations in which cells are strongly adherent, SFs do contribute to migration by generating the force necessary to release the tail of the cell and move the rear forward (Crowley and Horwitz, 1995).

SF assembly

Two model systems have been used to examine how SFs assemble. In one, quiescent cells that are essentially stationary and often in a confluent monolayer are stimulated with agents that activate RhoA. In the other, the formation of SFs is studied in actively migrating cells. Different results and conclusions have been reached, consistent with the view that the mode of assembly is influenced by the migratory state of the cell. In the first system, exploited by Ridley and Hall (1992), cells, such as Swiss 3T3 fibroblasts, were starved of serum, i.e., deprived of growth factors, for a few hours. Under these conditions, preexisting SFs and focal adhesions disassembled. Synchronous reassembly was triggered by the addition of serum or agents, such as lysophosphatidic acid, that activate RhoA (Ridley and Hall, 1992). Using this system, in which ventral SFs are rapidly assembled, it was shown that active RhoA drives assembly by stimulating myosin activity (Chrzanowska-Wodnicka and Burridge, 1996). It was suggested that active myosin induces the aggregation of actin filaments into SFs, both by generating tension and by cross-linking the filaments (Chrzanowska-Wodnicka and Burridge, 1996; Burridge et al., 1997). In turn, integrins that have been dispersed during the period of serum starvation and low RhoA activity are recruited to form the core of the focal adhesion. In this model, SF formation was proposed to occur by aggregation of preexisting actin filaments, and relatively little actin polymerization was anticipated. Consistent with this, Machesky and Hall (1997) found only low levels of polymerization occurred during the formation of ventral SFs in quiescent cells in which RhoA had been activated. It was also speculated that the low affinity of integrins for their ECM ligands facilitated clustering of dispersed but ECM-bound integrins (Chrzanowska-Wodnicka and Burridge, 1996). Recent work supports this model but with an interesting twist. Evidence was presented that the association of the critical linker protein talin with integrins occurs in the focal adhesion but not in the dispersed state. Away from focal adhesions, other interactions presumably maintain the connection between integrins and F-actin (Rossier et al., 2012).

The second model system involves studying the formation of SFs as cells spread and migrate (Hotulainen and Lappalainen, 2006; Oakes et al., 2012). Elegant analysis by Hotulainen and Lappalainen (2006) revealed a critical role for actin polymerization in the de novo assembly of SFs in migrating cells. Using cells that displayed dorsal SFs, transverse arcs, and ventral SFs (depicted in Fig. 1), they were able to demonstrate an interplay between the assembly of these filament bundles as well as conversion of dorsal SFs and arcs into ventral SFs. Dorsal SFs grew by formin-mediated actin polymerization from small adhesions developing just behind the leading edge. As the actin bundles polymerized, they became associated with the filament cross-linking protein α-actinin; myosin was incorporated later into regions where α-actinin was depleted. Earlier work revealed that the region of an SF emerging from a focal adhesion is characterized by unipolar actin filaments with their barbed ends closest to the adhesion (Cramer et al., 1997). However, in order for myosin to incorporate and generate force, filaments with the opposite polarity need to be generated. We will discuss how this may occur later. In parallel with the assembly of dorsal SFs, transverse arcs arose from the region behind the lamellipodium and moved centripetally toward the nucleus. They appeared to assemble from the annealing of short bundles of filaments generated in an Arp2/3 complex–dependent process within the lamellipodium. Some of these bundles were associated with myosin, whereas others were associated with α-actinin. Over time, annealing of these bundles gave rise to a sarcomeric alternating pattern of α-actinin and myosin that is characteristic of transverse arcs and ventral SFs. Hotulainen and Lappalainen (2006) observed examples in which much of a transverse arc fused with dorsal SFs on opposite sides of a cell, resulting in an SF anchored at each end. Contractions of the curved, anchored transverse arc generated a straight ventral SF. They also provided examples in which two dorsal SFs growing from opposite sides of the cell fused to give rise to a ventral SF.

