Chemical and mechanical micro-diversity of the extracellular matrix

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Abstract Interaction with the extracellular matrix (ECM) triggers multiple physiological responses in living cells, affecting their structure, function and fate. Recent studies have demonstrated that cells can sense a wide variety of chemical and physical features of the ECM, and differentially respond to them. Thus, cells cultured on flat surfaces coated with two different integrin-reactive adhesive proteins, fibronectin and vitronectin, display varying degrees of spreading on these matrices, and form morphologically distinct types of matrix adhesions, with variable prominence and spatial distribution of both focal and fibrillar adhesions. It was further shown, using labeling with different antibodies which bind to distinct sites on the fibronectin molecule, that even a "molecularly homogeneous" matrix displays spatial micro-heterogeneity, exposing distinct epitopes at different locations. Diversification of the adhesive surface can be induced by the application of mechanical force to the elastic fibronectin matrix, resulting in the formation of different patterns of fibrillar ECM arrays. Time-lapse monitoring of matrix fibrillogenesis by cells expressing fluorescently-tagged fibronectin demonstrated that the assembly of fibrils in such cell cultures occurs when the leading lamella of the cell advances, attaches to the substrate-bound fibronectin, and then retracts backwards, thus applying tensile forces to the attached fibronectin. These results indicate that the ECM is a highly complex cellular environment, whose chemical and physical properties are directly regulated by the attached cells.

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1 The cell-extracellular matrix interface and environmental signaling

The activities and fates of living cells are regulated by two major mechanisms: an "intrinsic," lineage-dependent control, and an "external," environmental control. The former mechanism defines the specific state of cellular differentiation, which is characterized by a particular gene expression profile and, hence, specific functional properties. However, cells with an identical lineage history can adopt very different modes of behavior, depending on the extracellular matrix (ECM) on which they grow, suggesting that signaling from the ECM has a profound effect on the adhering cells [1].

The ECM itself is composed of diverse classes of adhesive and scaffolding molecules, which form a rich variety of filamentous networks with distinct physical and chemical properties [2, 3]. Together, these ECM fibers play key roles in tissue scaffolding [2] and in adhesion-mediated signaling processes [4], both mediated via specialized adhesion receptors, mostly integrins [1,5]. Consequently, these interactions affect multiple cellular responses, including the regulation of cell survival, differentiation, proliferation and migration [6]. However, despite their crucial physiological importance, the mechanisms underlying signaling by cell-ECM adhesions are still largely unclear.

What features of the ECM can cells "sense," and respond to? Recent studies indicate that multiple properties of the ECM, both chemical and physical, can modulate its effects on the fates of cells adhering to it. Primarily, ECM networks may differ in their molecular composition, enabling them to interact with cells via distinct adhesion receptors and, as a consequence, trigger different signaling events. Less obvious are local or global differences in the physical properties of the ECM caused by its mechanical modulation, which, in turn, lead to its spatial diversification, and ultimately affect its signaling activity.

In this article, we discuss the chemical and mechanical micro-diversity of the ECM, and show that cells can sense such features and differentially respond to them, both locally and globally.

2 The varying responses of cells adhering to different extracellular matrices

Previous studies have indicated that adhesion to chemically-defined matrices, mediated via different cell-surface receptors, may exert varying effects on the at-

tached cells. For example, adhesion to fibronectin (via $\alpha_5\beta_1$ integrin) or to vitronectin (via $\alpha_V\beta_3$ integrin) can differentially activate Rac and Rho GTPase activity, driving lamellipodial protrusion or stress fiber formation, respectively [7]. Further studies indicated that the responses of different cell types adhering to these two matrix molecules can vary greatly, in line with the notion that lineage history and ECM specificity jointly affect the cellular response [8,9,10].

