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Sarcomere Length Fluctuations and Flow in Capillary Endothelial Cells

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Tensile force within non-muscle tissue cells is generated in actomyosin stress fibers, which are composed of contractile units called sarcomeres. The number of sarcomeres and sarcomere lengths dynamically change in the cell but the mechanisms by which these processes occur are not understood. Using live cell imaging of labeled sarcomeres, we show that sarcomere lengths continually fluctuate, with a fluctuation relaxation time of about 20 min. New sarcomeres are formed at focal adhesions and are convected into the fiber at a speed that is independent of focal adhesion size, suggesting that the speed is independent of tension. Furthermore sarcomeres were observed to disappear at specific points or "sinks" along the stress fibers. These results show that stress fibers are highly dynamic structures despite their relatively static morphology, with nascent sarcomeres forming and being incorporated into the fiber at a nearly uniform, tension-independent velocity throughout the cell. The fluctuating length of individual sarcomeres under constant tension is consistent with a model whereby sarcomere contraction/expansion speed, rather than sarcomere length, is modulated by tension. © 2011 Wiley-Liss, Inc.

Key Words: stress fibers, endothelial sarcomeres, stress fiber dynamics, stress fiber assembly

Introduction

Generation and maintenance of intracellular tension is necessary for endothelial cells to adhere to the basement membrane and resist external mechanical stresses such as those due to blood flow. Tension in endothelial cells is generated in stress fibers which are observed not only *in vitro,* but also *in vivo* [Drenckhahn and Wagner, 1986; Wong et al., 1983] in endothelial cells. Endothelial stress fibers are made from tens of repeating contractile units called sarcomeres assembled end to end [Langanger et al., 1986; Lazarides and Burridge, 1975]. Stress fiber sarcomeres consist of periodic actomyosin contractile elements separated by crosslinked regions of actin filaments referred to as "dense bodies" containing proteins such as α-actinin. Translocation of bipolar myosin filaments along the actin filaments generates force in the sarcomere. This tension is balanced by the extracellular matrix at focal adhesions [Geiger and Bershadsky, 2001].

Stress fibers are extremely dynamic structures that form as a result of continual generation of F-actin at focal adhesions and the cell membrane [Endlich et al., 2007; Hotulainen and Lappalainen, 2006] and subsequent crosslinking and bundling of microfilaments [Hirata et al., 2007]. Dynamic assembly and disassembly of stress fibers is crucial for the cell's ability to adapt to external mechanical stimuli [Hayakawa et al., 2001]. Recently it has been reported that stress fibers undergo force induced thinning events that result in stress fiber breakage [Smith et al., 2010]. Surprisingly, the dynamic behaviors of sarcomeres, which are building blocks of stress fibers, have not received such attention. It is known that sarcomeres respond dynamically on treatment with calyculin A with shortening of peripheral sarcomeres and lengthening of central sarcomeres [Peterson et al., 2004]. Nascent sarcomeres have also been shown to flow in at focal adhesions at the cell periphery [Endlich et al., 2007; Hotulainen and Lappalainen, 2006]. Using femtosecond laser ablation to sever individual stress fibers, we recently explained the measured sarcomere contraction length distributions by hypothesizing that sarcomeres in a stress fiber may continually fluctuate with time [Russell et al., 2009]. However,

Additional Supporting Information may be found in the online version of this article.

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we are not aware of studies which have actually demonstrated fluctuations in sarcomere lengths in living cells.

In this paper, we found that sarcomere lengths indeed continually fluctuate in living endothelial cells. Nascent sarcomeres were observed to flow into pre-existing fibers at focal adhesions throughout the cell with flow velocities that do not correlate with focal adhesion size. Frequently sarcomeres were observed to be consumed in 'sinks' at stress fiber junctions. Together, these results shed new light into the dynamics of stress fiber sarcomeres in endothelial cells.