Since the work of Ridley and Hall (1992), RhoA has been recognized as a dominant factor regulating SF assembly. For ventral SFs, the critical pathway downstream from RhoA appears to be mediated by ROCK regulation of myosin light chain phosphorylation and consequent contractility (Amano et al., 1996; Chrzanowska-Wodnicka and Burridge, 1996; Kimura et al., 1996). The inhibition of cofilin-mediated F-actin severing downstream from ROCK and LIM kinase may also contribute to SF assembly induced by RhoA (Maekawa et al., 1999). RhoA activation of mDia driving actin polymerization is more critical for dorsal SF assembly (Hotulainen and Lappalainen, 2006; Oakes et al., 2012). Watanabe et al. (1999) showed that both of these arms of the RhoA signaling pathway are required to generate a "normal" pattern of SFs. They expressed constitutively active ROCK or mDia, or a ratio of these two effectors, in cells in which endogenous RhoA activity was inhibited. High ROCK activity in the absence of mDia induced large condensed SFs, often exhibiting a contracted starlike configuration. In contrast, high mDia activity without ROCK activity induced the assembly of many thin filaments that were organized in a sheetlike array with few, if any, bundles being seen (Watanabe et al., 1999). These results demonstrate that the pattern of SFs seen in a particular cell reflects not only the level of RhoA activity but also the ratio of these different effectors. They also confirm the importance of myosin as a protein that cross-links actin filaments into bundles (Chrzanowska-Wodnicka and Burridge, 1996), which has also been shown through the use of myosin mutants that retain actin binding but which are unable to generate force (Choi et al., 2008).

In migrating cells, dorsal SFs emerge from focal adhesions, but contrary to the prevailing dogma, Oakes et al. (2012) have provided evidence that the tension they generate is less important in adhesion maturation than their role as structural templates. When tension was reduced by as much as 80%, adhesions matured as judged by their growth and accumulation of phosphorylated FAK and paxillin. However, inhibiting assembly of dorsal SFs by blocking mDia-induced actin polymerization or by knocking down α-actinin expression resulted in adhesions that failed to mature even though tension on the adhesions was unaffected and derived from myosin activity within the lamella driving rearward movement of transverse arcs. These results

raise questions concerning the mechanosensitivity of dorsal SFs. Currently, we know of no direct studies on this topic, which may reflect that dorsal SFs have been much less studied than ventral SFs.

Organization of SFs

Although SFs have long been described as having a sarcomeric type of organization, there are many questions that remain about how the SF sarcomere is organized. Certainly, many of the components exhibit a periodic distribution along SFs, including myosin, tropomyosin, zyxin, caldesmon, and α-actinin (Fig. 3). However, it is striking that actin rarely displays a detectable periodicity. This raises the question of how the actin filaments are organized. In general, the lack of a periodic actin distribution has been attributed to variable lengths of actin filaments, with many of the filaments extending more than one sarcomeric unit. In early work, using heavy meromyosin decoration of F-actin, Sanger and Sanger (1980) were able to follow individual filaments in SFs extending for distances of up to four sarcomeres. They observed filaments with uniform polarity at their sites of attachment to membranes, but in the middle of SFs, they noted overlapping filaments of opposite polarity. To explain the apparent lack of actin periodicity, they concluded that the sarcomeres in an SF are visualized in a contracted state. In their model, contraction occurs until the myosin filaments essentially collide with the α-actinin–containing Z disk–equivalent structures. Because the actin filaments are much longer than myosin filaments, in this contracted state, they extend beyond the borders of a single sarcomere (Sanger and Sanger, 1980). This explanation is supported by the observation that SFs are under tension as indicated by traction force measurements at focal adhesions (Balaban et al., 2001; Beningo et al., 2001) and by the rapid shortening of SFs commonly observed when they are released from their attachment to the substratum at focal adhesions. However, this explanation does not account for the periodicity of proteins such as tropomyosin. This usually parallels the distribution of myosin along the SF and has the counter periodicity to α-actinin (Lazarides, 1976; Gordon, 1978). In muscle sarcomeres, the distribution of tropomyosin essentially parallels the distribution of actin, except at the Z disks where α-actinin is present.