To demonstrate this feature of the ECM; namely, the different roles of two distinct integrins ($\alpha_5\beta_1$ and $\alpha_V\beta_3$) in the adhesion response, we plated primary human foreskin fibroblasts (HFF) on either fibronectin- or vitronectin-coated surfaces, and monitored both cell spreading, and the development of focal adhesions. In addition to plating cells on different matrices, it was necessary, in this case, to block clustering of the $\alpha_5\beta_1$ integrins in cells plated on vitronectin, using $\alpha_5\beta_1$ inhibitory antibodies (JBS5, kindly provided by Prof. Martin Humphries, Wellcome Trust Centre for Cell-Matrix Research, School of Biological Sciences, University of Manchester, UK) [11]. As shown in Figure 1, HFF cells adhering to fibronectin indeed displayed specific clustering of the $\alpha_5\beta_1$ integrin in defined focal adhesions, leaving the $\alpha_V\beta_3$ receptor largely homogeneously distributed. Conversely, cells plated on vitronectin-coated surfaces developed $\alpha_V\beta_3$-rich focal adhesions along the cell periphery, while the $\alpha_5\beta_1$ integrin remained uniformly distributed.

Examination of cellular behavior on these two surfaces indicated that attachment to fibronectin via its specific receptors induced much more extensive spreading, compared to cells adhering to vitronectin. Moreover, integrin adhesions formed with the fibronectin ECM (visualized by paxillin and vinculin labeling) were widely distributed throughout the cells' ventral surfaces, while vitronectin adhesions were particularly enriched along the cell periphery. It is interesting to note that particularly conspicuous differences were noted between the distributions of tensin, which was highly enriched along fibronectin adhesions (mostly "fibrillar adhesions" [12]) in the cell's central area; and zyxin, whose recruitment to focal adhesions was reported to be force-dependent [13,14], and primarily associated with definitive, stress fiber-associated focal adhesions.

The various types of ECM assemblies can also induce diverse forms of integrin adhesions, as shown in Figure 2. Double labeling of cultured fibroblasts for fibronectin and different focal adhesion components provided insights into the molecular diversity of these adhesion complexes. The pair of images showing a cell labeled for vinculin and fibronectin (the chosen cell is associated with a moderately sized network of extracellular fibronectin; Figure 2, upper panel) indicate that vinculin-rich focal adhesions are mainly found at the cell periphery, in the form of large adhesion patches, whereas fibronectin tends to accumulate under the cell center. Careful examination of the merged image reveals that vinculin and fibronectin only partially overlap. More often, fibronectin fibers tend to be excluded from large focal adhesions, apparently displaced from it centripetally, toward the cell center (see also [15]). This "spatial diversification" of integrin

adhesions appears to differ, depending on the components of the adhesion-associated cytoplasmic plaque. Thus, tensin, another focal adhesion component, overlaps with the fibronectin matrix to a far greater extent than vinculin (Figure 2, lower panel). These images, as well as time-lapse movies showing the rearrangement of integrin adhesions [15,16] provide a glimpse into the morphological, dynamic and molecular diversity of integrin adhesions. Surely, the overall molecular and functional diversity of these adhesions is much greater, though our current understanding of its nature is still limited.

The results presented herein indicate that integrin-mediated adhesions are "chemosensitive;" namely, capable of distinguishing between matrices consisting of different molecules, and organizing the adhesion sites accordingly. Thus, the differentially sensed ECM networks can induce varying degrees of cell spreading, and morphological differences in adhesion complexes; e.g., "classical focal adhesions," or fibrillar adhesions. These differences indicate that signaling via different integrins can trigger distinctly different signaling events.