Materials and Methods

Cell Culture and Transfections

Bovine capillary endothelial (BCE) cells were maintained at 37°C in humidified 10% CO_2. The BCE cells were cultured on tissue culture dishes in complete medium consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM; Mediatech, Manassas, VA) supplemented with 10% donor bovine serum (Gibco, Grand Island, NY), 1% 1 M HEPES (Mediatech), glutamine (0.292 mg/ml)/ penicillin (100 U/ml)/ streptomycin (100 g/ml) (Sigma, St. Louis, MO) and basic FGF (2 ng/ml; Sigma). This culture method was described previously in Russell et al. [2009].

For experiments, cells were transiently transfected with an EGFP-α-actinin-1 plasmid (kindly provided by Prof. Carol Otey) using the Effectene (Qiagen, Valencia, CA) reagent. Previous studies have rigorously shown that the fusion construct is functional and localizes with the endogenous protein [Edlund et al., 2001]. Cells were transfected for 6 h in 12-well cell culture dish. After 24 h, the cells were trypsinized (Gibco) and plated sparsely on glass bottom dishes (MatTek, Ashland, TX). Cells were imaged approximately 24 h after seeding on glass bottom dishes.

Micropatterning

To determine if sarcomere dynamics were dependent on cellular shape changes, BCE cells were seeded onto micropatterned islands of fibronectin. Microcontact printing was done according to previously published methods [Fink et al., 2007]. Briefly, molds for the stamps were produced using standard UV lithography techniques. PDMS (Sylgard 184 kit, Dow Corning, MI) stamps were created by casting photo-resist mold using a 10:1 ratio (w/w) of elastomer to hardener and cured at 60°C for 2 h and postcured at 100°C for 1 h. Human fibronectin (50 μg/ml solution) (BD Biocoat^{TM}, Franklin Lakes, NJ) was then adsorbed to PDMS stamps. Stamps were then dried and placed onto the substrate, Ibidi dishes (Ibidi), for 5 min. The uncontacted area was blocked with PLL-*g*-poly(ethylene glycol) (SuSoS AG), preventing protein adsorption and cell attachment. After treatment the surface was washed and cells were plated.

Imaging

For epi-fluorescence imaging experiments, culture media was replaced with imaging media consisting of low-glucose phenol red-free Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% donor bovine serum (Gibco), 1% 1 M HEPES (Mediatech) and glutamine (0.292 mg/ml)/ penicillin (100 U/ml)/ streptomycin (100 g/ml) (Sigma). Imaging was done on an inverted Nikon TE-2000 equipped with a temperature- and CO_2-controlled environmental chamber. The environmental chamber allowed experiments to be conducted at the culture conditions described above (37°C and humidified 10% CO_2). Time-lapse live cell widefield epi-fluorescent imaging was conducted with a 63×, 1.49 NA or a 40× 1.4 NA oil immersion objective. Images were captured at 3-min intervals with a cooled CCD camera controlled by Nikon Elements software.

Image Analysis

To measure sarcomere lengths in stress fibers during time lapse imaging, custom tracking software described previously [Russell et al., 2009] was used to determine the positions of GFP-α-actinin labeled dense bodies to subpixel accuracy. Image sequences from the Nikon Elements software package were exported to ImageJ (NIH) for image processing. Tracking a simulated particle indicated that the error was minimal, less than 1%.

Particle Image Velocimetry was performed in MATLAB using MatPIV [Sveen, 2006] for all images (20) of square cells collected over 1 h. The individual velocity fields were then averaged to obtain a time averaged velocity field for the cell. Vectors were plotted at 12 pixel increments (5 μm) on the first image to illustrate the time averaged flow field.

