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Live imaging of cell protrusive activity, and extracellular matrix assembly and remodeling during morphogenesis in the frog, *Xenopus laevis*

Lance A. Davidson^{1,2,3,*}, Bette D. Dzamba¹, Ray Keller², and Douglas W. DeSimone¹

¹ Dept. of Cell Biology, University of Virginia Health System, Charlottesville, VA 22908

² Dept. of Biology, University of Virginia, Charlottesville, VA 29904

Abstract

Cell motility and matrix assembly have traditionally been studied in isolation because of a lack of suitable model systems in which both can be observed simultaneously. With embryonic tissues from the gastrulating frog *Xenopus laevis* we observe stages of fibronectin fibrillogenesis coincident with protrusive activity in the overlying cells. Using live confocal time-lapse images collected from Cy3-tagged fibronectin and plasma membrane tethered GFP, we describe the movement and the elaboration of a complex fibrillar network undergoing topological rearrangements of fibrils on the surface of an embryonic tissue. Discrete processes of annealing, polymerization, stretching, breaking, and recoiling are recorded. Elaboration and maintenance of the complex topology of the ECM appears to require filamentous actin. These findings support a mechanical-model in which cell tractive forces elaborate the complex topological fibrillar network and are part of a homeostatic mechanism for the regulation of the extracellular matrix.

Keywords

fibrillogenesis; cell rearrangement; protrusions; fibronectin; polymerization; annealing; remodeling

Introduction

Fibrillogenesis, or the assembly of the extracellular matrix (ECM), involves both active cell-mediated polymerization processes as well as cell-mediated remodeling processes (McKeown-Longo and Mosher, 1983; Wierzbicka-Patynowski and Schwarzbauer, 2003). Protein-protein interactions that mediate the polymerization of fibrils from individual proteins or protein oligomers have been subject to intensive biochemical analysis (Sechler et al., 2001; Takahashi et al., 2007). Additionally, the earliest steps of fibril formation have been elucidated in controlled cell culture systems (Ohashi et al., 1999; Ohashi et al., 2002). With few exceptions, histological approaches have been used to study the macroscopic processes of ECM assembly during embryonic development, wound healing, organogenesis, and oncogenesis.

On the molecular level, multimerization of two fibronectin dimers requires physical stretching of at least one of the interacting dimers (Halliday and Tomasek, 1995; Baneyx et al., 2002; Krammer et al., 2002). While sites have been identified in fibronectin that mediate

*author for correspondence: (email) l davidson@engr.pitt.edu, (phone) 412-383-5820, (fax) 412-383-5918.

³currently at Dept. of Bioengineering, University of Pittsburgh, Pittsburgh, PA 15260

this interaction, a precise molecular mechanism is still lacking. The physical properties of fibronectin molecular subunits have been studied with both molecular dynamics simulations as well as biophysical observations of single molecules under tension (Craig et al., 2001; Gao et al., 2002; Krammer et al., 2002; Oberhauser et al., 2002; Abu-Lail et al., 2006). Thus, the principles of fibril assembly involve both specific protein-protein interactions as well as the physical application of force and strain to fibril networks. The magnitude of the forces required for polymerization exceed those available from thermal or chemical processes (Baneyx and Vogel, 1999).

In general, the large forces required for fibronectin oligomerization can only be generated by active cellular processes linked to the cytoskeleton. Attachment of cells to the ECM is largely accomplished through the integrin family of receptors (for review see (Dzamba et al., 2002)). ECM-specific integrin heterodimers link the cytoskeleton within the cell to the ECM. These linkages can control both the timing and location of cell-ECM interactions by integrating the cytoskeleton to both intracellular and extracellular signaling pathways. Once a mechanical linkage is formed, cells may generate force on the ECM through a number of different mechanisms: through the application of traction forces commonly associated with myosin II-based contraction found at the rear of lamelliform protrusions (Horwitz and Parsons, 1999), through transport processes associated with retrograde flow of subcortical actin meshworks (Suter et al., 1998), or through the directed transport of cargo and membrane associated proteins by microtubule- and actin-based motors such as dynein, kinesin, or various myosins (Sheetz, 1999). Thus, fibril assembly requires the integration of cell-ECM adhesion and cell-force generation with the biophysical and biochemical processes of ECM components.