3 Molecular diversity of the fibronectin ECM

A closer look at the cell-ECM interface indicates that the molecular diversity of the ECM depends not only on the different types of molecules associated with it, as indicated above, but also on the conformational state of the particular molecules. It was previously demonstrated that one of the major ECM networks, namely fibronectin fibrils, is strongly affected by mechanical forces exerted via the adhesion sites on the ECM proper. Such effects can be manifested by varying degrees of ECM stretching and, as a consequence, changes in the extent to which the fibronectin fibril unfolds [17,18,19,20]. To illustrate this point, we double-labeled a cell-generated ECM with four pairs of antibodies, each consisting of a "broad specificity antibody" and a "domain-specific monoclonal antibody (mAb)" reactive with either the collagen binding site (cat. #42037; QED Biosciences, San Diego, CA, USA), the interchain disulfide region near the fibronectin's C-terminus (cat. #42038; QED Biosciences), the cell-binding region (cat. #42040; QED Biosciences), or the fourth repeat of the type III module (cat. #F0791, Sigma, St. Louis, MO, USA). Our results are shown in Figure 3. While comparison of black-and-white image pairs indicated a largely similar pattern (not surprising, given the fact that the antibodies were all fibronectin-specific), intensity ratio imaging of each pair, shown in the colored panel, revealed major differences in the local intensity of both the general and the domain-specific labeling. The dramatic differences in epitope presentation indicate that the fibronectin-based ECM is spatially heterogeneous, presenting a rich variety of molecularly distinct microenvironments to the attached cells.

4 Mechanical forces affect the organization and adhesive properties of fibronectin fibrils

How is the molecular diversity of the ECM generated? Recent studies indicate that multiple processes may act together to modulate the structure and function of the matrix. These include the expression and secretion of the constituent molecules, regulated cross-linking ECM fibers [2,21], degradation of the matrix by cell-associated enzymes [22,23], and force-induced matrix rearrangement [3,5,24,25]. All these mechanisms apply to a wide variety of matrix components, and to fibronectin in particular. As shown above, fibronectin forms elaborate fibrillar networks, both *in vivo* and in cell culture [26]. This process of fibrillogenesis depends on specific self-binding sites, such as repeats 2-5 in the fibronectin-III domain and in the fibronectin-I domain, close to the N-terminus [27,28,29]. Vogel et al. [30] observed aggregation and fibrillogenesis of fibronectin upon adsorption to a water-suspended lipid monolayer, and showed that these processes are accompanied by substantial deformation of the fibronectin's original globular structure. Moreover, it was shown in cell culture that the development of mechanical tension is necessary for efficient fibronectin fibrillogenesis [31,32,25,20].

In order to test the hypothesis that polymerization of fibronectin and its assembly into fibers, *in vitro*, is dependent on the application of external or cell-generated forces, we undertook an experiment in which the forces applied to the fibronectin matrix, inducing its organization into fibers, could be quantified. For that purpose, we performed the fibronectin assembly assay on hydrophobic microfabricated pillars (Figure 4; see also [17]). In these studies, we applied water droplets containing different concentrations of fibronectin to the pillar tops, and applied forces of varying strengths to the droplets. The forces applied to the fibronectin fibrils, which interconnected neighboring micropillars, were quantified by measuring the bending of the pillars.

The shape of the fibronectin network formed in this system was subsequently examined by both fluorescence microscopy and high-resolution scanning electron microscopy [17]. In this study, we found that fibronectin fiber formation is a two-step self-assembly process, initiated by the formation of a stable fibronectin sheet composed of globular particles, at the air-liquid interface of the fibronectin-containing droplet. This is followed by shear force-driven fibrillogenesis along the superhydrophobic surface made up of elastic and hydrophobic micropillars. The fibronectin network attached to the tops of the micropillars and interconnecting them appears to be highly ordered, consisting of fiber bundles running between the micropillars. The diameter of these bundles can be controlled by the concentration of fibronectin in the water droplet, as well as by the duration of the contact between the droplet and the micropillar tops.