One of the unexpected observations concerning SF organization is that the dimensions of the bands corresponding to myosin and α-actinin vary, not only between cells but even along a single SF (Peterson et al., 2004). Variable dimensions of α-actinin periodicity are visible in Fig. 3. The widths of the myosin and α-actinin bands were noted to be shorter toward the periphery of the cell and longer in the middle (Peterson et al., 2004). Even more surprisingly, in response to factors stimulating contraction, some SF sarcomeres shortened as expected (typically at the periphery), whereas those in the center elongated. In contrast, in striated muscle, myosin thick filaments do not change their length during contraction or stretching. Similarly, α-actinin appears to remain confined to the Z disk and be unaffected by the contractile state of the muscle. These results indicate that although their structures are similar, there are significant differences in behavior between muscle and SF

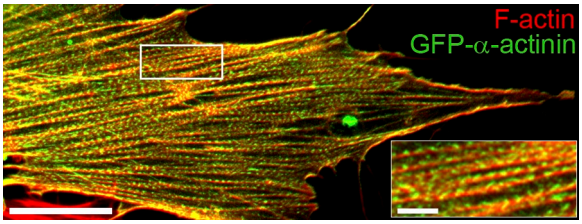

Figure 3. **Periodicity of α-actinin within SFs.** Swiss 3T3 cells stably expressing GFP–α-actinin (Edlund et al., 2001) were fixed and labeled with Texas red–phalloidin to label F-actin. Note the variable dimensions and spacing of the periodic fluorescence of GFP-tagged α-actinin along the length of SFs (red). Bar, 25 µm. (inset) Enlarged view of the boxed area. Bar, 10 µm.

sarcomeres. With respect to myosin, one possibility is that the number of myosin molecules polymerizing into myosin filaments varies and may be affected by the contractile demands of the cell. For α-actinin, the situation must be different, as this protein primarily cross-links actin filaments. Curiously, in striated muscle, α-actinin has always been depicted as cross-linking antiparallel F-actin at the Z disk, whereas in nonmuscle cells, it is usually considered to be cross-linking parallel filament bundles. However, in vitro studies have revealed that it is a highly flexible cross-linker (Courson and Rock, 2010) and can cross-link parallel (Taylor et al., 2000) as well as antiparallel bundles (Liu et al., 2004) and even that both ends of an α-actinin dimer can interact simultaneously with a single actin filament (Hampton et al., 2007), although this may be an in vitro artifact. Regardless of the dimensions of the SF sarcomeres, whether they are long or short (stretched or contracted), the α-actinin and myosin bands appear to maintain a complementary periodicity. α-Actinin has a relatively high rate of exchange on and off SFs as determined by fluorescence recovery after photobleaching (Edlund et al., 2001), and it was speculated that there is competition (either direct or indirect) between myosin and α-actinin for binding to the actin filaments (Peterson et al., 2004). During contraction of the SF sarcomeres, it was suggested that myosin displaces α-actinin, except from the Z disk–equivalent structures. Conversely, when myosin is released from F-actin, α-actinin reassociates, generating a banding pattern that is longer in regions of low tension but shorter in contracted regions (Peterson et al., 2004).

The least understood region of an SF is where it attaches to a focal adhesion. Many protein–protein interactions have been identified biochemically, with several proteins binding integrin cytoplasmic tails and some of these proteins also binding F-actin directly (Zaidel-Bar et al., 2007; Zaidel-Bar and Geiger, 2010). Most attention has focused on talin, which binds both integrins and actin, and its binding partner vinculin, which binds actin, as well as many other components in focal adhesions. The very high protein density in focal adhesions has limited the value of conventional EM analysis. However, Patla et al. (2010) used cryo-electron tomography to reveal a stratified organization at the inner face of focal adhesions. Just above the plasma membrane, they visualized doughnut-shaped particles that interacted with short tangential fibers. In turn, these associated with SF F-actin at the highest level. This organization is

intriguing, but the significance of the particles remains elusive, although they were shown to be mechanosensitive, disassembling when the RhoA–ROCK pathway was inhibited. Using super-resolution fluorescence microscopy, Kanchanawong et al. (2010) revealed the architecture of multiple components in focal adhesions in nanometer resolution. They found that the integrins in the plasma membrane are vertically separated from actin by a zone of ∼40 nm. Within this zone, they identified several layers; closest to the membrane is a signaling layer containing FAK, paxillin, and the integrin cytoplasmic domains, and then there is a layer involved in force transduction, including talin and vinculin, and finally, there is a layer containing actin filaments as well as vasodilator-stimulated phosphoprotein (VASP), α-actinin, and zyxin. Interestingly, the N and C termini of talin were spatially separated, with the N terminus closer to the membrane and the C terminus extending into the actin-rich domain, consistent with talin linking the cytoplasmic domains of integrins to F-actin in SFs. Much remains to be determined about the organization of this critical region, but these techniques provide an encouraging start.