Uncontrolled fibril assembly is prevented through a balance between cell traction forces and the physical-mechanical properties of the ECM (Harris, 1987). The early steps of matrix assembly proceed as a cell protrudes into the network and samples the stiffness of local fibronectin fibrils with focal attachments. Fibrils that are oriented along the direction of traction and anchored by other attachments resist the traction generated by the attachment and strengthen the contact (Riveline et al., 2001). Forces applied through the attachment by the cell then pull and stretch the fibril. Strain in the fibril, rather than stress, controls the rate of polymerization (Baneyx et al., 2002; Krammer et al., 2002). Cells may then either release the fibril or remain attached to the fibril following maturation (Zamir et al., 2000). Continuing rounds of cell protrusion, traction, fibril strain, and polymerization add to fibril thickness and increases its mechanical stiffness. Forces generated by cell traction may increase during these rounds as the thicker, stiffer fibrils attract and strengthen nascent attachments (Sheetz et al., 1998; Galbraith et al., 2002). However, at some point the forces generated by the cell are no longer able to stretch the fibril to a degree required for additional polymerization. Thus, the coupling of cell protrusions, formation of attachments to the ECM, cell traction, fibril strain, and strain-dependent polymerization form a feedback control system for the homeostatic maintenance of the ECM.

The integration of cellular, biophysical, and biochemical processes that modify the ECM dominate the macroscopic processes of oncogenesis, wound healing, organogenesis, and morphogenesis. Of these macroscopic processes, morphogenesis in amphibians involves some of the most dynamic changes in the ECM. In a matter of hours, entire fibrillar networks are laid out and remodeled (Nakatsuji et al., 1985; Winklbauer and Keller, 1996; Darribere and Schwarzbauer, 2000). Sequential assembly of ECM defines tissue boundaries, establishes the embryonic body plan (Lee et al., 1984; Wedlich et al., 1989; Marsden and DeSimone, 2003; Davidson et al., 2004b), and migratory tracks for neural crest and growth cones (Epperlein et al., 1988; Krotoski and Bronner-Fraser, 1990). Furthermore, dynamically assembled ECM plays a key role during branching morphogenesis in the

salivary gland (Sakai et al., 2003) and is likely to contribute to shaping incipient organs such as the heart, liver, vasculature, and kidneys.

In this paper we present visualization techniques that allow the direct observation of both cells and the extracellular matrix as fibronectin fibrils form in embryonic tissue explants. We characterize several macroscopic processes that remodel the fibrillar fibronectin matrix with different timescales and demonstrate the role of active cellular processes that maintain and localize the fibronectin matrix to specific tissue interfaces.

Results

Embryonic tissue explants develop complex fibrils

To visualize fibril dynamics within a live tissue we modified previous explant preparations and visualization techniques (DeSimone et al., 2007). Histological analysis had identified a phase of rapid assembly and remodeling of a fibronectin-rich fibrillar ECM during early stages of gastrulation in *Xenopus laevis* (Davidson et al., 2004b). To visualize fibrils dynamics at these stages we turned to using well characterized explants of the frog embryo's animal cap or marginal zone (figure 1A; (Davidson et al., 2004a)). Animal cap explants contain prospective ectoderm whereas marginal zone explants contain prospective ectoderm and mesoderm. *In vivo* both ectoderm and mesoderm appose a fibrillar fibronectin matrix. When cultured on artificial rigid substrates (e.g. plastic, glass, or fibronectin-coated glass/plastic) these explants continue to secrete fibronectin which adsorbs onto these non-deformable substrates but do not develop a fibrillar ECM (see supplementary figure S1). In order to visualize matrix remodeling under conditions more representative of deformable substrates *in vivo*, we cultured either marginal zone or animal cap explants between sheets of agarose (figure 1B) supplemented with 0.1 mg/ml of FN in the media. Tissue explants do not adhere to agarose sheets and develop a complex fibrillar ECM. A key difference between these explants and previous preparations is that we have removed a single layer of epithelial cells to prevent a wound healing response and allow direct observation of forming fibrils using time-lapse laser scanning confocal microscopy (see also (Davidson and Wallingford, 2005)). If the epithelial layer of cells is not removed wound healing begins and the epithelial sheet engulfs exposed deep cells rapidly blocking the view of forming fibrils. The fibrillar ECM that forms under these conditions is confined to the surface of the explant. With the exception of the early to mid gastrula stage animal cap ectoderm that assemble fibrils along a surface that faces the fluid-filled blastocoel, most ECM is assembled at interfaces between two tissues. Fibril assembly on exposed mesodermal tissues in explants takes place without either the endoderm or ectoderm, tissues that would normally face the mesoderm at these interfaces.