By means of high-resolution scanning electron microscopy, we found that the initially formed fibrils, displaying "rough" surfaces with globular sub-domains,

were transformed into “smooth” fibers with a characteristic diameter of 14 nm, upon stretching. It was further demonstrated that mechanically, the fibronectin fiber network is quite robust, and can support cell attachment and focal adhesion assembly. The location of focal adhesions is correlated with the location of fibronectin fibers. Clearly, the cellular forces transmitted by the actin cytoskeleton to the fibronectin fibers can bend the attached micropillars [17]. These studies further showed that fibronectin fibers are indeed elastic, and that their stretching can modulate their strain. We propose that cells fine-tune the biological activity of the underlying matrix by modulating the contractile forces applied to it via their adhesion receptors. The force-dependent exposure of new sites on the fibronectin matrix, may, in turn, affect the physiological responses of the cells, including their contractile and signaling activities.

5 Involvement of lamellar retraction in fibronectin fibrillogenesis by means of cultured fibroblasts

As shown above, the ECM appears to be molecularly and mechanically diverse, largely due to the extensive, two-way interactions between the cells and the underlying adhesive environment. Thus, cells adhere to the matrix and apply force to it, thereby modulating it spatially (e.g., inducing filament formation); mechanically (e.g., altering matrix rigidity); and molecularly (e.g., exposing new binding sites on the filament surface). To visualize this process in real time and in live cells, we expressed YFP-tagged fibronectin (kindly provided by H. Erikson, Department of Cell Biology, Duke University Medical Center, Durham, NC, USA; [25]) in Hela-WJ cells, and monitored the process of fibronectin fibrillogenesis using time-lapse microscopy. Careful examination of the resulting “fibronectin movies” revealed the formation of fluorescent, fibronectin-rich vesicles around the cell center, their transport toward the cell periphery, the deposition of their contents on the substrate, and the formation of nascent fibronectin fibrils. A detailed description of this complex process is beyond the scope of this article. Here, we would like to focus only on the formation of definitive fibers at the cell periphery. As shown in Figure 5, and in the Supplementary Movie, nascent fibronectin fibers become apparent beneath the peripheral lamellae of the cultured cells. Comparison of individual frames selected from the time-lapse movie demonstrates that initially, multiple spot-like fibronectin deposits appear under the cell periphery which, upon retraction of the leading lamella, are apparently “pulled” by the contracting lamella centripetally, forming novel, elongated fibrillar structures. This observation of fibronectin fibrillogenesis by living cells in real time corroborates the physiological relevance of the force-dependent fibrillogeneic process discussed above.

6 Conclusions

In this article, we addressed the mechanisms whereby adherent cells sense the chemical and physical properties of the adhesive surfaces to which they are attached. We demonstrated herein that different ECM molecules can induce radically different responses in the attached cells; moreover, even a molecularly uniform ECM, composed of a single matrix component (e.g., fibronectin), can be spatially heterogeneous, presenting on its surface microdomains exposing different fibronectin epitopes that may differ in their adhesive properties. Such diversification of the fibronectin ECM can be attributed to various degrees of force-dependent unfolding of fibronectin fibers. Furthermore, we demonstrated that force-dependent fibronectin fibrilligenesis, whereby cells secrete fibronectin at the cell periphery and convert these fibronectin deposits into fibers upon retraction of the leading lamella, is a common biological phenomenon.

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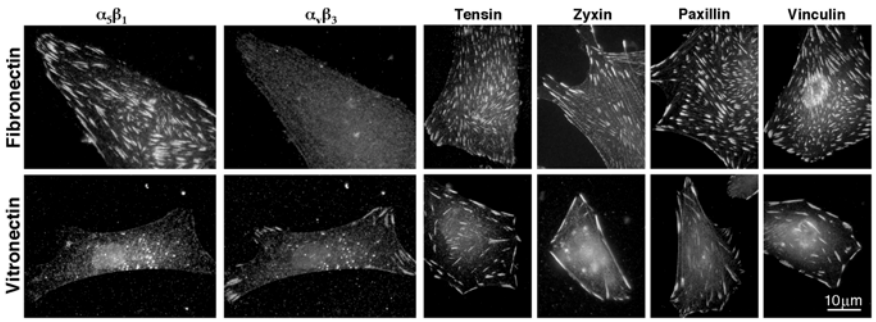