Results

Fluctuations in Sarcomere Length

To investigate sarcomere dynamics in endothelial cells, we imaged the dynamics of EGFP- α-actinin transfected BCE cells. Sarcomeres in the same fiber were observed to undergo significant changes in length on the time scale of several minutes (Figs. 1A and 1B; Supporting Information Movie 1) in cells cultured in normal growth medium. Individual sarcomeres in a given fiber exhibited different behaviors, with some sarcomeres shortening or lengthening while others fluctuated around the mean (Fig. 1B). The length changes were random because the deviation from the mean length remains close to zero throughout the time course of the measurement (Fig. 1C). The autocorrelation of the pooled population of measured sarcomeres revealed that correlations in sarcomere length changes die out over a time scale of approximately 20 min. The mean sarcomere length from the autocorrelation was

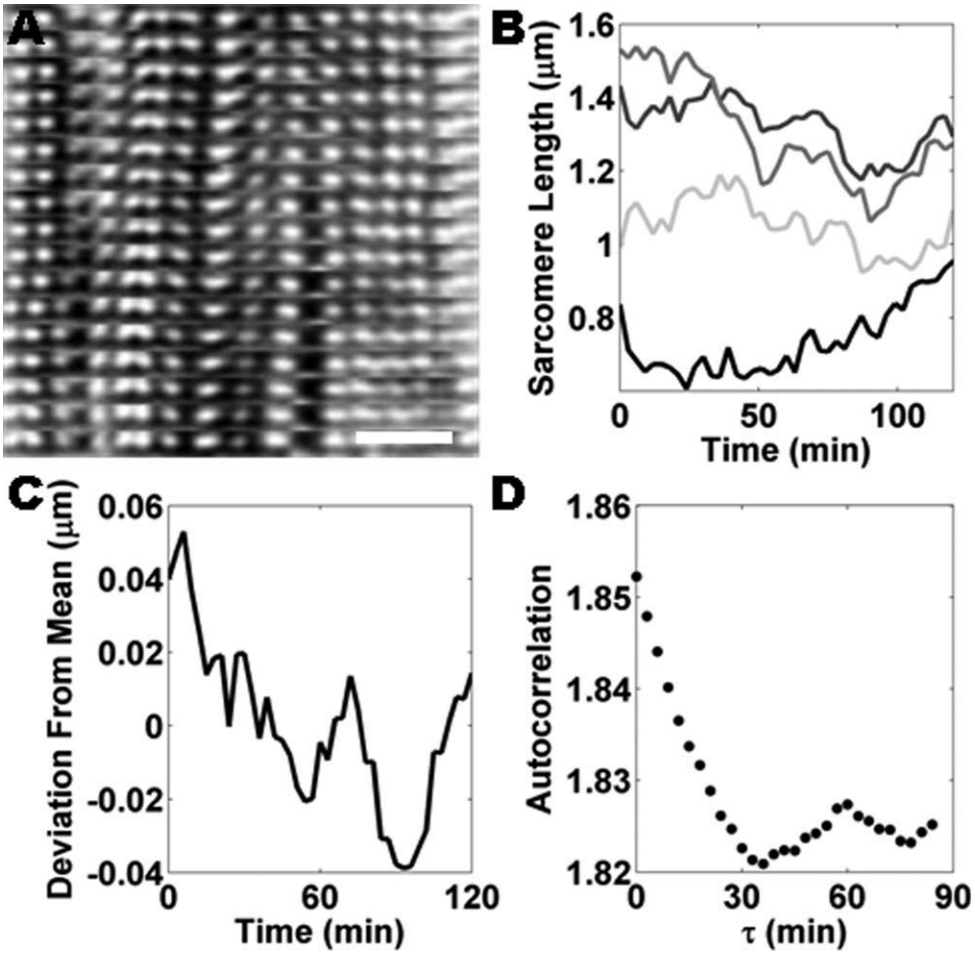

Fig. 1. Sarcomeres undergo dynamic length fluctuations. A: A kymograph showing sarcomeres undergoing length fluctuations during time lapse epi-fluorescence imaging (Scale bar is 1.4 μm and time between frames is 3 min). Note that sarcomeres do not behave uniformly during the time course. **B:** Four sarcomere lengths from the same stress fiber are plotted with respect to time. The trajectories show the heterogeneous nature of sarcomere fluctuations. **C:** The deviation from the mean for 13 sarcomeres is plotted with respect to time. The fluctuations can be assumed to be random because the deviation fluctuates around zero. **D:** The pooled autocorrelation was calculated for 13 sarcomeres indicating that correlations in the fluctuations die out after approximately 30 min.

found to be 1.3 μm and a standard deviation of the fluctuations was 0.2 μm (Fig. 1D).