Generation of sarcomeric periodicity and the recruitment of myosin II

Cramer et al. (1997) showed that SFs have uniformly oriented actin filaments emerging from focal adhesions with their barbed ends at the adhesion. One of the challenges is to understand how bundles of unipolar filaments are converted into sarcomeres with alternating polarity. Antiparallel arrays of F-actin are necessary for myosin to generate force. When Hotulainen and Lappalainen (2006) considered how dorsal SFs incorporate myosin and convert from a uniform bundle of filaments into a sarcomeric arrangement, they suggested that actin filaments might rotate through 180°. Although theoretically possible, rotation of filaments seems both unlikely and energetically unfavorable. Here, we propose an alternative mechanism (Fig. 4). We suggest that the filaments are cleaved and that the protein that severs and caps the newly exposed barbed ends either contains actin-nucleating activity itself or recruits a nucleating protein or complex that induces polymerization. The critical requirement is that polymerization occurs in the opposite direction of the preexisting filament so that a filament of opposite polarity is generated. Many proteins that nucleate actin polymerization have been identified (Campellone and Welch, 2010), and at least one formin, FRL-α (FRL1), has actin-severing activity (Harris et al., 2004), raising the possibility that a single protein may fulfill the required characteristics (severing/capping/nucleation) for this model. Much has been learned from striated muscle sarcomere assembly (Sparrow and Schöck, 2009), and one of the actin-nucleating proteins implicated in sarcomere organization is leiomodin (Chereau et al., 2008). As several leiomodin family members exist, it will be important to determine whether any of these molecules function similarly in nonmuscle sarcomeres (Conley et al., 2001).

Related to the question of how sarcomeric organization is generated is the question of how myosin is recruited to SFs. Several studies point to the importance of tropomyosin in this process. Tropomyosin is an actin-binding protein that exists

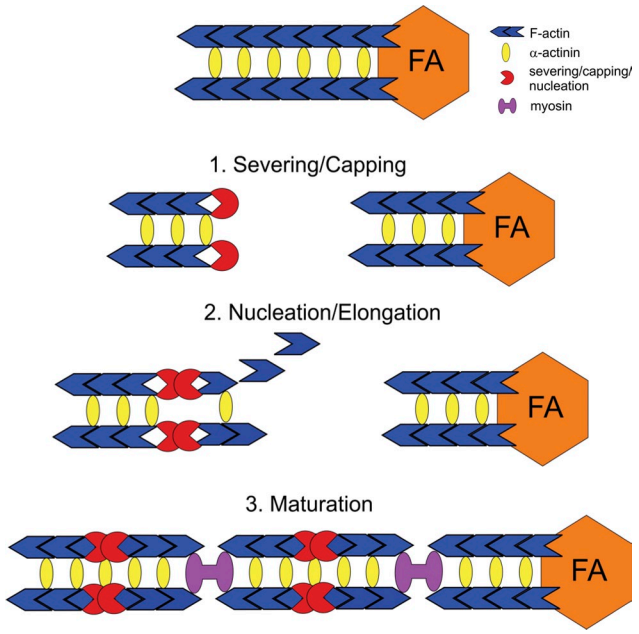

Figure 4. **Proposed model for conversion of unipolar to bidirectional actin filaments during the maturation of SFs.** Unipolar, α-actinin cross-linked actin filament bundles oriented with their barbed ends facing the focal adhesion are first severed and capped (step 1). This severing protein then either recruits another protein or protein complex that nucleates actin filament polymerization in the opposite orientation, or alternatively, a single protein possessing severing/capping/nucleation activity may fulfill this role (step 2). The final stage involves incorporation of myosin filaments into the maturing SF with its characteristic periodic distribution (step 3).