Fibrils form and remodel in close association with underlying cells

To visualize both cells and fibronectin fibrils we expressed mRNA encoding a plasma membrane localizing EGFP (GAP43-GFP) in embryos and cultured explants in media containing a Cy3-conjugated monoclonal antibody directed against frog fibronectin (Cy3-4H2). Fibronectin fibrils on mesodermal cells in time-lapse confocal sequences appear as an interconnected meshwork that forms between the cellular tissue and the agarose sheet (figure 1D). Like the assembly of fibrils *in vivo*, fibrils rarely penetrate between cells (see supplemental figure S1). Both lamellipodia and filopodia can be tracked in scattered-labeled individual mesodermal cells (figure 1E; scattered-labeled cells are surrounded by unlabeled cells). Both cells and the fibrillar matrix assembled at their surfaces move dynamically in this sequence (see supplementary time-lapse movie S1A). Since these time-lapse sequences are collected with a confocal microscope small fibers occasionally "disappear" but are in fact simply moving out of the confocal microscope's plane-of-focus. Fibrils can transiently

disappear because the uneven surface of the explant or the agarose gel provide spaces for fibrils to occasionally escape the plane of focus. Similar fibronectin fibril dynamics are seen at the surface of ectodermal tissues (see supplementary time-lapse movie S1B).

Growth and topological elaboration of fibrillar network

Time-lapse sequences of fibronectin fibrils on both ectoderm and mesoderm reveal that the network undergoes distinct topological changes and that each type of change occurs with its own characteristic timescale. The most obvious change in the network is growth (figure 2A; see supplementary time-lapse movie S2). Over the course of hours the fibrillar matrix grows in density and complexity. Most fibril growth may reflect polymerization rather than simple “thickening” after fibrils are released and shorten in length (see fibril ‘loop’ between arrowheads in figure 2B; see supplementary time-lapse movie S3). Both polymerization and *shortening-and-thickening* produce a denser fibrillar array, however, they do so on different timescales. Polymerization takes hours to increase the density of fibrils while individual fibrils can shorten and thicken in as little as 10 minutes (compare timescales in figure 2A to 2B). The “connectivity” of the fibrillar network, that is the number and location of connections between fibrils, changes on the timescale of minutes. Single fibrils can move, contact, and anneal to neighboring fibrils during this time (arrow in figure 2C; see supplementary time-lapse movie S4). These new connections then appear to integrate immediately into the mechanical fabric of the matrix (e.g. new fibril links stretch by last frame in figure 2C). The most rapid change in the network occurs in seconds as fibrils stretch and break (arrow indicates intact fibril in figure 2D; see supplementary time-lapse movie S5). The free ends of these broken segments then recoil into globular particles (arrowheads after fibril segment breaks in figure 2D) perhaps through a process of self-annealing. Thus, fibronectin fibrils form a highly dynamic network that is undergoing constant remodeling by a series of topological processes in which the fibrils polymerize, anneal, stretch, and break.

Elaboration of the network is driven through cell contact with fibrils

Movement of the fibrillar network occasionally correlates with cell protrusions. Lamellipodia in these explant preparations extend from one cell under the ventral surface of neighboring cells. The majority of lamellipodial movements occur without apparent contact with fibrils, however, some appear to contact, bind, and drag fibrils over the course of a protrusive duty cycle (supplementary time-lapse movie S6A). Several fibril segments (within yellow circles; figure 3A and C) move from the surface of one cell (hash mark in figure 3B) after contact with a lamellipodia (arrowhead at 6 minutes in figure 3B) and move to the boundary between two cells (between the asterisk and hash marked cells in figure 3B) as the lamellipodia retracts. Fibrils that run along cell-cell boundaries are subject to additional modification as small fibril “spines” frequently form perpendicular to the main fibril (see arrowheads in figure 1B). Additional fibril movements coincide with filopodia (data not shown), however, many fibril movements are not associated with either lamellipodia or filopodia.