We next asked if sarcomere length fluctuations resulted in perturbations to the stress fiber network from cellular shape changes. To control cell shape, we confined cells on 40 μm square micropatterned islands of fibronectin, which has been shown to minimally affect cellular viability [Chen et al., 1997]. BCE cells transfected with EGFP- α-actinin displayed an ordered network of stress fibers with most fibers aligned along the diagonal (Fig. 2A). Sarcomere lengths fluctuated continuously even in confined patterned cells (Fig. 2B; Supporting Information Movie 2), suggesting that fluctuations were not dependent on overall cell shape changes.

Flow of Nascent Sarcomeres from Focal Adhesions

Previous studies have shown that nascent sarcomeres flow out from focal adhesions at the cell periphery [Endlich et al., 2007; Hotulainen and Lappalainen, 2006]. In BCEs, we found that nascent sarcomeres flowed out of adhesions at the periphery as well as at adhesions in the interior of the cell (Figs. 3A and 3D). In many cases, the sarcomeres did not have coherent dense bodies, but became more organized as they flowed into the cell (Fig. 3A). Focal adhesions remained stationary as the dense bodies moved inward which established that the sarcomeres were indeed flowing in [and not just sliding because of focal adhesion movement (Supporting Information Movie 3)]. In the majority of cases, the velocity of nascent dense bodies was constant with increasing distance from the focal adhesion (Fig. 3E). When the trajectories for successive emerging sarcomeres from a single focal adhesion were tracked, the velocity was found to be remarkably uniform (Fig. 3E). The measured mean sarcomere velocity in endothelial cells was 0.075 ± 0.0066 μm/min which was roughly three times less than that measured in U2OS cells [Hotulainen and Lappalainen, 2006] and podocytes and fibroblasts [Endlich et al., 2007].