as many alternatively spliced isoforms (Gunning et al., 2008). Different isoforms are recruited to different populations of actin filaments (Gunning et al., 2005). When some tropomyosin isoforms are overexpressed, SFs are induced, whereas knockdown of all tropomyosin leads to SF disassembly (Gimona et al., 1996; Bryce et al., 2003; Bakin et al., 2004; Gupton et al., 2005). Studying U2OS osteosarcoma cells, it was shown that different tropomyosin isoforms associated with different regions of dorsal SFs. Tm2 distributed along the whole length of dorsal SFs, whereas Tm1 and Tm5NM1 or 2 concentrated in focal adhesions. In contrast, Tm3 and Tm4 were excluded from the ends of SFs (Tojkander et al., 2011). In this same study, Tm4 was implicated in recruiting myosin to transverse arcs and hence to ventral SFs arising from arcs. In another cell type, however, Tm5NM1 was found to selectively recruit myosin IIA, but not IIB, to SFs (Bryce et al., 2003). Together these results reveal the importance of tropomyosin in determining the recruitment of myosin filaments to SFs but raise additional questions concerning the factors regulating tropomyosin distribution and the subtleties of tropomyosin–myosin interactions.

Disassembly

In comparison with the large amount of work studying how SFs assemble, much less attention has been paid to their disassembly, even though it is equally important during dynamic morphological changes involving the actin cytoskeleton. Disassembly occurs when cells go into mitosis, are detached from

their adhesions, or when RhoA is inhibited. Elevating cAMP also causes the rapid loss of SFs (Lamb et al., 1988; Lampugnani et al., 1990); this is a result of inhibiting RhoA activity via PKA-dependent phosphorylation of RhoA, which enhances its binding to Rho guanine nucleotide dissociation inhibitor (Lang et al., 1996; Sauzeau et al., 2000; Ellerbroek et al., 2003) as well as PKA phosphorylation and inhibition of the myosin light chain kinase (Lamb et al., 1988). Most of the situations in which SFs disassemble are associated with a loss of tension. Notably, a direct effect of decreased tension on cofilin-mediated severing and disassembly of F-actin was recently discovered (Hayakawa et al., 2011). These investigators proposed that tension is sensed directly in the actin filament protecting it from cofilin severing. Using laser tweezers to manipulate single filaments, they demonstrated that F-actin under tension bound less cofilin than filaments under resting conditions. Although relaxation-induced cofilin severing of F-actin is likely to be an important mechanism, we suspect that other pathways also contribute to SF disassembly.

Although loss of tension and decreased myosin contractility lead to SF disassembly, complete severing of SFs with a laser surprisingly did not (Colombelli et al., 2009). When SFs were cut in the middle, there was rapid retraction away from the cut site. The SF sarcomeres close to the cut site shortened, but then, the severed SF segments stabilized, and the ends developed new adhesions. This was accompanied by loss of zyxin from the original focal adhesion and SF as well as redistribution to distinct regions along the new SF fragments. The foci of zyxin were immobile, suggesting that they were linked to the underlying ECM. Analyzing the behavior of laser-severed SFs led Colombelli et al. (2009) to conclude that before the severing, SFs were already associated along their length with the substratum via integrins in adhesions too small to detect by light microscopy. As the severed SFs contracted, these small preexisting adhesions would rapidly become reinforced in response to the increased tension. In turn, as they strengthened, isometric tension would be reestablished between these new adhesions, thus preventing continued disassembly of the severed SF.