Fig. 1: Differential effects of fibronectin and vitronectin matrices on cell spreading and focal adhesion formation. Human foreskin fibroblasts were plated onto glass cover slips coated with either fibronectin (10 μg/ml) or vitronectin (10 μg/ml), and allowed to spread for 3 hrs. To block the binding of the $\alpha_5\beta_1$ integrin to vitronectin or to cell-secreted fibronectin, the cells were treated with an $\alpha_5\beta_1$ inhibitory antibody (JBS5). The cells were then fixed and immunolabeled for $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins, as well as various focal adhesion plaque proteins such as tensin, zyxin, paxillin and vinculin. As shown, cells plated on fibronectin were considerably more spread out than those growing on vitronectin, and their adhesions were prominent both at the cell periphery and around the cell center. Careful examination of the distribution of the various focal adhesion-associated molecules confirmed that the adhesions formed with the fibronectin matrix

were enriched with $\alpha_5\beta_1$, while those that associated with vitronectin predominantly contained the $\alpha_V\beta_3$ integrin. (Adapted from Baruch Zimerman's Ph.D. thesis).

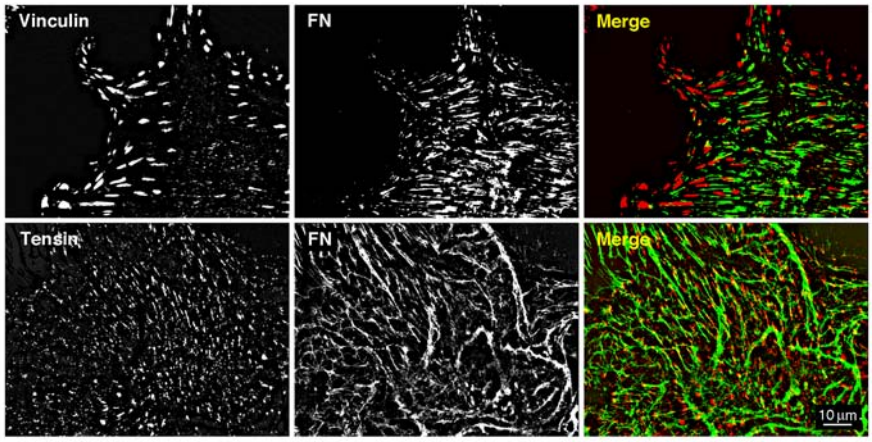

Fig. 2: Different spatial relationships between fibronectin fibrils and the focal adhesion proteins tensin and vinculin. Pig aortic endothelial cells (PAEC) were cultured on glass cover slips for 16 hours, and then fixed and double-immunolabeled for tensin and fibronectin, or for vinculin and fibronectin. Merged images (green-red) are presented in the right column. Note that fibronectin fibers tended to be excluded from large, vinculin-rich focal adhesions, but rather accumulated in the vicinity of focal adhesions. Tensin, on the other hand, tended to be associated with the fibronectin matrix.