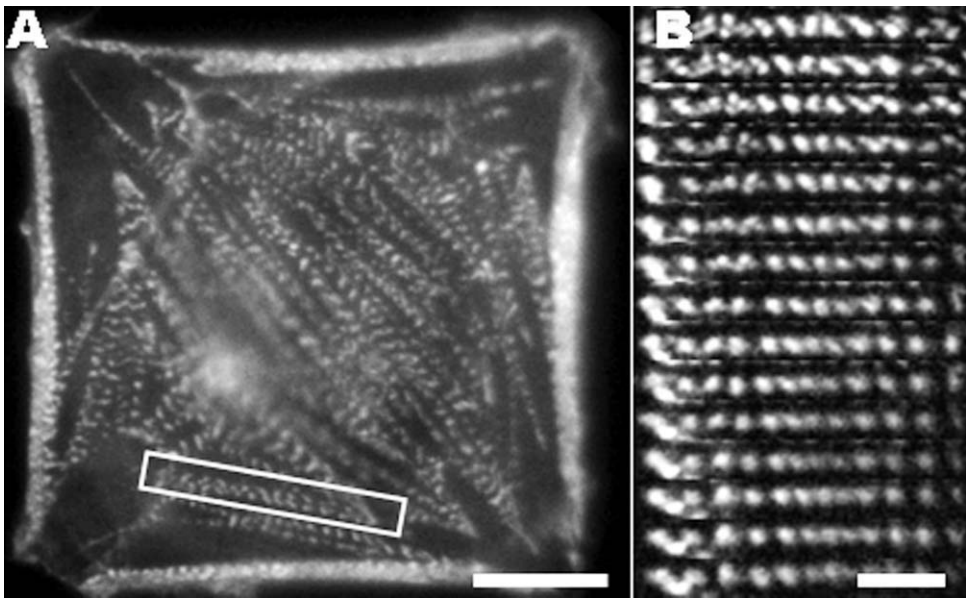

Fig. 2. Sarcomeres undergo length fluctuations in cells with confined shaped. A: BCE cells transfected with EGFP-α-actinin were seeded onto square micropatterned islands of fibronectin. Shown here is an epi-fluorescence micrograph illustrating the stress fiber network of micropatterned BCE cells (scale bar 10 μm) (**B**). This kymograph shows the dynamics of a stress fiber (labeled with box in A) in a micropatterned BCE cell. The fiber undergoes qualitatively similar fluctuations to stress fibers from unpatterned cells suggesting that transient shape changes are not the cause of fluctuations (scale bar 4 μm and 18 min).

Focal adhesions in cells confined to square micropatterned islands of fibronectin were found to exist at both the periphery as reported before [Parker et al., 2002] and in the interior of the cell (Fig. 3B). Particle image velocimetry (PIV) was used to determine the flow pattern of dense bodies in the micropatterned BCE cells. The results indicated that there was continual flow of nascent dense bodies from all focal adhesions towards the center of the cell (Fig. 3C). Flow of dense bodies from an interior focal adhesion is illustrated in the kymograph of Fig. 3D.

Sarcomere Flow Velocity from Focal Adhesions is Tension Independent

Dense bodies were observed to flow from focal adhesions of all sizes throughout the cell. It has been previously established that focal adhesion size is correlated with the net tensile force on the adhesion [Balaban et al., 2001; Chrzanowska-Wodnicka and Burridge, 1996; Tan et al., 2003]. To determine whether the rate of sarcomere production was dependent on focal adhesion size and thus tension, the flow velocity was plotted against size of the associated focal adhesion (Fig. 4). No correlation was found between dense body flow rate and focal adhesion size suggesting that flow does not depend on tension.

Sarcomeres are Consumed at "sinks"

Since new sarcomeres are continually incorporated at focal adhesions, it stands to reason that sarcomeres must be consumed somehow along the fiber length. We observed sarcomeres being consumed at discrete points, or "sinks," along the stress fibers, most often (but not always) at junctions between adjacent stress fibers (Fig. 5A; Supporting Information Fig. 1 and Movie 4). In some cases sarcomeres were seen to join end-on as illustrated in Fig. 5B. Taken together these results indicate that multiple mechanisms for disassembly of sarcomeres exist in capillary endothelial cells.

Discussion

While stress fibers have been shown to be dynamic and responsive to mechanical stimuli, little is known about the dynamics of the sarcomeric subunits of stress fibers. In this article, we present observations of a rich array of dynamic behaviors exhibited by sarcomeres in living cells. Stress fiber sarcomeres were observed to fluctuate in length, flow from focal adhesions and be consumed at "sinks." Identical behaviors were found in cells confined to micropatterned islands of fibronectin. The rate of new sarcomere formation and incorporation from focal adhesions was found not to be correlated with focal adhesion size.

Interestingly, within the same stress fiber, some sarcomeres elongated while other nearby sarcomeres simultaneously shrunk. Since tension is commonly assumed to be uniform along the length of the stress fiber, such fluctuations are difficult to explain with models which assume that sarcomere length is elastically coupled to tension. On the other hand, if tension and length were weakly coupled and instead only contraction/expansion speeds were governed by tension, then small changes in local myosin