In most cases fibril movements cannot be correlated with cell protrusions but instead move toward focal points far from cell-cell boundaries (asterisk; figure 3B; see supplementary time-lapse movie S7; for additional examples see fibril movements in supplementary time-lapse movies S2A, and S2B). These fibrils are gathered to a central, perinuclear location on the ventral cell surface (asterisk in figure 3E, or within yellow circles in figure 3D and F). Fibril gathering focal points are relatively static structures, however, fibrils can join or leave them on the timescale of minutes (e.g. see fibril movements at the end of supplementary time-lapse movie S2). Movements of the fibrillar network, distinct from cellular protrusions,

continue even after a dense fibrillar network is assembled (see last 10 minutes of elapsed time in supplementary time-lapse movie S1).

Maintenance of the network depends on F-actin

Since the fibrillar network was so closely associated with the cell surface we wondered whether the cortical actin cytoskeleton was essential to maintain the matrix. Cellular protrusions, as well as other force generating machinery in the cell, require polymerization of the actin cytoskeleton. We inhibited actin polymerization by adding cytochalasin D (CD) to explants that had already assembled a fibrillar network. Twenty minutes after addition of 4 μ M CD to cultured explants the fibrillar network collapses to large thick cables, cells in the explant become round and cease protrusive activity but remain attached to one another (figure 4A; see supplementary time-lapse movie S8). These cables remain associated with the exposed cell surface of the explant and are not found deeper within the explant even after cells round up and cell-cell contacts are significantly reduced. When intact fibril networks are incubated in 1 mM of the divalent cation chelator EDTA, the fibrils also collapse to cables, however, these cables are no longer held to the outer surface of the explant, penetrate between cells, and move into the middle of the explant (figure 4B; see supplementary time-lapse movie S9). Thus, the F-actin cytoskeleton assists in maintaining the open network topology but is not required to hold the fibrils to the outer surface of the explant.

Discussion

Multiple Steps for Fibril Assembly and Remodeling may form the basis of *Homeostatic ECM Maintenance*

Distinct processes of growth, annealing, stretching, breakage, and elastic recoil shape the ECM that forms on the exposed surface of frog embryonic tissue explants. These observations support a three-step process for the formation of the fibrillar matrix (figure 5A) in which FN-puncta are stretched both across apposing cell-cell boundaries or within the membrane of single cells (Dzamba et al, submitted). These short fibrils can then anneal to one another to form dense contiguous networks that span multiple cells. The incipient network is then remodeled as fibrils are both *gathered* toward the cell's perinuclear region on the ventral surface of cells and as fibrils are *swept* to cell-cell junctions (figure 5B). Thickening of individual fibrils may occur through both polymerization or as released fibrils shorten and thicken (figure 5C). Further remodeling of the network can occur through cycles of annealing-breaking-recoiling (figure 5D). This study is just the first step in describing these apparently chaotic topological changes in the ECM and future studies will be needed to determine the precise role of cell-adhesion and cell-traction in both polymerizing and regulating fibrillar ECM assembly.

The physical coupling of ECM and cells' actin cytoskeleton that underlies the mechanics, assembly, and remodeling of the ECM may be responsible for the homeostasis of the ECM in both developing embryos and in adult tissues. The concept of ECM homeostasis was first suggested by Harris (1987) to explain the capacity of sponge tissues to maintain an unchanging ECM despite the processes of growth and disturbance. In the current study we report live changes in the fibronectin-ECM as the matrix assembles and remodels in frog embryonic tissue explants. Earlier studies related the effects of molecule-scale strain and force in exposing cryptic sites within FN required for fibril assembly (Schwarzbauer and Sechler, 1999). Thus, our findings support a direct role for cell attachment in assembling and remodeling the fibrillar FN-ECM in tissues as part of a strain-dependent feedback mechanism for the regulation of ECM density and topology (McKeown-Longo and Mosher, 1983; Schwarzbauer and Sechler, 1999).