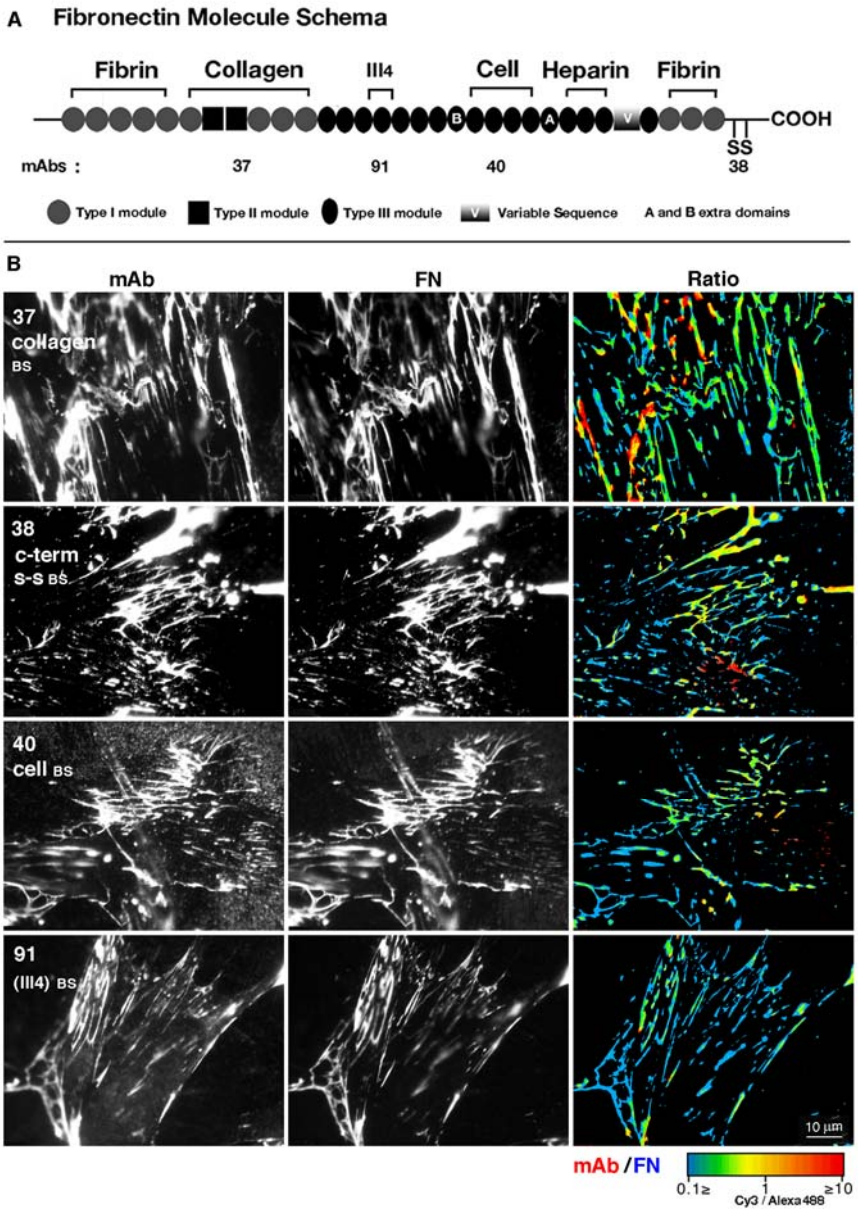

Fig. 3: Mapping of specific subdomains on the fibronectin molecule. A. Schematic drawing of the fibronectin molecule, illustrating its multi-domain structure, as well as its various binding sites (BS). mAb 42037 was shown to bind to the collagen-binding region, mAb 42038 to the interchain disulfide region, mAb 42040 to the cell-binding region, and mAb F0791 to the fourth repeat of the type III module. B. WI 38 cells were cultured on fibronectin-coated (25µg/ml) glass cover slips overnight, and then fixed and double-immunolabeled with each of the mAbs to

various binding sites (BS) on fibronectin molecules, and with fibronectin polyclonal Ab. The intensity ratio was computed per pixel, as previously described [12]. Ratio images are presented in a logarithmic spectrum scale (right column). Notice the partial exposure of the different regions identified by the mAbs along the fibronectin fibrils.