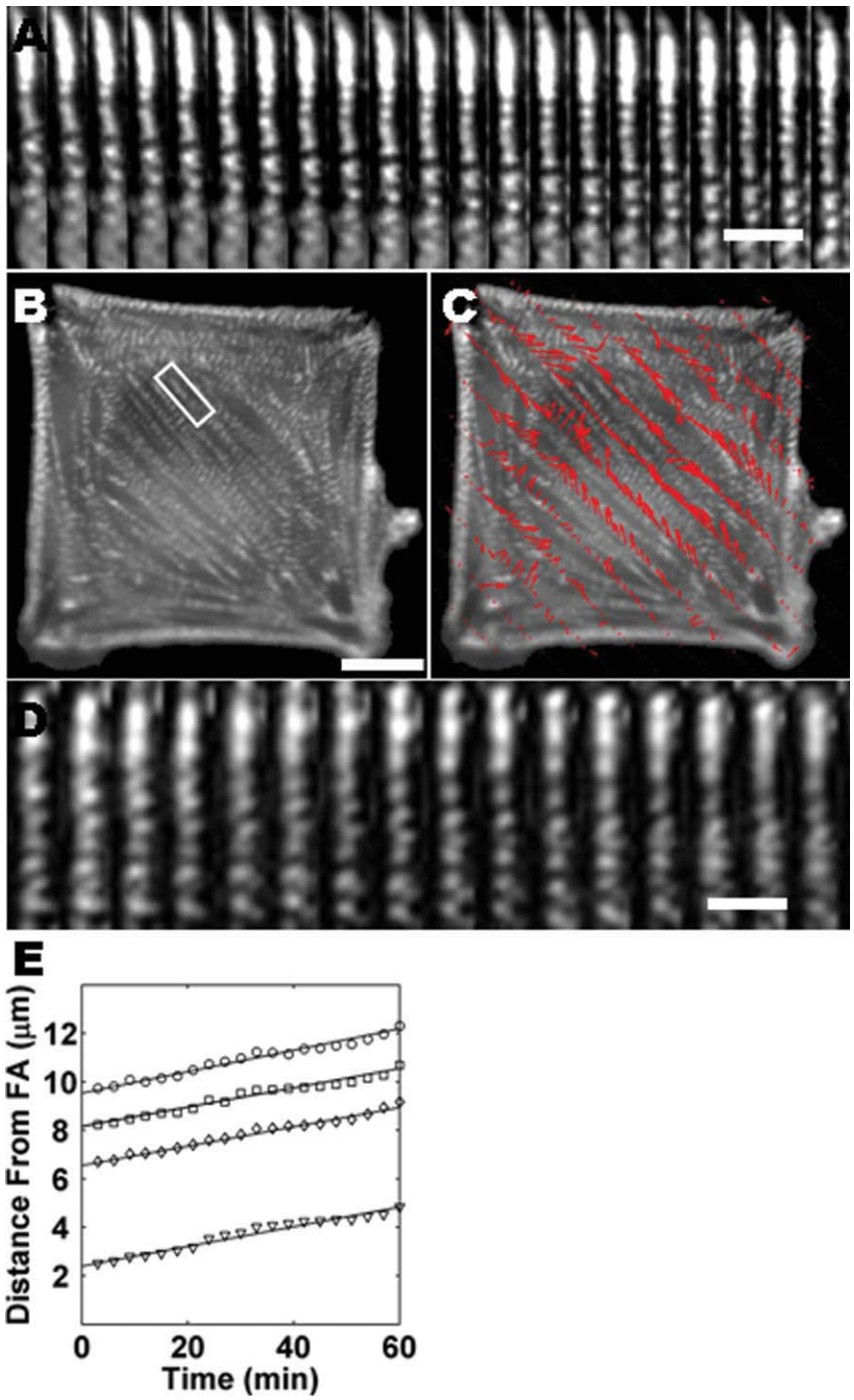

Fig. 3. Nascent sarcomeres flow from all focal adhesions. A: A kymograph illustrating the continual flow of nascent sarcomeres from focal adhesions in BCE cells. The focal adhesion remains stationary and approximately the same size ruling out focal adhesion sliding (scale bar 5 μm and 6 min). **B:** A fluorescence micrograph of EGFP-α-actinin transfected BCE cells confined to square micropatterned islands of fibronectin (scale bar 10 μm). **C:** Particle image velocimetry was used to determine bulk flow patterns throughout the entire micropatterned BCE cell. The PIV results indicate that nascent dense bodies flow from focal adhesions throughout the entire cell and not just at the periphery. **D:** A kymograph of flow of dense bodies from an interior focal adhesion (labeled with box in B) illustrates that flow occurs from interior focal adhesions (Scale bar 5 μm and 6 min). **E:** The trajectories of four dense bodies flowing from a single focal adhesion are plotted with respect to time. Sarcomeres were observed to flow from focal adhesions at constant velocity as evidenced by the constant slope of each dense body. Successive sarcomeres displayed almost identical velocities as evidenced by the parallel trajectories.

activity could lead to large changes in sarcomere length, while maintaining a uniform overall tension. We recently proposed such a model to explain the constant contraction speed and the exponential distribution of contraction distances following removal of tension by laser-severing of stress fibers [Russell et al., 2009].

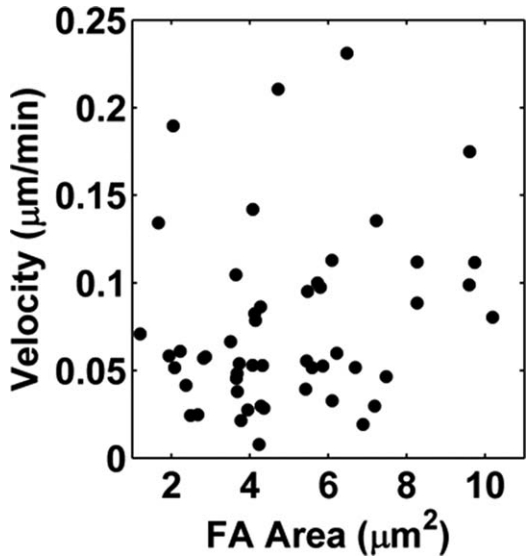