Our time-lapse sequences of cell and ECM dynamics during morphogenesis should dispel the perception that the extracellular matrix is static during morphogenesis. Recent work has shown that fibronectin fibrils are extensively remodeled during gastrulation in the frog (Davidson et al., 2004b) and chick embryos (Czirok et al., 2004; Zamir et al., 2006) even as cells undergo migration and rearrangement. The timescale of matrix remodeling is nearly the same as the timescale of the cell rearrangements that drive convergence and extension (Keller et al., 2000). Fibronectin fibrils may be remodeled on similar timescales during bone remodeling by osteoblasts (Dallas et al., 2006; Sivakumar et al., 2006) and during branching morphogenesis in the submandibular salivary gland (Sakai et al., 2003; Larsen et al., 2006). In all these examples cells may migrate on, remodel, and move ECM with them. Potentially, information embedded in the ECM such as bound growth factors will be likewise moved or remodeled with consequences for cell signaling and behaviors. Future advances in image processing, co-localization of focal adhesion proteins, and biophysical analyses of the mechanics of the ECM will be needed to understand the cell biology underlying the complex topological changes in the ECM and their role in developing tissues. The interplay between fibrils, which may mechanically couple adjacent cells, and the protrusive activity that drives these cell rearrangements is not well understood but can now be clearly resolved.

Experimental Procedures

Frogs, Eggs, and Explant Preparation

Fertilized frog eggs were obtained using standard methods, dejellied, and cultured in 1/3X Modified Barth's Solution (Sive et al., 2000) to the desired stage (Nieuwkoop and Faber, 1967). At early gastrula stages, embryos were selected and transferred to explant culture media (Danilchik's For Amy, DFA; (Sater et al., 1993)) for microsurgery. then cultured for longer periods in DFA.

Explants consisting of deep ectoderm or mesoderm were isolated using hairloops and eyebrow knives. Embryos were manually removed from their vitelline with forceps. Marginal zone explants are prepared as described (Davidson et al., 2004a) with slight modification. The outer, single cell-layered epithelium is removed once the mesendoderm is pulled away from the interface that forms the Cleft of Brachet (see figure 1B in (Davidson et al., 2004a)). The explant is then separated from the remainder of the embryo and transferred to confocal viewing chambers containing DFA supplemented with 0.1 mg / ml bovine plasma fibronectin (Roche Molecular Biochemicals), antibiotic/antimycotic (Sigma), and 5 μ g / ml Cy3-4H2 (see antibody preparation).

Once cut, explants were cleaned of debris and mounted in chambers for confocal imaging. Chambers were made from rectangular-shaped custom pieces of acrylic milled to provide small volume for culture media. Acrylic chambers were glued to large coverslip fragments using silicone grease (Dow-Corning) and sealed with small square glass coverslips. For experiments where media needed to be added a small corner of the top coverslip was removed leaving a small opening. Explants were transferred to the well along with two thin rectangular sheets of agarose. The explant was then sandwiched between the agarose and held in place by a small coverslip fragment and silicone grease.

Expression of membrane-localized GFP

Synthetic capped RNA encoding a plasma-membrane localizing GFP (GAP43-GFP) was transcribed (Epicentre) from plasmid (Moriyoshi et al., 1996). To obtain embryos expressing GAP43-GFP evenly throughout the explant, embryos were injected 4 times around the marginal zone with a total of 0.2 ng of RNA before the first cell division after fertilization. To obtain scatter-labeled samples, embryos at early cleavage stages of 16 to 128 cells were

injected in single blastomeres in the marginal zone with the same equivalent concentration of RNA.

Antibody Isolation and Conjugation

For live imaging of fibronectin fibrils, the mAb 4H2 (a non-function blocking antibody raised against the central cell binding domain of frog fibronectin (Ramos and DeSimone, 1996) was conjugated to the Cy3 fluorophore using an NHS-ester form of Cy3 (FluoroLink; Amersham Pharmacia Biotech). Cy3-4H2 was dialyzed against DFA (without BSA) and concentrated with cutoff filters (Centricom YM-30 centrifuge filters; Amicon).

Imaging and Image Processing

Confocal time-lapse sequences were collected using a confocal laser scan head (PCM2000, Nikon, Melville, NY) mounted on an inverted compound microscope (Nikon). High resolution images were acquired with a 1.40 n.a. 60X oil-immersion plan apochromat objective. The GFP signal was excited using the 488 nm line from an argon laser and collected with the 515 nm emission filter. The Cy3 signal was excited using a 543 nm Green HeNe laser and collected with the 565 nm emission filter. Independent neutral density filters were used to reduce the excitation intensities to minimal levels to reduce photodamage to the samples. Furthermore, the confocal pinhole was opened to the “middle” position to collect from a larger z-slab and to increase brightness. Samples were exposed to both excitation wavelengths and the emission signals collected from the same scan. Multi-wavelength, multi-Z-position, and time-lapse sequences were collected and stored as uncompressed animation stacks on a computer (PC) running image acquisition software (Compix Inc., Cranberry Township, PA). These stacks were transferred to a computer (PC) and processed using ImageJ (ImageJ; Wayne Rasband, NIMH; see <http://rsb.info.nih.gov/ij/>). Processing was limited to linear adjustments to the lookup table and to one round of smoothening and one round of sharpening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Live imaging of fibrillogenesis