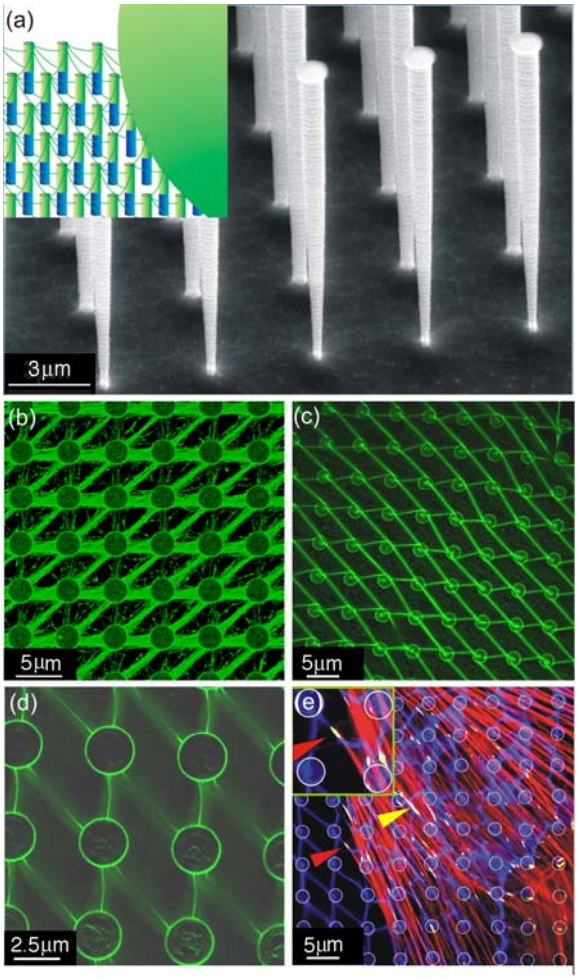

Fig. 4: Force-induced fibronectin fibrillogenesis *in vitro*. (a) A sheet of fibronectin was formed at the air-water interface of a water droplet suspended on top of hydrophobic, microfabricated pillars, shown in the scanning electron microscope image. The water droplet formed a contact angle of close to 50°– 75° on top of the pillars (data not shown). The fibronectin molecules were bound to the pillar tops, due to hydrophobic interactions. Due to the rolling of the droplet, force was applied between the pillar top and the sheet of fibronectin at the air-water interface. The pillars bent, due to the force exerted by the rolling droplet. These forces drove the formation of fibronectin fibers, which finally bridged the tops of the micropillars, as schematically illustrated in the inset. **(b-d)** Fluorescent optical micrographs of ordered arrays of fibronectin fibers, suspended between the pillar tops. The diameters of the fibers were mainly controlled by the concentration of fibronectin dissolved in the droplet, and the incubation time of the droplet on

the pillar tops. **(e)** Rat embryo fibroblasts stably expressing YFP-paxillin were cultured on the tops of such suspended fibronectin fiber networks (blue). Focal contacts formed along the fibronectin fibers, and are seen here as yellow clusters (inset). The fibronectin fibers bent, due to the forces applied by the focal adhesion to the fiber (inset). The actin cytoskeleton is shown in red.

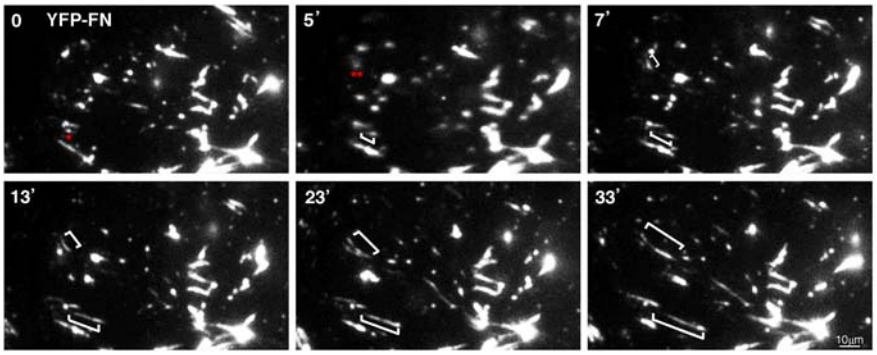

Fig. 5: Fibronectin fibrillogenesis by living cells. Hela-JW cells expressing YFP-tagged fibronectin were photographed at intervals of 60 sec between images. Six frames from the movie were selected to demonstrate stages in fibronectin fibrillogenesis. Two such developing fibrils were traced (starting points are marked with red asterisks). The movie is provided online in the Supplementary Material.

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