Fig. 4. Sarcomere flow rate is independent of mechanical stress. Sarcomere flow velocities from single focal adhesions are plotted with respect to the focal adhesion size (n = 51 FA from 7 cells). There is little correlation between sarcomere flow rate and focal adhesion size (correlation coefficient of 0.23).

Previous studies have reported that sarcomeres are assembled and flow out of focal adhesions in podocytes, fibroblasts [Endlich et al., 2007], and U2OS cells [Hotulainen and Lappalainen, 2006]. Because stress fibers are anchored to the substrate at focal adhesions and are under isometric tension, any new sarcomeric units added to the fiber must form under tension. We found that sarcomere flow velocities are independent of focal adhesion size; it has been shown that focal adhesion size correlates with tension [Balaban et al., 2001; Chrzanowska-Wodnicka and Burridge, 1996; Tan et al., 2003]. It has been previously demonstrated that inhibition of myosin stops the flow of sarcomeres into the fiber [Endlich et al., 2007]. Taken together these findings suggest that while myosin activity is necessary for flow, the flow is not influenced by the magnitude of tension. This independence of actin assembly rate on tension is consistent with an end-tracking motor model for insertional polymerization of focal adhesion-attached filaments plus-ends [Dickinson et al., 2004; Dickinson and Purich, 2002] but not models where actin assembly of attached filaments is enhanced by tension [Kozlov and Bershadsky, 2004; Mogilner and Oster, 2003]. Moreover, the slow, uniform speed of actin assembly that is independent of focal adhesion size argues against an assembly mechanism limited by the rate of monomer diffusion and suggests that the assembly rate is governed by a molecular timer with a rate constant of around 0.5 s^{-1} (based on the measured speed divided by the 2.7 nm added length per actin monomer).