A) Epithelia-free explants are microdissected from either the animal cap (e, ectoderm) or the marginal zone (n, notochord; s, somite) of gastrulating embryos (d, dorsal anterior midline; bc, blastocoel) and cultured between two sheets of pre-cast agarose (B). C) In order to collect high resolution confocal time-lapses the tissue is gently compressed under a glass coverslip fragment (g), sandwiched between two sheets of agarose (the lower sheet as thin as 10 microns), glued in place with silicone grease (s). D) A sequence of frames from a confocal time-lapse of fibronectin-rich fibrils (red; labeled by incubation with a non-function blocking monoclonal antibody to *Xenopus laevis* fibronectin, 4H2, conjugated to the fluorophore Cy3) on the ventral surface of the explant and E) a sequence of frames from the same time-lapses showing a scatter-labeled group of cells expressing RNA encoding a plasma membrane localizing GFP (green; GAP-43 GFP). Small fibronectin-rich spines can be seen forming perpendicular to thick fibrils (arrowheads).

Figure 2. Remodeling the network topology of fibrils

Frames from representative time-lapse sequences showing dynamic changes to the organization and topology of the fibrillar network showing fibril growth (A), fibril shortening and thickening (B), trans-fibril annealing (C), and a sequence demonstrating stretching, breaking, and subsequent recoil of the fibril fragments (D). All scalebars are 10 μm . Fibrils are labeled by incubation of explants with a Cy3-coupled mAb to frog fibronectin.

Figure 3. Movements of fibrils associated with lamellipodia and perinuclear regions

A) Frames from a representative time-lapse of the ventral surface of the ectoderm showing lamellipodia (green; GAP43-GFP) moving a cluster of fibrils (red; Cy3-coupled mAb). B) A lamellipodia (arrowhead at 6 min.) extends from one cell (asterisk) to the ventral surface of the adjacent cell (hash mark) and retracts fully by 8 minutes elapsed time. C) A group of fibrils is moved by the lamellipodia between 4 and 6 minutes and subsequently anneals by 8 minutes. D) Frames from a time-lapse sequence showing fibrils gathered to the perinuclear mid-body of a cell (asterisk in E). No lamellipodia are seen near these fibrils although they move with the cell over the course of 14 minutes (F). The cluster of fibrils moved by the lamellipodia (A and C) and those associated with perinuclear regions of the cell (D and F) are enclosed by yellow circles at the start and end of each sequence.

Figure 4. Disrupting cortical actin or cell adhesion rapidly alters fibril topology

A) Frames from a representative time-lapse sequence show cell plasma membrane and fibronectin-rich fibrils on the surface of animal cap ectoderm after addition of 4 μM Cytochalasin D. Within 10 minutes the complex network is reduced to a few thick cables. These cables remain on the ventral surface of the explant even after the cells have rounded up at 20 minutes. B) Frames from a time-lapse sequence where 1 mM EDTA is added. EDTA also reduces network complexity but remaining cables penetrate deep into the tissue where the fibrils are held between cells (arrows in panel on far right in B).

Figure 5. Phases of assembly, categories of movements, and topological changes in the fibronectin fibril network

A) Fibronectin-rich puncta form on the surface of cells. These puncta can be physically stretched to form short fibrils either bridging cell-cell junctions or between attachments at the cell-cell junction and the cell center. If a short fibril is released it can relax, returning to a puncta. Short fibrils may anneal once they contact one another to form contiguous fibrils that span multiple cells. B) Once fibrils span multiple cells they may be remodeled by cell protrusions that can gather fibrils to cell centers or sweep fibrils to cell-cell boundaries. C) Fibrils may thicken by polymerization or by shortening and thickening. D) The overall topology of the fibril network can be remodeled after a cycle of "stretch-anneal-break". For example, two separate fibrils can be swept to cell-cell junctions where they anneal. One of the segments of the new network can stretch and break, thus, altering both the network topology and the mechanical properties of the matrix.