SFs and the generation of force

Not only is tension an important factor in the assembly of SFs, but SFs are themselves recognized as tension-generating structures. Originating from the pioneering work of Harris et al. (1980) who used flexible rubber surfaces to visualize the traction forces generated by cells, many techniques have been developed for quantifying these forces (Lee et al., 1994; Galbraith and Sheetz, 1997; Pelham and Wang, 1999; Balaban et al., 2001; Tan et al., 2003). The resolution of these techniques has been developed to the point at which the force exerted at single focal adhesions, i.e., generated by a single SF, can be calculated. Balaban et al. (2001) and Schwarz et al. (2002) determined that the focal adhesions in their fibroblasts were transmitting forces of $\sim$5.5 nN/μm^2 and that the force at a focal adhesion was proportional to its area. Given that the area of a focal adhesion is usually related to the diameter of the SF and that larger SFs would be expected to contain more myosin molecules, this relationship between the area of focal adhesions and the transmitted force made intuitive sense. Unexpectedly, however, a very different result was obtained by Beningo et al. (2001). Investigating migrating cells, they discovered that the highest forces were transmitted to the substrate by small adhesions in the front of the cell, and these forces were greater than those exerted by the large focal adhesions away from the cell front. Similar results were also obtained by Stricker et al. (2011). Reconciling these opposite results has been difficult, but Tan et al. (2003) suggest a possible explanation. They cultured cells on flexible microneedles and measured cell-generated traction forces by quantifying the bending of the microneedles. For most adhesions, they found a linear relationship between the area of the adhesion and the force exerted at it, and they calculated that the force was $\sim$4–5 nN/μm^2, similar to the values obtained by Balaban et al. (2001) and Schwarz et al. (2002). However, they also detected a set of small adhesions that did not fit this relationship between size and force; these were associated with unexpectedly high forces. These small adhesions were <1 μm^2 and most likely are equivalent to the high traction, force-generating nascent adhesions found at the front of migrating cells. The force transmitted by SFs will generally be proportional to the level of active myosin within that SF. With dorsal SFs, however, additional force derives from the contraction and rearward movement of transverse arcs that are coupled to dorsal SFs. This extra force generated by arcs likely accounts for the high forces associated with small adhesions at the front of some cells (Beningo et al., 2001; Stricker et al., 2011).

Remodeling, reinforcement, and repair

Although the response of SFs to mechanical force has been known for many years, most attention has focused on the mechanotransduction properties of focal adhesions, in which SFs interact with the ECM (Chen et al., 2004; Shemesh et al., 2005; Bershadsky et al., 2006; Vogel and Sheetz, 2006; Lessey et al., 2012). As structures that are load bearing but also force generating, it is to be expected that SFs have mechanisms to adapt to changes in force allowing them to strengthen as the tension increases. As discussed earlier, mechanical force exerted on integrins activates RhoA (Zhao et al., 2007; Guilluy et al., 2011), which contributes to the cellular stiffening response (Matthews et al., 2006; Guilluy et al., 2011). Several pathways downstream from RhoA should strengthen SFs, including myosin light chain phosphorylation (promoting myosin filament assembly and ATPase activity), actin polymerization via mDia1, and inhibition of F-actin severing by cofilin, via cofilin phosphorylation.

Cyclic stretch is a unique type of mechanical perturbation that cells must adapt to in many physiological settings; an example is the pulsatile stretching and shear forces encountered by endothelial cells and vascular smooth muscle. The response to cyclic stretch involves several concurrent steps: SF reorganization and remodeling, ultimately leading to cell reorientation, and SF reinforcement and repair, as means of adaptation to cyclic strain. In contrast to endothelial cells exposed to uniform shear forces, which reorient parallel to the direction of flow (Tzima, 2006), fibroblasts exposed to cyclic stretch rearrange their SFs perpendicular to the direction of force (Hayakawa et al., 2001), with the temporal dynamics of

cytoskeletal and cell reorientation occurring in an amplitude-, frequency- (Jungbauer et al., 2008), and substrate stiffness–dependent manner (Faust et al., 2011). Mathematical modeling of cell reorientation has suggested that the adhesive forces within the SF and associated focal adhesions oscillate around an optimal isotonic value until a critical force amplitude and frequency threshold is reached, at which point the destabilization of focal adhesion catch bonds results in focal adhesion slipping, resulting in SF contraction or rotation to a more stable configuration, i.e., perpendicular to the direction of stretch (Chen et al., 2012). SF reorientation in response to cyclic stretch requires Src/Fyn/Yes kinase signaling as well as the mechanosensory protein p130Cas (Niediek et al., 2012).