The PIV analysis in patterned cells suggests that there is continual flow of dense bodies from all focal adhesions directed toward the diagonal of the square cell. Because the steady state length of the stress fiber is approximately constant, in the absence of any sinks the length of each sarcomere would decrease continuously along the

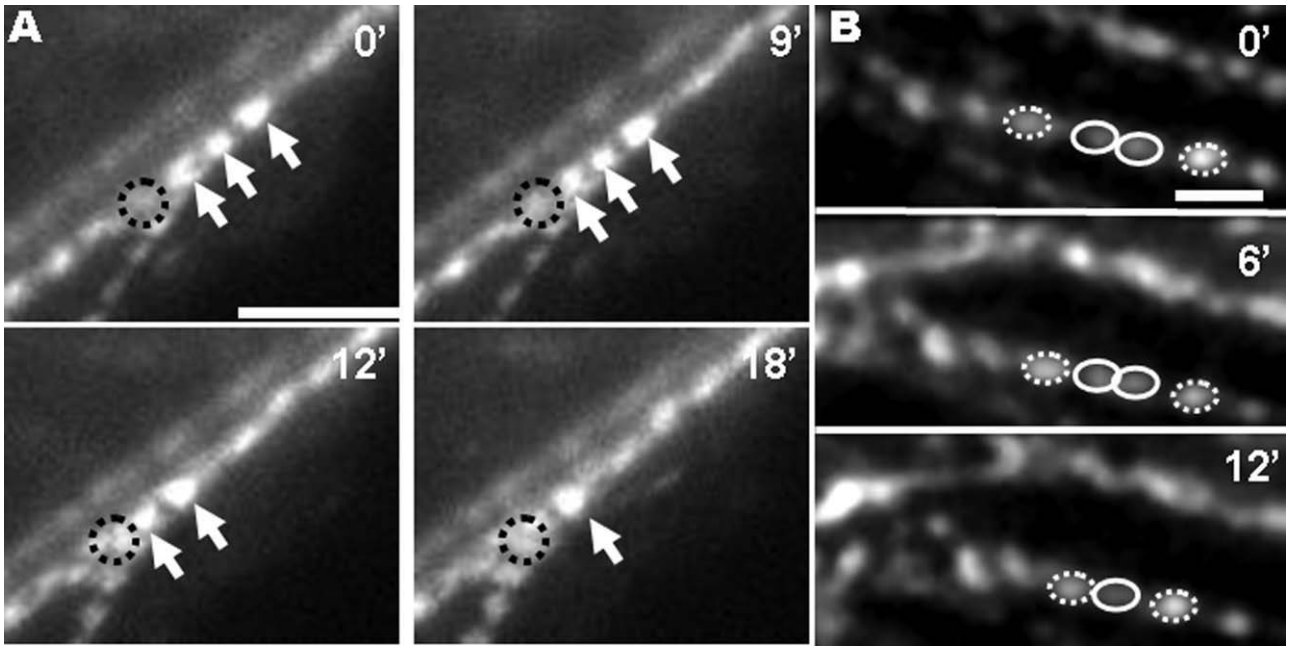

Fig. 5. Sarcomeres are consumed to maintain tension. A: Time lapse images of EGFP-α-actinin transfected BCE cells reveal that sarcomeres are consumed in "sinks" that exist at the junction of two stress fibers. Dense bodies (arrows) move toward the "sink" (black dotted circle) until two disappear during the course of 18 min (scale bar 5 μm). **B:** Dense bodies were observed to join end-on in stress fibers during time lapse imaging. A sarcomere shortens continuously until the two dense bodies (solid white circles) are indistinguishable while the neighboring sarcomeres (between solid and dotted white circles) maintain their integrity (scale bar 2.5 μm).

fiber. A simple number balance would require the existence of "sinks" to maintain a steady state stress fiber length. Our finding of sarcomere fusion along the stress fiber length, therefore suggests a mechanism by which the cell controls sarcomere length at optimal values.

Acknowledgments

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