In addition to the cell reorientation response to cyclic stretch involving SF remodeling and reorganization, reinforcement and repair of SFs subjected to mechanical strain must also occur. Studies from several groups have shown that the focal adhesion protein zyxin redistributes in response to mechanical force (Yoshigi et al., 2005; Lele et al., 2006; Guo and Wang, 2007; Colombelli et al., 2009), and zyxin has been implicated in the reinforcement and repair of SFs responding to mechanical strain. In cells undergoing cyclic stretching, zyxin redistributed from focal adhesions to along SFs, and the SFs became thicker in a zyxin-dependent manner (Yoshigi et al., 2005). Interestingly, this zyxin-mediated SF thickening did not require ROCK signaling but instead involved MAPK-dependent phosphorylation of zyxin (Hoffman et al., 2012). This group also noted that zyxin is not required for cell reorientation in response to cyclic stretch but is involved predominantly in the reinforcement phase. Using live-cell imaging, it was noticed that SFs exhibit regions of spontaneous elongation and thinning in the absence of external stimuli (Smith et al., 2010), similar to the stretching of sarcomeres previously observed when cells were stimulated to contract (Peterson et al., 2004). Zyxin was recruited to sites of strain-induced breaks or damage and was necessary for repair of the SF and restoration of force transmission. VASP and α-actinin were also recruited to these sites and depended on zyxin for their redistribution. Both VASP and α-actinin strengthened the SF at these sites of damage, whereas α-actinin also contributed to restoring actin integrity (Smith et al., 2010). Notably, new sarcomeres developed at strain sites, and zyxin was implicated in this process (Chapin et al., 2012). The addition of new sarcomeres within the stretched region of an SF would be predicted to strengthen it. These findings are particularly interesting because previous work had pointed to focal adhesions and the ends of SFs as the regions of sarcomere addition (Hotulainen and Lappalainen, 2006; Endlich et al., 2007; Guo and Wang, 2007; Russell et al., 2010). Chapin et al. (2012) observed frequent changes in the lengths of SF sarcomeres in unstimulated cells even though the overall length of the SFs remained constant. Indeed, as one sarcomere lengthened, the sarcomeres on either side frequently shortened, leading the authors to suggest a "tensional homeostasis" mechanism and communication between sarcomeres along an SF. The cause and effect relationship of the contracting and lengthening sarcomeres has yet to be resolved, but these are provocative observations. When muscles contract, their resting length is restored by the action of opposing muscles that stretch the contracted sarcomeres. With SFs, there is no equivalent counteracting mechanism. One possibility is that the tension generated in an SF by one sarcomere shortening may be important for stretching neighboring sarcomeres so as to counter their contracted state. Such a mechanism might prevent individual sarcomeres from becoming "locked" irreversibly into a fully contracted state and would allow repeated cycles of contraction to occur.

Conclusions

The adage that familiarity breeds contempt holds true for SFs; this familiarity obscures unresolved and fundamental questions about these often taken for granted cytoskeletal arrays. One key question concerns the organization of SF sarcomeres, which at first glance appear similar to, but which, in fact, differ in several key aspects from the sarcomeres of striated muscle. This is illustrated by the unexpected shortening or lengthening of myosin and α-actinin bands as SF sarcomeres contract or stretch, a radically different behavior from muscle, in which the equivalent bands remain constant regardless of sarcomere stretching or contraction. Understanding how new sarcomeres are added in regions of strain is a challenge that is only beginning to be approached. Already, one protein, zyxin, has been identified contributing to this process, and it will be important to learn how zyxin is recruited to sites of tension and high stress where reinforcement and repair of SFs occur in part by the addition of new sarcomeres. The idea that tensional homeostasis exists along an SF is intriguing and deserves further investigation, as does the implication that adjacent sarcomeres communicate. Another unanswered question relates to how bundles of unipolar actin filaments are converted into sarcomeres with alternating polarity, which is necessary for myosin-based force generation. Although much has been learned about the pathways governing SF assembly, numerous questions remain about their disassembly, which is equally important but rarely studied. Addressing questions such as these should provide a deeper understanding of how cells remodel and fine tune their cytoskeletons not only in response to the chemical signals that cells receive but also in response to the physical state of their extracellular environment and the forces that they encounter.

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